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Structure-function analysis of SOCSI mediated Growth arrest

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Structure function analysis of SOCS1-induced growth arrest

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Résumé

Les suppresseurs de signalisation des cytokines (SOCS) sont une famille des protéines qui contrôlent la signalisation des cytokines par l'inhibition des JAK/STATs. Ils fonctionnent comme un adapteur pour la formation d'une ligase E3. De cette façon, des protéines SOCS aident à la dégradation des JAK et d'autres cibles, incluant d'autres SOCS. SOCS1 est la protéine la plus étudiée de la famille. Elle a été identifiée comme étant nécessaire pour la fonction normale du développement des lymphocytes. Aussi SOCS1 est hyperméthylé dans beaucoup des cancers et fonctionne comme un suppresseur de tumeurs. Les mécanismes précis de SOCS1 comme suppresseur de tumeurs ne sont pas connus. La plupart des études se concentrent sur le rôle de SOCS1 dans la dégradation des kinases JAK et l'inhibition de la voie de signalisation de JAK/STAT.

Dans cette étude, nous avons démontré que la surexpression de SOCS1 induit un arrêt permanent dans le cycle cellulaire par l'induction des mécanismes de sénescence. SOCS1 prévient la formation de colonies dans la lignée de cancéreuse des U2OS et rend active la voie de signalisation de p53, peut-être par des interactions directes. Nous avons découvert un résidu nécessaire, le N198, qui reste dans le « Cul box » de SOCS1. Quand ce résidu a subi une mutation ou une perturbation, ceci inhibe la capacité de SOCS1 à induire l'arrêt de croissance. Le résidu N198 est impliqué dans la capacité de recrutement des cullins, ceci suggère que des séquences différentes dans le « Cul box » de SOCS1 peuvent lui donner la capacité de se lier avec d'autres protéines ce qui lui donne la capacité d'induire un arrêt de croissance et de rendre active la voie de p53. D'autres membres de la famille des SOCS ne sont pas capables d'induire un arrêt de croissance, alors que SOCS5 semble fonctionner comme oncogène.

Mots Clés : SOCS : suppresseur de signalisation par les cytokines, JAK : kinase Janus, STAT : capteur de signal et activateur de transcription

Abstract

The suppressors of cytokine signaling are a powerful family of proteins shown to negatively regulate cytokine signaling by inhibiting the JAK/STAT pathway. By acting as adaptor proteins and recruiting the modules of an active E3 ligase, SOCS proteins can degrade JAKs and a variety of other target substrates including other SOCS molecules. SOCS1 has been the most studied of the family and has been shown to be vital to normal lymphocyte development. As well it has shown to be silenced via hypermethylation in a variety of cancers and been shown to contain the properties of a tumor suppressor. The exact mechanisms of SOCS1 as a tumor suppressor are not known though most theories focus on the degradation of JAK's and inhibition of JAK/STAT signaling.

Here we demonstrate SOCS1 overexpression is able to induce permanent cell cycle arrest in primary cells by induction of the senescence machinery. It also prevents colony formation in the U2OS sarcoma line and leads to activation of the p53 pathway, possibly through direct interactions. Also we have identified an essential residue, N198, which when mutated or deleted leads to loss of ability to promote growth arrest. This residue is unique to SOCS1 and lies within the Cul box domain of SOCS1 and is involved in Cullin recruitment. This suggests that differential sequences in the Cul box of SOCS1 compared to other SOCS family proteins may allow it to bind to different cullins or other proteins that give it the ability to promote growth arrest in a variety of cells and activate the p53 pathway. They also hint towards the fact that SOCS1 induced senescence and growth arrest is independent of its actions on the JAK/STAT pathway. Other members of the SOCS family investigated were not able to induce premature senescence, while some such as SOCS5 promoted cell proliferation.

Key words: SOCS: suppressor of cytokine signaling, JAK: Janus kinase, STAT: Signal transducer and activator of transcription

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List of Symbols and abbreviations

%	Percentage
°C	Degrees Celsius
μ	Micro (10^{-6})
μg	Microgram
μL	Microlitre
™	Trademark
A	Alanine
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine 5'-triphosphate
bp	Base pair
BSA	Bovine serum albumin
C-	Carboxy
cAMP	Cyclic adenosine monophosphate
CIS	Cytokine inducible suppressor
c-Kit	Cytokine stem cell factor
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cDNA	Complementary DNA
Cul	Cullin
D	Aspartic Acid
DA	Dalton
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	Glutamic acid
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor

EST	Expressed sequence tags
EPO	erythropoietin receptor
Erk2	Extracellular signal-regulated kinase 2
et al.	And collaborators
F	Phenylalinine
FGF	Fibroblast growth factor
FBS	Fetal bovinse serum
FBXO11	F-box protein 11
G	Glycine
g	Gram
G-CSF	Granulocyte colony-stimulating factor
GAS	Gamma activated sequences
GH	Growth hormone
GST	glutathione *S*-transferase
HIF	Hypoxia inducible factor
HPV	Human papillomavirus
I	Isoleucine
IRS	Insulin Receptor substrates
IFN	Interferon
ISRE	IFN response elements
IL	Interleukin
JAB	Janus kinase inhibitor/Janus kinase binding protein
JAK	Janus kinase
JH1	Janus homology domain 1
K	Lysine
KIR	Kinase inhibitory region
KDa	KiloDalton
Kbp	Kilo base pair
L	Litre
LIF	Leukemia inhibitory factor
LOH	Loss of heterozygosity

LPS	Lipopolysaccharide
M	Molaire (mol/litre)
mM	millimolaire
ml	millilitre
MAPK	Mitogen-activated protein (MAP) kinases
MHC I	Major histocompatibility complex I
mRNA	Messenger RNA
MTOC	Microtubule-organizing center
N	Asparagine
N-	Amino
n	nano (10-9)
nm	nanometer
NKT	Natural killer T-cell
ORF	Open reading frame
P	Proline
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-OH-kinase
PMBL	primary mediastinal B-cell lymphoma
PML	Promyelocytic leukemia protein
R	Arginine
Rb	Retinoblastoma protein
Rbx	Ring-finger catalytic protein
RNA	Ribonucleic acid
S	Serine
SA-β-Gal	Senescence-associated β-galactiosidase
SH2	Src homology 2 domain
SH3	Src homology 3 domain
shRNA	short hairpin RNA
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacryladmide gel electrophoresis

STAT	Signal transducer and activator of transcription
SOCS	Suppressor of cytokine signaling
SSI-1	STAT-induced STAT inhibitor-1
SUMO	Small Ubiquitin-related modifier
T	Threonine
TCR	T cell receptor
Tkip	Tyrosine kinase inhibitor peptide
UBC	Ubiquitin conjugating enzyme
TNF	Tumor necrosis factor
TYK2	Tyrosine kinase 2
VHL	Von Hippel-Lindau tumor suppressor
X-Gal	5–Bromo–4–Chloro–3– indolyl–β-D-galctopyranoside
Y	Tyrosine

Cytokine Signaling & Janus kinases

Cytokines are powerful signaling ligands used to communicate a signal from one cell to another and are capable of commencing multiple signaling cascades within a cell [1]. Cytokine signaling has been shown to play a large role in growth and differentiation though it is largely known for its role in immune responses such as wound healing and inflammation. They have also been demonstrated to play roles in development of the nervous system, and development during embryogenesis. Cytokines act in a similar manner to hormones as they are secreted and pass signals from one cell to the next, binding to a membrane bound receptor to commence their cascade within that cell. Most cytokines are small water-soluble proteins or glycoproteins and can be secreted by many different cell types. Four groups of cytokines have been classified based on structure. The first group is classified as having a four α-helix bundle and can be further divided into subfamilies. The three subfamilies are; the interferons (IFN), activated in viral response, the interleukin-2 (IL-2) subfamily, which mediates signals to activate lymphocytes, and the interleukin-10 (IL-10) subfamily, which acts as an inhibitory molecule and prevents activation of other cytokine pathways such as the IFN and IL-2 pathways [2-7]. The a-helix bundle group makes up the largest and most well known group of cytokines. The other three groups include the interleukin-1 group (among the first cytokines discovered), interleukin-7 group and a group of cytokines simply classified as chemokines [8-11].

Each specific cytokine has its own receptor that in turn is associated with a tyrosine kinase from the Janus kinase family (JAKs) [12-15]. The JAK family of kinases

consists of JAK1, JAK2, JAK3, and a fourth member, TYK2 [12, 13]. It is the JAKs that are responsible for downstream activation of all other kinases seen involved in cytokine signaling. All JAKs are similar in size, 120-130Kda. They are characterized by having a carboxy terminal kinase domain and pseudo kinase domains that precedes it. JAKs are almost ubiquitously expressed except for JAK3, which is found primarily in haematopoietic cells.

JAK binding to cytokine receptors can take a variety of forms, which are generally classified into three patterns. JAK2 can bind constitutively, or in response to ligands, to single-chain receptors which then aggregate and cause a subsequent aggregation of JAK2 [16, 17]. This leads to transphosphorylation of the kinase activation loops, activating the JAKs and increasing their catalytic activity. The now activated JAK2 is able to phosphorylate the receptor and target substrates recruited by the receptor, as well as autophosphorylate itself. A second model involves a β_c-chain associated with a ligand binding α–chain, JAK2 associated with the β_c-chain, which leads to its activation and signal transduction [17-19]. This is commonly seen in the IL-3 and IL-6 families. The interferons and IL-2 family require two chains to induce JAK signaling [20-24]. IFN-α & β have a β-chain, which associates with JAK2 and a α-chain that associates TYK2 [21-24]. IFN-γ has an α-chain that associates with JAK1 and a β-chain that associates with JAK2 [23]. This is similar to the IL-2 receptor, where JAK2 binds to either a ligand specific α-chain or the proximal region of the β-chain and JAK3 binds to a shared γ_c chain, the receptor aggregates bring the two JAKs together [20].

Though the mechanisms may vary slightly, the end goal of cytokine binding to a receptor is to cause aggregation of the JAKs which leads to their subsequent cross

activation and commences the signaling cascade [1, 12, 13, 18, 20]. JAKs can activate many signaling cascades by means of recruitment using SH2 domains of target molecules. Such target molecules include RAS, phosphatidylinositol-3-OH-kinase (PI3K), as well as the signal transducers of activation and transcription (STATS).

STATS

The STATS make up a well-known family of transcription factors heavily linked to cytokine/JAK signaling. STATS act upon target genes through different mechanisms as well. The first mechanism was found in studies involving INF-α/β, these showed STAT1 was phosphorylated in response to IFN and bound in a complex with p48 [25]. This caused STAT1 to enter the nucleus and cause transcription of genes containing IFN response elements (ISRE). Later it was shown IFN-γ caused phosphorylation of STAT1 which caused it to form a dimer which than moves into the nucleus and binds to gamma activated sequences (GAS) in target genes and drives transcription [26]. As more STAT molecules were cloned, this model became the widely accepted model for STAT activation, and it has since been shown that that almost all cytokines activate at least one STAT [13].

All STAT members contain conserved SH2 domains as well as an SH3-like domain and a DNA binding domain [13, 27]. Phosphorylation of tyrosine residues in the carboxy terminus mediates both hetero and homodimer formation via the SH2 domains [28]. Dimer formation leads to entry into the nucleus where transcription follows [26, 28]

STATS can also be activated by other serine/threonine kinases and have been linked to MAPK and the RAS signaling cascade [26, 28-32].

Discovery of a SOCS Family of proteins

Obviously, cytokines are involved in a variety of important signaling cascades and therefore must be carefully regulated. Constitutive activation of any growth or proliferation signal, as well as a chronic inflammation response, can have dire consequences for the cell, the worst being malignant transformation. As such, another family of proteins known as the suppressors of cytokine signaling (SOCS) exist to negatively regulate the effects of cytokines through a variety of mechanisms including direct inhibition of their receptors or the JAKs which activate them [33-37]. The first protein of this family was discovered in 1995 and named cytokine induced suppressor (CIS) [35].

Currently, there are eight known members of the SOCS family in humans (SOCS1-7 and CIS) [38]. By studying the primary amino acid sequences of the SOCS family members it has been revealed that there exists a high degree of similarity between pairs of proteins. For example, SOCS1 is very similar to SOCS3 and thus they form a pair, SOCS2 and CIS make another, while SOCS6&7 and SOCS4&5 round out the pairings. SOCS family members all share a SH2 domain as well as conserved C-terminal SOCS box motif, but have variable N-terminals. The SOCS box domain has since been found in a variety of non-SOCS family proteins, over twenty so far [39]. The mechanisms

of inhibition by SOCS proteins varies from protein to protein, some even display multiple mechanisms.

SOCS1 has become the most studied of the group. It was cloned and described by three different groups under different studies, which speaks of its versatility as a negative regulator of cytokine signaling. Naka et al. were trying to find other STAT family members using an antibody for a sequence in the STAT3 SH2 when they identified both CIS and a new gene, which they cloned and named Stat-induced STAT inhibitor-1 or SSI-1 [37]. SSI-1 showed a 36% homology to the SH2 domain of CIS, but none to STAT3 or STAT6, except for the phospho-tyrosine recognition site. A second group, looking for proteins that could bind to the JAKs directly through the JH1 domain, also managed to clone SOCS1, which they at the time had named JAB for Janus kinase binding protein [34]. They too reported a protein with a SH2 domain sharing similarity to that of CIS (35%). A third group named the protein SOCS1 after finding it by screening for genes encoding proteins that could inhibit IL-6 signaling [33]. They performed a screen of library of genes from the factor dependent cell line FDC-P1. Upon infecting M1 cells with a retrovirus containing the cDNAs from the FDC-P1 cells they found a 1.4 Kbp insert that rendered the M1 cells unresponsive to the IL-6 signal. They cloned the insert and also found it to be a relative of CIS and named it the suppressor of cytokine signaling 1 (SOCS1).

The early studies on SOCS1 all lead to similar results that helped elucidate its role in cytokine signaling and shed some light on the mechanisms through which it works. The *SOCS1* gene rests on a single exon and is located in mice on chromosome 16, close to the protamine gene cluster [33, 34]. It shares no homology to any of the protamine

genes, but rather corresponded to what was a then unknown ORF at the 3' of these genes [34]. It encodes a 212 amino acid protein in mice and rats, 211 in humans [33, 34, 37]. The human, mouse and rat copies of the protein are highly homologous sharing a 95-99% amino acid sequence [33]. SOCS1 contains a SH2 domain at amino acids 79-167 and a SOCS Box domain at its C-terminus [33, 34]. Using the sequence from SOCS1 and expressed sequence tags (ESTs) Starr et al., were able to clone the first two other related SOCS proteins SOCS2 and SOCS3, which have similar SH2, and SOCS box domains as SOCS1 [33]. *SOCS1* expression is ubiquitous in most tissue types though it has been repeatedly shown to have higher levels of expression in the thymus, spleen, testes and lung [33, 37].

Role of SOCS1

Investigations into the role of SOCS1 in cytokine signaling clearly show its role as a negative regulator. When expressed in myeloid leukemia (M1) cells that also expressed a Thrombopoietin receptor, SOCS1 conferred a general resistance to cytokine signaling [33]. Furthermore these cells continued to grow and proliferate when expressing SOCS1, even when treated with a variety of cytokines including IL-6, IFN, Leukemia inhibitory factor (LIF) and Thrombopoietin (in M1.mpl cells). Such treatment caused parental M1 cells to stop proliferation and form differentiated colonies [33, 37]. Treatment with dexamethasone did lead to differentiation in cells expressing SOCS1, implying it acted only in the cytokine signaling pathways, and not the general differentiation process [33]. Expressed in NIH3T3 cells, SOCS1 was able to block many

of the antiviral activities of IFN [34]. Since SOCS1 seemed to inhibit cytokine signaling, it was suspected that it acted as a negative feedback mechanism so the effects of cytokine signaling on SOCS1 induction were studied. In growth factor dependent cell lines such as M1 and the hybridoma MH60 cell line, *SOCS1* was induced in response to treatment with IL-6 and soluble IL-6 receptor [37]. This was repeated in the IL-4 dependent line CT4S after treatment with IL-4 as well as the G-CSF dependent NFS60 line with G-CSF treatment. Bone marrow cells stimulated with different cytokines are capable of not only inducing *SOCS1*, but also a variety of SOCS family members, depending on the cytokine [33]. Also some cytokines are capable of inducing certain SOCS genes in specific cell lines but not others. IL-3 and activation of the erythropoietin receptor (EPO) are unable to induce *SOCS1* in Tf-1 and NFS60 cells. IL-6 induces *CIS*, *SOCS1-3*, in mice liver cells but only *CIS* and *SOCS1* in M1 cells, displaying a cell specific cytokine response in the induction of SOCS family members.

Since SOCS1 inhibits many cytokines, and most cytokine signaling cascades signal through the JAK/STAT pathway, the interactions between SOCS1 and the JAK/STAT pathway were further studied. IL-6 and IL-4 are inhibited by SOCS1 and are both capable of inducing *SOCS1*. Both act through the JAK/STAT pathway, IL-6 through STAT3 and IL-4 through STAT6. Studies of the SOCS1 promoter revealed binding sequences for STAT3 and STAT6, implicating *SOCS1* as a target gene for STAT signaling. It has since been shown that *SOCS1* also has binding sites for STAT5 as well in its promoter. M1 cells transfected with *SOCS1* and *STAT3* show induction of SOCS1 mRNA in response to IL-6. However when SOCS1 is co-expressed with a STAT3 mutant, in which a tyrosine reside phosphorylated by a JAK (Y705) is replaced with a

phenylalanine, no induction takes place, showing a requirement for activated STAT signaling as a requirement for the induction of *SOCS1* [37].

Tyrosine phosphorylation of STAT3 and the Gp130 protein, which is part of the cytokine receptor and is phosphorylated by JAKs, were both reduced in M1 cells expressing *SOCS1*. IL-6 treatment is able to phosphorylate both STAT3 and Gp130 in normal M1 cells, though expression of *SOCS1* reduces this. STAT3 and STAT5 tyrosine phosphorylation was also reduced in 293 cells expressing *SOCS1*. General STAT activation in response to many cytokines is greatly inhibited by SOCS1; STAT5 activation by EPO and STAT3 activation by IL-6 is almost completely abolished in *SOCS1* expressing M1 cells [34]. As well several STAT target genes such as the immunoglobin fragment Fcγ recptor (*FcγR*) show a large reduction in expression in *SOCS1* M1 cells.

SOCS1 also prevents STAT molecules from forming dimers that enter the nucleus, preventing their activation of DNA transcription [33]. In M1 cells treated with IL-6, the most common dimers observed are the SIF-A (STAT3 homodimer) and SIF-B (STAT1/STAT3 heterodimer), the formation of these complexes is observed using electrophoretic mobility shift assays. In *SOCS1* expressing cells IL-6 treatment fails to induce formation of these dimers, as well it also blocks formation of the SIF-C STAT1 homodimer induced by IFN-γ. Hence SOCS1 is able to prevent tyrosine phosphorylation of STATs, preventing them from forming an active heterodimer and blocking transcription of STAT target genes in the process.

SOCS1 does not directly bind and inhibit the STAT transcription factors. Instead it targets the JAKs that activate them and inhibits their signaling cascade. SOCS1 directly

interacts with the JH1 domain of JAK2 via its SH2 domain and has been shown to interact with TYK2 as well [34, 37]. However, JAK activity is needed for this interaction as SOCS1 does not interact with a K882D kinase defective mutant copy of JAK2. When co-expressed in 293 cells JAK2 tyrosine phosphorylation levels are much lower than in control 293 cells not overexpressing *SOCS1*. However, phosphorylation at a key residue Y1007, is necessary to activate the JAK2 and needs to occur before SOCS1 can bind. This implies that upon activation of the JAK2, SOCS1 is able to binds to JAK2 and inhibit its autophosphorylation and subsequent phosphorylation of cytokine receptors, STAT molecules and their target genes. Therefore, it is through inhibition of the activity of the JAKs that SOCS1 confers a resistance to cytokines and negatively regulates their signaling cascades.

When JAK2 and STAT3 are co-expressed in 293 cells, STAT3 undergoes a rise in tyrosine phosphorylation. The same occurs when STAT5 is co-expressed with JAK2. If JAK2, SOCS1 and STAT3/STAT5 are all expressed together, no phosphorylation of STAT3 or STAT5 occurs. Furthermore, SOCS1 is capable of preventing phosphorylation of JAK1 and JAK3 in 293 cells as well, indicating SOCS1 is capable of regulating a broad spectrum of JAK/STAT signaling pathways other than just JAK2. SOCS1 is able to inhibit IL-2 and IL-3 activation of the *c-fos* promoter as well [34]. Since STATS are not required to activate c-fos but JAKS are, it demonstrates that SOCS1 directly targets the JAKs not the STATs themselves [34, 40].

The inhibitory mechanisms of SOCS1 seem to be restricted specifically to the JAK/STAT pathway. Whereas SOCS1 inhibits IL-2 and IL-3 activation of c-fos, it does nothing to prevent c-fos activation by cAMP (which follows a non cytokine activation

and does not involve the JAKs) [34]. When expressed in NIH3T3 cells SOCS1 does not alter tyrosine-phosphorylation of Erk2 and Shc in response to fibroblast growth factor (FGF). SOCS1 binds the kinase domain of c-kit in yeast cells but fails to inhibit c-kit or the epidermal growth factor receptor. This demonstrates that SOCS1 targets specifically the JAK tyrosine kinase activity and not tyrosine kinases in general.

SOCS1 and the immune system

SOCS1 plays a central role in the development of the immune system. SOCS1 knockout mice show many phenotypes, among them T-cell associated lymphoma and large scale infiltration of macrophages, lymphocyte and eosinophils [41-43]. These mice die very young due to overactive IFNγ signaling, the majority coming from T-cells [43]. They have been shown to suffer symptoms such as liver necrosis, myocarditis, polymyositis and fatty degeneration. SOCS1 has been shown to induce resistance to IFNγ when overexpressed [44]. In SOCS1 knockout mice lacking T-cells or containing a knock out of IFNγ, the same rates of young lymphocyte-dependent lethality are not seen as they are in normal SOCS1 -/- mice [43, 45]. Macrophages isolated from SOCS1 -/- mice require little IFNγ to become activated, as compared to normal wild type mice [46]. As well these mice show higher levels of natural killer T-cells (NKT), which are implicated in the necrosis of the liver [42].

Regulation of T cell development has been shown to be controlled directly by SOCS1. SOCS1 has been shown to be expressed in the thymus, especially during thymocyte development [33, 34, 37, 47, 48]. Expression of SOCS1 is critical for the

proper development of $CD4^+$ T cells [49]. Deficiencies of SOCS1 lead to increases in $CD8^+$ $CD4^-$ cells and $CD4^+CD8^+$ double positive cells, but leads to decreases in $CD4^+CD8^-$ cells [47, 49]. This is caused through increased levels in IL-7 signaling, which protects double positive cells, and IL-5 which stimulates the proliferation of $CD8^+$ $CD4^-$ cells, both cytokines are usually negatively regulated by SOCS1 [47, 50, 51]. The number of total T lymphocytes in SOCS1 deficient mice is also increased [52].

SOCS3 has also been shown to play a large role in regulation of macrophages and helper T-cells, placing the SOCS family of proteins in the middle of T-cell development and regulation of normal immune system development [53, 54].

Other immune cells such as dendritic cells are also under SOCS1 control. Dendritic cells deficient in SOCS1 are hypersensitive to interferon signaling and promote aberrant B-cell proliferation leading to the production of autoreactive T-cells [55, 56]. Silencing of SOCS1 can enhance dendritic cell response and antigen presentation, this is beneficial for anti-tumor responses by enhancing cytotoxic t-cell responses [55-57]

SOCS1 also helps in signaling within the innate immune system. Many foreign proteins such as v-E7 and bacterial LPS strongly induce SOCS1 [58, 59]. SOCS1 induction helps curtail the levels of inflammatory cytokines and chemokines secreted by activated antigen-presenting cells as well as negatively regulating LPS-induced macrophage activation. SOCS1 deficient mice have been shown to be unable to form a tolerance to LPS which can be lethal to them in certain cases [60]. Tumor necrosis factor alpha (TNF-α) is also regulated by SOCS1, giving SOCS1 another branch of control over inflammation responses, such as TNF-α induced apoptosis, and regulation of cells within the immune system [61].

Regulation of the immune system is therefore dependant on SOCS1 at many levels. SOCS1 plays a central role in the development of T-cells as well as their homeostasis. It also plays a role in reducing macrophage activation due to external stimuli and reduces dendritic cell activation and their ability to present antigens, thereby inhibiting autoimmune responses. As well SOCS1 directly controls the levels of many inflammatory signaling pathways such as TNF-α giving it direct control over inflammation and innate immune responses.

SOCS1 acts through its SH2 domain

SOCS1 acts through multiple mechanisms to inhibit the activity of JAKs. There are multiple lines of evidence supporting different mechanisms, which make use of the different structural domains of SOCS1. Such diversity of mechanisms may seem redundant but is perhaps necessary to ensure proper regulation of signaling cascades that control growth and proliferation as well as many components of the immune system. It is also known that the different SOCS family member act through different mechanisms from each other. For example CIS inhibits cytokine signaling in a mechanism different from that of SOCS1 and does not bind directly to the JAKs [36, 62].

One of the mechanisms through which SOCS1 exerts control over JAKs is through direct binding to the autophosphorylation site through interactions with the JH1 domain of JAK2 and the SH2 domain of SOCS1 [34, 63]. Once cytokines bind to their receptors they induce phosphorylation of JAK2 at Y1007, a critical step in JAK2 activation [64]. Subsequent downstream JAK2 signaling in turn induces SOCS1, which

regulates the pathway in a negative feedback manner. SOCS1 is able to bind to JAK2 phosphorylated at Y1007 with a high affinity but does not bind to unphosphorylated JAK2 or JAK2 mutants which contain mutations in the JH1 domain (K882D) [34, 63]. Mutational analysis of SOCS1 has revealed 3 regions necessary for complete binding and inhibition of JAK2 [63]. In order for SOCS1 to bind to Y1007 of JAK2 the SH2 domain, specifically a phospho-tyrosine-binding residue Arg 105, and a stretch of twelve amino acids (I68-G79) immediately N-terminal to the SH2 subdomain, dubbed the extended SH2 subdomain, are both required. A second set of twelve amino acids just N-terminal before the extended SH2 subdomain (N56-67) are required for SOCS1 to bind to JH1 with high affinity and are necessary to inhibit JAK2 signaling. This region has been named the kinase inhibitory region (KIR).

The extended SH2 subdomain contains three amino acid residues I68, L75 and G79, which are highly conserved throughout the SOCS family and appear at the same position relative to the SH2 domain of STATs as well. Similarity between the SH2 domains of SOCS and STAT family members may be important in SOCS1 inhibition of JAK/STAT signaling as perhaps SOCS1 could compete directly with STATs for binding sites with JAKs to reduce STAT signaling. Mutating these conserved residues in (I68E and L75E) is enough to prevent the interaction between SOCS1 and JH1 and Y1007 as well as reduce EPO-dependant STAT5 signaling. Crystal structures of STAT molecules show these conserved residues are involved in phospho-tyrosine binding in the SH2 domain [65].

The Kinase inhibitory region is not important to binding to Y1007 but is essential for inhibition of JAK2 signaling and mutations to the region greatly hamper JH1 binding

[63]. Mutations to individual amino acids in the region prevented SOCS1 from inhibiting EPO-dependent STAT5 signaling. Furthermore eight of the twelve amino acids are also present in SOCS3, which can also bind JH1 domains and inhibits the JAKs, further implying their importance in SOCS/JAK interactions [63, 66]. Among these conserved amino acids were F56, F59, D64 and Y65. Mutations to these residues in particular greatly hampered SOCS1 from binding to JH1. Mutations to F59 showed the greatest effect suggesting it is perhaps the more critical residue [63].

The current mechanism for SOCS1 interaction with JAK2 involves the SH2 of SOCS1 recognizing the phosphorylated Y1007 of JAK2. The classical SH2 domain is involved in binding to this region. The extended SH2 subdomain aids in further binding to JAK2. Binding to Y1007 allows the KIR to bind to the JH1 with high affinity and disrupt Jak2 signaling.

A 12 mer synthetic tyrosine kinase inhibitor peptide (Tkip) is able to mimic SOCS1 by also inhibiting JAK2 signaling through binding of the autophosphorylation site [67]. Tkip is able to bind to JAK2 at Y1007 with a higher affinity than SOCS1 and can bind to unphosphorylated JAK2 as well. Its binding to JAK2 is able to suppress IFN-γ activity, such as the upregulation of MHC Class I molecules, induction of growth arrest, and EGFR autophosphorylation. Tkip actually acts much like the KIR region of SOCS1 [68]. By acting in a mechanism very similar to that of SOCS1 Tkip is also able to inhibit constitutive STAT3 and IL-6 activated STAT3 in prostate cancer cells (LNCaP and DU145) [69]. Injecting mice with Tkip also reduces allergic responses by inhibiting overactive cytokine signaling [70].

SOCS1 as an E3 Ligase

Another mechanism commonly used to control cellular signaling is the degradation of cellular receptors or other proteins involved in the cascade. Targeting of specific proteins for degradation is a tightly controlled process that requires complexes of enzymes working in unison. One of the better known pathways to protein degradation is the ubiquitin-proteasome pathway, which uses long chains of ubiquitin molecules to mark proteins for degradation [71]. Degradation is a two-step process. The first step requires the target protein to be covalently flagged with a long chain of ubiquitin, usually accomplished by a complex of proteins. The second step is the actual degradation of the protein by the 26 proteasome. The actual attachment of ubiquitin to a target substrate is a three-step process and involves many proteins. The first is an E1 activating enzyme, which activates the ubiquitin molecules. Once activated an E2 ubiquitin carrier or ubiquitin conjugating enzyme (UBC), of which there are several, facilitates the transfer of the activated ubiquitin from the E1 to one of the E3 protein ligases. The substrate usually contains specific sequences to ensure proper and specific binding to the E3 ligase. The E3 ligase is responsible for completing the process and creating the covalent bond between the ubiquitin and the target protein. Usually, the first moiety is attached to a NH_2 group on a lysine residue, generating an isopeptide bond. Once the first moiety is attached the rest can attach to the proceeding moiety via its Lys48 residue. This allows the formation of a long poly-ubiquitin chain that serves as a flag, marking the protein for later degradation by the 26S proteasome or lysosome. The number of E3 ligases are unknown

as many more proteins and/or protein complexes that have E3 ligase activity are still being discovered.

Though the SH2 domain of SOCS1 is responsible for binding to Jak2, it alone is not enough to inhibit JAK signaling [34]. A mutant copy of SOCS1 lacking both C- and N- terminal domains also has no inhibitory affects on cytokine signaling demonstrated by its inability to inhibit *c-fos* activation by IL-2 or IL-3, which suggests that one or both of these domains is necessary for complete SOCS1 activity. The C-terminus of SOCS1, like all SOCS family members, contains a domain known as the SOCS box [33, 34, 37, 39, 72-76]. The SOCS box was once thought to be a unique domain to SOCS family members but has since been shown to be present in the C-terminal of a variety of different protein families including the Ras, WD-40 repeat, ankyrin repeat families and SPRY domain containing families [39, 77].

Within the SOCS box, there is a T/SL/MxxxC/SxxxV/L/I Elongin BC binding consensus sequence named the BC Box [77]. Elongin BC is a heterodimer protein complex made up of the ubiquitin like Elongin B, and Elongin C, a protein that resembles the adaptor protein Skp in sequence [78, 79]. Elongin BC was first shown to be an activator of the RNA Pol II elongation factor A (Elongin A) [80]. It has since been shown to also take part in the von Hippel–Lindau tumor suppressor complex (VHL) [81]. Mutations within, or deletions of the consensus sequence render Elongin A and VHL unable to bind Elongin BC [81-83]. Elongin BC is much more abundant in the cell than either Elongin A or VHL, which means it must have other activity or possible binding partners unrelated to the two [77]. It is now known that the Elongin BC complex can bind

not just SOCS1, but the BC box of all proteins containing a SOCS box and that deletions or point mutations of the BC box inhibit the interaction [76, 77].

Endogenous levels of Elongin B and C can be immunoprecipitated from the lysates of cells stimulated with interferon and IL-6 against antibodies for the C- and N-terminals of SOCS1, proving that this is a physiological interaction [77]. As well expressed Elongin B and C can be can be immunoprecipitated with antibodies against JAK2 and SOCS1 in cells co-expressing JAK2 and SOCS1. This Proves that Elongin BC is part of the SOCS1 complex that binds to JAK2 and inhibits its signaling. Only a form of SOCS1 with an intact BC box is capable of making the Elongin BC-JAK2 complex, although mutations in the BC Box do not disrupt SOCS1-JAK2 binding.

The VHL tumor suppressor acts as an active E3 ligase [84]. In this complex Elongin BC acts as an adaptor to link VHL to a Cullin and a RING finger-containing protein (Rbx). The cullins are a family of proteins that assemble with an Rbx molecule to form a module capable of catalyzing the transfer of ubiquitin from an E2 ubiquitin conjugating enzyme to the target substrate [85, 86]. There are currently five known cullin members in mammals (Cul1-5) [87]. The VHL protein acts as a subunit of a multi-protein complex able to recognize substrates for ubiquitination [84]. The VHL tumor suppressor shows similarity to the SKP-Cul1-F-box E3 ligases (SCF), and both show some similarity to an Elongin BC-Cul2-SOCS1 complex. SOCS1 also binds to Elongin BC, Elongin B is an ubiquitin like molecule, and Elongin C shares sequence similarity to the adaptor protein SKP1 [78, 79]. The F- box is a similar domain to the SOCS box. Studies into SOCS1 showed that it too was able to bind to a Cullin/Rbx module [88]. This Elongin B/C-Cul2-SOCS1 complex is capable of forming GST-polyubiquitin chains by the E2

GST-ubiquitin conjugating enzyme Ubc5 when ATP, E1 activating enzyme Uba1 and GST-ubiquitin^{K48R} are present, proving it acts as an active E3 ligase. The crystallized structures of a similar complex involving SOCS2 also defined an E3 ligase [75]. As well SOCS3 also forms such a complex, and upon activation with IL-6 sees its protein expression peak within 60 minutes only to be near depleted by 120 minutes [76]. This drop off in SOCS3 levels is abolished by treatment with proteosomal inhibitors such as LLnL. This suggests that SOCS proteins form active E3 ligases that play a role in their subsequent degradation after induction.

The specificity of the cullin binding is said to be due to a small stretch of amino acids located at the C-terminal of the SOCS box known as the Cul box [89]. Differences in the Cul box between VHL and SOCS family members lead VHL to specifically bind to endogenous Cul2-Rbx1 while SOCS-box proteins bind to endogenous Cul5-Rbx2. These differences allow VHL and SOCS family members to be grouped accordingly according to their Cul Box. VHL has a Cul2 box while the members of the SOCS family of proteins contain a Cul5 box; they bind to endogenous Cul5-Rbx2 and not Cul2-Rbx1.

A specific conserved amino acid sequence of LPxP within the Cul5 box appears necessary for Cul5 binding as mutating it abolishes it. While all SOCS members have a Cul5 box, SOCS1 oddly has an incompletely conserved Cul5 box. The Cul box of SOCS1 contains the amino acid sequence lPLN instead of LPxP; this leads SOCS1 to bind to Cul2-Rbx1, not Cul5-Rbx2, making it an exception within the SOCS family as the only member to do so. Changes in Cul5 expression cause no change in the degradation of VHL target substrate HIF-2a, however reducing Cul2 expression by usage of shRNA cause increases of HIF-2a. This suggests that each Cul member has a distinct

purpose or possibly has the ability to recognize different substrates. If this is the case there could be some unique function conferred upon SOCS1 as the only SOCS member that binds to Cul2.

There is plenty of evidence to suggest that the mechanism by which SOCS proteins inhibit cytokines lies in their ability to form ECS E3 ligases. SOCS1 localizes to the microtubule organizing complex that is associated with the 20 S proteasome itself in a manner dependent on the SH2 domain [90]. SOCS1 also binds directly to the microtubule organizing complex (MTOC). Such a direct link to the proteasome suggests SOCS1 may help localize targets to the proteasome for degradation. SOCS1 does in fact cause JAK1 and VAV1 to localize to the MTOC and MTOC-associated 20S proteasome.

Many studies have suggested that SOCS1 directly targets proteins for degradation. SOCS1 was shown to bind to the N- terminus of the guanine nucleotide exchange factor VAV, and reduce the formation of VAV-induced foci in NIH 3T3 cells [91]. SOCS1 also diminished the levels of VAV within NIH 3T3 and COS cell lines, and was ultimately found to induce ubiquitination of VAV and onco-VAV. IFN-γ induces expression of SOCS1 and leads to a degradation of the viral oncogene E7 in HeLa and CaSki tumor lines [59]. E7 mRNA levels remain the same when SOCS1 alone is expressed in these cells though E7 protein levels are diminished. SOCS1 co-localizes with E7 in the nucleus and can immunopreciptate with E7, suggesting a physical interaction. This interaction is dependent on the SOCS Box, but is uninterrupted by mutations of the SH2 domain. Like the interaction of SOCS1 and VAV, tyrosine phosphorylation does not seem to play a role. SOCS1 is also seen to promote ubiquitination of the viral oncogene E7 upon interaction and inhibits HPV-E7 mediated

transformation by leading to E7 degradation. JAK kinases such as JAK2 are sometimes involved in oncogenic fusions, such as the fusion protein TEL-JAK2 [74]. In Ba/F3 cells transformed with TEL-JAK2 expression of SOCS1 or induction of SOCS1 by treatment of IL-3 leads to apoptosis. This response is not seen however in Ba/F3 cells transformed with p210 Bcr-Abl, showing SOCS1 activity to be specific to JAKs. SOCS1 mutants lacking the SOCS box cannot suppress the growth of TEL-JAK2 cells, despite the SOCS box not being necessary to bind to JAK2, suggesting that the SOCS box still plays a role in inhibition of JAKs [74, 92]. In 293 cells full length, WT SOCS1 can reduce the levels of TEL-JAK2 and suppress TEL-JAK2 activation of STATs. Treatment of proteasome inhibitors MG132 and lactacystin protected TEL-JAK2 from SOCS1 induced degradation, suggesting it is proteasome dependent. Further studies showed that phosphorylation of the JH1 domain led to binding with SOCS1 and led to degradation of JAK2 preceded by SOCS1 induced ubiquitination of TEL-JAK2. The SH2 domain of SOCS1 is necessary for degradation of TEL-JAK2. The SOCS box domain can be replaced with that of the protein CIS, but not the SOCS3 SOCS box. SOCS3 binds and inhibits TEL-JAK2, but does not induce its degradation, this is odd as both SOCS3 and CIS contain a different Cul5 box from SOCS1 [66, 89]. A dominant negative Cul-2 inhibits the Tel-JAK2 degradation, suggesting SOCS1 induced degradation must work in an E3 ligase manner.

SOCS1 also acts in the insulin signaling pathway and degrades insulin receptor substrates (IRS). In HEK293 cells, SOCS1 associates with IRS1 and IRS2 via interactions that are significantly increased in response to insulin signaling. SOCS2 and SOCS3 were also found to interact with IRS1 and IRS2. Interactions between SOCS1 and

IRS leads to subsequent falls in the levels of IRS1 and IRS2 in HEK293, MCF7 breast cancer cells and ETE-L1 adipocytes. Induction of SOCS1 by treatment with proinflammatory cytokines such as IL-6, TNFα, and IFN-γ also led to reductions in levels of IRS1 and IRS2. Expression of SOCS1 in the liver of mice via adenovirus infection causes IRS1 and IRS2 levels to fall until the infection is no longer detectable at which point IRS levels return to normal. A SOCS1 mutant missing the SOCS box is unable to regulate the same control over IRS1/2 as can wild type SOCS1. Mutations to key residues of the BC box cause a decrease in degradation of IRS but do not prevent SOCS1 binding. This led Rui et al. to search for ubiquitination of IRS1/2. They found that SOCS1 was indeed able to lead to ubiquitination of IRS1/2, although this could be prevented with treatment of MG132 or lactcystin.

Colony formation induced by the granulocyte-colony stimulating factor, G-CSF, as well as its activation of STAT3 and STAT5, are also suppressed by SOCS1 and SOCS3. However, both ΔSOCS Box mutants of SOCS1 and SOCS3 fail to cause any changes to colony formation or STAT activation in response to G-CSF [94]. This also suggests a possible role of SOCS box mediated degradation of G-CSF or its downstream targets as well. Stabilization of phospho-STAT5 by proteasomal inhibitors supports this model. The Apoptosis signal-regulating kinase1 (ASK1) is also a target of SOCS1 induced degradation [95]. ASK1 binds to SOCS1 through its SH2 domain and is both ubiquitinated and degraded. It is known that TNF signaling stabilizes ASK1 levels and prevents degradation while SOCS1 acts as a negative regulator of TNF and promotes ASK1 degradation.

These results have shown two powerful properties of SOCS1. The first is to act on target substrates by forming an E3 ligase with Elongin BC and a Cul-Rbx subunit in the SOCS box domain [76, 77, 88, 89]. It suggests the SH2 domain is used to recognize substrates which it than marks for proteosomal dependent degradation [59, 74, 91, 96]. Localization with the MTOC associated with the 20S proteasome only strengthens this argument [90]. It also shows another important property of SOCS1, the property of a tumor suppressor. It has already been established that SOCS1 inhibits the JAK, a family of tyrosine kinases. Receptor tyrosine kinases are implicated in many cancers as mutations which confer constitutive activity to them cause an over stimulation in many signaling cascades that promote growth and proliferation. However the results mentioned above also show SOCS1's ability to directly target other oncogenes such as EP7, onco-VAV and the TEL-JAK2 fusion protein and directly lead to their destruction by targeting them for proteosomal degradation.

SOCS1 in Cancer

Receptor tyrosine kinases have been identified as being proto-oncogenes. The JAKs as well can serve as oncogenes should their signaling go astray, or should they become involved in fusion proteins. SOCS1 meanwhile demonstrates tumor suppressor properties [97]. Tumor suppressors are often inactivated in a variety of cancers either through gene silencing or mutations that encode a non-functioning protein. SOCS1 can actually prevent transformation of Ba/F3 cell lines caused by Tel-JAK2 by inhibiting JAK2 and leading to its degradation [92]. Gene methylation of CpG islands and other

epigenetic modifications are seen in a variety of cancers, this often leads to silencing of tumor suppressor genes. *SOCS1* methylation was first demonstrated in HCC cell lines (65%) [98]. Several groups have since repeated similar studies showing a high level of SOCS1 methylation in HCC, corresponding to a low level of expression and accompanied with constitutive JAK2 activation [99-102]. Since then it has been shown that *SOCS1* is methylated in a variety of cancers. In 2003, it was shown that over half the patients sampled (53%) with newly diagnosed acute myeloid leukemia (AML) showed methylation of the promoter region of *SOCS1* [103]. Multiple myeloma cell lines such as the IL-6 dependant XG1 and U266 lines show a low level of *SOCS1* expression even in the presence of IL-6 compared to control lines [104]. These cell lines showed high levels of STAT3 phosphorylation and a higher sensitivity to a chemical JAK inhibitor, AG490, which induced apoptosis. Samples taken from primary tumors of multiple myeloma also confirmed the results shown in cell culture as one study showed 62.9% of patients sampled to have *SOCS1* methylation. This is also confirmed by the fact that SOCS1 deficient mice die within 3-4 days due to a lymphoma [41, 42]. Many human pancreatic cancers have also shown reductions in expression of *SOCS1* [105]. This usually correlated with a methylation of the 5' promoter region for *SOCS1*. While one group showed methylation of the *SOCS1* promoter region in tumor samples, they found no mutations to markers within *SOCS1*. Breast and ovarian cancer displayed *SOCS1* methylation as well but showed a pattern of differential hypermethylation between other members of the SOCS family [106]. *SOCS1* was found to be hypermethylated in 4 out of 6 studied ovarian cancer cell lines and 8 out of 11 breast cancer lines. When actual tumors were studied, 23% of ovarian cancers had hypermethylated *SOCS1* CpG islands,

while only 9% of the breast cancers showed the same result. Cells from the colorectal cancer line Hep3B, which have methylated DNA, also do not express *SOCS1* [107]. Also, SOCS1 deficient mice showed a higher rate of developing tumors within their colon due to over inflammation in response to uninhibited IFN-γ/Stat1 signaling [108]. Other groups have shown that SOCS1 hypermethylation and subsequent loss of SOCS1 expression confers growth-promoting effects in pancreatic cancers such as pancreatic ductal adenocarcinomas and intraductal papillary mucinous neoplasms [109]. Silencing of *SOCS1* can occur due to loss of heterozygosity (LOH) and can lead to an increase of carcinogenesis in mice livers [110]. Hypermethylation of *SOCS1* in all these cancers leads to a large reduction in expression, this effectively allows for constitutive JAK/STAT signaling. Constitutive signaling of both the Janus kinases and STAT molecules has been largely linked to the formation of cancers in the past [111]. Silencing of *SOCS1* in these tumors can often be reversed by introducing agents that reverse DNA methylation, such as 5-azadeoxycytidine (5-aza-dC), mimic SOCS1 activity (Tkip), or directly inhibit the JAKs [69, 104, 106, 112]. Restoration of *SOCS1* expression or overexpression of *SOCS1* in these cancers often leads to growth inhibition or induction apoptosis. *SOCS1* mutations have been found in a significant number of tumors from Hodgkin, Reed-Sternberg, Hodgkin and classical Hodgkin tumors as well as in primary mediastinal B-cell lymphomas [113]. Mutations can range from out of frame mutations which lead to premature stop codons cutting off the SOCS box domain, to deletions in amino acids of the SH2 domain. These mutations lead to loss of function of *SOCS1* and allow JAK2 signaling to persist, eventually resulting in an accumulation of active phospho-STAT5 in the nucleus. In primary mediastinal B-cell lymphoma (PMBL) cell

lines, MedB-1 and Karpas1106P, biallelic mutations to the coding region of SOCS1 allow JAK2/STAT5 signaling to go unchecked [114, 115]. In this cancer, JAK2 remains constitutively active although it shows only a normal physiological level of expression. Instead JAK2 degradation is greatly impaired, hence the JAK2/STAT5 signaling cascade is allowed to persist. Overexpressing SOCS1 in MedB-1 cell lines with this mutation leads to growth inhibition [114].

Our lab focuses on the study of premature senescence and growth arrest. Premature senescence is a permanent cell cycle withdrawl that can act as a tumor suppressor mechanism [116-118]. Senescence can be induced in response to oncogenic stress as a barrier to transformation. Previously, our lab demonstrated that a constitutively activated form of STAT5 (STAT5A1*6) can induce premature senescence [119]. *SOCS1* is highly upregulated in STAT5 senescent cells, implicating its involvement in senescence. Other SOCS1 activators such as β-IFN can also induce senescence [120]. Members of our lab have demonstrated that overexpression of *SOCS1* in the IMR90 fibroblast line induces premature senescence. It is established that SOCS1 is able to induce a growth arrest in cells. However, the mechanisms by which it acts are completely unknown. SOCS1 can induce *p53* and SOCS1 induced senescence in the IMR90 cell line was shown to be p53 dependent. SOCS1 has the ability to bind to and inhibit JAKs and can form E3 ligases. Whether either of these functions is necessary for the induction of senescence or growth arrest is unknown. Also, the role of the different domains of SOCS1 is unknown at this time.

Regulation of SOCS1

The mechanisms by which SOCS1 is regulated are not well known. While many different stimuli for SOCS1 induction are well known and documented, the negative regulation of SOCS1 remains for the most part a mystery. It is known that SOCS1 can be targeted by other SOCS family proteins for degradation [121]. SOCS2 has been shown to have the ability to bind and inhibit SOCS1 by targeting it for proteosomal degradation. Both SOCS6 and SOCS7 were later shown to have the ability to interact with all other SOCS molecules. Other reports suggest that SOCS1 leads to its own auto degradation and that binding to the machinery of its E3 ligase such as the Elongin B/C complex lead to reductions in SOCS1 protein stability [76, 122]. However other reports have shown it is necessary to have binding of SOCS1 to the Elongin B/C to promote stability and that interruptions in binding lead to proteasomal degradation [77, 123, 124]. The interconnecting web of a family of SOCS proteins, each capable of forming active E3 ligases acting on one another and each with the ability to potentially self-regulate makes the exact mechanisms of regulation by means of how SOCS1 is regulated by proteasomal degradation hard to elucidate, especially considering how little is known about the majority of the other SOCS family members. Other groups have pointed to protein kinases that may regulate SOCS1. The Pim family of protein kinases was shown to phosphorylate SOCS1 which leads to stabilization of protein levels [122]. It is possible that SOCS1 undergoes other modifications which are used to regulate it at the protein level, including phosphorylation by other kinases, however as of yet these mechanisms remain mostly unknown.

Materials and methods:

Cell culture

All IMR90 primary cell lines and U2OS sarcoma cell lines were cultured in Dulbecco's Modified Eagle Media (DMEM) (GIBCO) supplemented with 10 % FBS (GIBCO) and 1% Penicillin G-streptomycin sulfate (GIBCO).

Retroviral vectors and gene transfer

The following retroviral vectors were used, pLPC and its derivatives expressing *SOCS1, SOCS3, SOCS5, SOCS6, SOCS1 ΔBOX, SOCS1 ΔBC, SOCS1 ΔCul, SOCS1 ΔN198* and *SOCS1 N198P* as well as the retroviral vector in pBABE and its derivatives *hRAS* and *STAT5A1*6*. Phoenix cells were plated to a density of $2.5x10^4$ cells / ml in a 10 mm culture plate (Corning) (10 ml total volume) and transfected with retroviral vectors (20 μg) and treated with 200 μl of 5 mg/ml sodium butyrate the next day. Medium was changed 12 hours after sodium butyrate addition. 12 hours after the medium was changed and retroviral soups were collected and supplemented with 10% FBS and 4 mg/ml polybrene. The supplemented soups were immediately added to primary cells which had been previously plated to a density of 8.0×10^5 cells / ml in 10 cm culture plates (Corning). Cells were treated with new retroviral soups every 6 hours for a total of 3 infections. Infected cells were selected in puromycin 2.5 μg/ml (Bioshop) for 2 days.

SA β-Gal activity

IMR90 cells were infected as described above. Cells were given 3 days recovery period after selection and plated at a density of 2.5 $x10^5$ cells / ml 6 days post selection. Cells were than incubated in 5-bromo-4-chloro-3-indolyl-β-D-galctopyranoside (X-Gal) at pH 6.2 at 37°C until the negative control showed approximately 10% positive staining. The percentage of cells expressing SA-β-Gal was quantified by inspecting 100 cells per 10 mm plate three times.

Immunofluorescence microscopy

IMR90 cells were plated on cover slips in a 6-well plate at a density of $2.5x10^4$ cells / ml and fixed using 4% paraformaldehyde (Fisher Scientific) in PBS for 15 minutes at room temperature. Cells were washed several times in PBS and permeabilized using 0.2% Triton X-100 (Fisher Scientific) in PBS with 3% bovine serum albumin (BSA) on ice for 5 minutes. Cells were than washed again several times with PBS/BSA and than treated with the primary antibody, anti-PML (Rabbit) prepared by Marie-France Gaumont-Leclerc for 1 hour at room temperature in a humidified chamber. After three more PBS/BSA washings cells were stained with Alexa-Flora Red, anti-rabbit secondary antibody (1:2000) (Molecular Probes) for 1 hour in a humidified chamber. Cells were than washed several times with PBS/BSA and counterstained with 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.1 μg/ml in PBS/BSA. Fluorescence microscopy was performed using an inverse fluorescence microscope (Nikon TE2000) and the Metamorph software (Molecular Devices). Images were prepared using Metamorph and Canvas X (Deneba).

SOCS1 mutagenesis

The *SOCS1 ΔBox* construct had already been prepared by Vivianne Calabresse. The rest of the *SOCS1* mutants were made using PCR techniques. All reactions were performed in a Biometra T-gradient PCR Machine. Reactions were performed in 100 µl total volume using 50 ng of pLPC SOCS1 as a template, 200 µm dNTP, 50 µM of both primers, and 5% DMSO and Deep Vent Polymerase (New England Biolabs) and it's Thermopol Buffer (New England Biolabs). *SOCS1ΔCul* was created using a PCR using a sense primer (referred to as SOCS1 sense primer) of *5'GCGAATTCTGATGGTAGCACGCAACCAGGTG3'* and an antisense primer *5'GCGGGCTCGAGTCAGTTCTCGCGACCCACGGC3'* that introduced a premature stop codon before the Cul Box.

SOCS ΔBC was created using a two step process. First two small fragments were made. Fragment A was created using the SOCS1 sense primer as used for *SOCS1ΔCul* and an anti-sense fragment that started at the beginning of the BC Box and contained a 15 bp overlap for fragment B, *5'ACCCACGGCGGC CACCCGCACGCGGCGCTG3'*. Fragment B was created using a sense primer that had a 15 bp overlap to the tail of Fragment A and started after the BC Box, *5'CAGCGCCGCGTGCGGGTGGCCGCCGTGGGT3'* and a normal antisense primer for *SOCS1, 5'CGCTCGAGTTCAGATCTGGAAGGGGAAGGA3'*. The two fragments were than used as a template for a reaction using the normal SOCS1 sense and antisense primers.

SOCS1ΔN198 and *SOCS1 N198P* were made in a similar fashion. First a fragment was made using the aforementioned SOCS1 sense primer and an antisense primer

introducing a deletion of the 198P residue, *5'GTCACGGAGTACCGGAAGAGG GATGCGCGC3'* this fragment was not full length *SOCS1*. A second fragment was made using a forward primer that contained a 15 bp overlap to the first fragment and introduced a sense strand deletion mutation to the 198 P residue, *5'GCGCGCATCCCTCTTCCGGTACTCCGTGAC3'* and an antisense primer that started after the SOCS1 insert in the pLPC vector back bone, to produce a larger insert than 100 bp to facilitate cloning, *5'CAGCTG TTCCATCTGTTCTTGGGC3'*. The two fragments were than used as template for a PCR reaction using the normal SOCS1 sense primer and a second SOCS1 antisense primer (as the first did not work well with this reaction) *5'GGGCCTCGAGTCAGATCTGGA AGGGGAAGGA3'* to give a full length SOCS1 product with the ΔN198 mutation. The N198P mutant was made in similar fashion using the SOCS1 sense primer and a N198P antisense primer *5'GTCACGGAGTACCGGGGGAAGAGGGATGCGCGC3'*. Fragment B was generated using a sense primer of *5'GCGCGCATCCCTCTTCCCCCGGTACTCCGTGAC3'* and the pLPC antisense primer mentioned above. All mutants were sequenced at l'Institut de Recherche en Immunologie et en Cancérologie (IRIC) to make sure mutants were in the right places and that no other errors were introduced by the PCR process.

Colony assays

U2OS cells were transfected with 15 μg of DNA using the calcium phosphate method. Transfected cells were selected in puromycin 1μg / ml for 6 days. After which cells were given a 2 day recovery period for colonies to grow. Cells were then stained in 0.5 % Crystal violet to show colony formation. Cells were de-stained using 10% Acetic acid.

100 µl of each sample was loaded to a 96 well plate and measured for absorbance at the 960 nm wavelength in a plate reader. Values were plotted on a bar graph and normalized to the control to show relative differences in growth. Standard error represents 1 standard deviation from the mean value.

Protein analysis

Immunoblots were preformed using whole-cell lysates obtained by first making cell pellets and than boiling them in Laemmli sample buffer. Samples of 20 µg of protein were resolved in SDS-polyacrylamide gel electrophoresis and transferred using the wet-transfer method to Immobilion-P membranes (Millipore). Antibodies used in the immunoblots include anti-SOCS1 (4H1; 1:1000 Upstate), anti-p53 (catalog number 9282 1:1000 Cell Signaling Technology), anti-PS-15p53 (catalog number 9284 1:1000 Cell Signaling Technology), anti-p21 (catalog number 2949 1:1000 Cell Signaling Technology) , anti-RB (C-19, 1:250 Santa Cruz Biotechnology), anti-Mdm2 (2A10; 1:250; donated by A. Levine), anti-Tubulin (B-5-1-2; 1:2,000; Sigma). Western blot assays were performed using ECL detection (Amersham) or Lumilight detection system (Roche Applied Science).

Results:

We were interested in exploring the full extent and mechanisms of SOCS1-mediated growth arrest. First, we explored SOCS1 growth arrest in primary cells. When infected into IMR90 fibroblasts SOCS1 induces a permanent cell cycle arrest known as premature senescence. Senescent SOCS1 cells show a larger, flatter morphology and stain positive for the senescence associated β-galactosidase akin to oncogenes such as RAS and STAT5A1*6 (Figure 1A) [125]. As well these cells show accumulation of PML bodies within the nucleus (Figure 1B). Accumulation of PML bodies in the nucleus is often used as a marker of senescence [125].

Since premature senescence is a tumor suppressor program it was interesting to see the effects of SOCS1 on the growth of cancer cells directly. The sarcoma cell line U2OS was transfected with SOCS1 and an empty vector. As shown in Figure 2 *SOCS1* expression abolishes the ability of U2OS cells to form colonies and greatly hinders their growth. Hence SOCS1 is also able to lead growth arrest within tumor cell lines as well as inducing permanent cell cycle arrest in primary cells.

Members of the SOCS family of proteins exist in similar pairs, SOCS1 and SOCS3 for example are more similar to each other than to other family members [38]. Some other SOCS members such as SOCS3 have also been demonstrated to play roles within a variety of cancers as well [106]. We wanted to see the effects of other SOCS family members on growth arrest so we repeated the colony assay in U2OS using SOCS3, SOCS5, CIS4/SOCS6 as well. As demonstrated above *SOCS1* completely

abolishes the ability of U2OS cells to form colonies as the SOCS1 transfected cells didn't form colonies as did the control plate (Figure 3B).

Although very similar in sequence to *SOCS1*, *SOCS3* does not inhibit colony formation in the U2OS cell line. Though the number of relative colonies is slightly lower than that of the control plate, it is significantly higher than that of the *SOCS1* plate (Figure 3B). Surprisingly, *SOCS5* over-expression actually causes a near two fold increase in the number of colonies formed (Figure 3B). This result was repeatable and suggests *SOCS5* may actually play the role of an oncogene. The full role of SOCS5 is largely unknown though it may function by actually interacting with the activity of other SOCS molecules, as some crosstalk and interference between SOCS family members has been previously shown. [121]. *SOCS6* was the only other member tested that showed the ability to prevent colony formation that was significantly similar to *SOCS1* (Figure 3A & 3B). When tested in primary cells however, *SOCS6* is unable to induce premature senescence (Figure 4). This suggests that SOCS6 maybe capable of inducing growth arrest, but unable to induce a permanent cell cycle arrest such as SOCS1 through induction of the senescence program.SOCS2 and SOCS7 as well as CIS constructs were unavailable at this time.

Many roles of SOCS1 are dependent on its SOCS box domain and its ability to form an active E3 ligase [73, 74, 91, 93]. E3 ligase activity of SOCS1 requires an intact SOCS box to assemble the E3 ligase machinery. We wanted to investigate whether SOCS1 induced growth arrest was dependent on the SOCS Box as well. We made a series of SOCS box mutant lacking both the BC and Cul box subdomains, labelled as *SOCS1 ΔBC* and *SOCS1 ΔCul* respectively. As well a SOCS construct lacking the entire

SOCS box, *SOCS1 ΔBox,* was donated to our lab and sub-cloned into a pLPC vector. These mutants were then transfected into U2OS cells (Figure 5).

Deletions of the entire SOCS box have only minor effects on SOCS1 induced growth arrest in U2OS (Figure 5). This points to a possible role of SOCS1 in growth arrest that independent of its SOCS box and its E3 ligase activity. SOCS1 constructs containing deletions of the BC and Cul box also show little effect on growth arrest (Figure 5). However these mutants provide no real information to this regard as the levels of SOCS1 in the cells are poorly expressed and cannot be detected through western blots (Figure 6A).

Some studies have shown SOCS1 can target itself for auto degradation or that binding to the Elongin BC complex is necessary for stability of SOCS1 [77, 123, 124]. To see if this is the case with our SOCS mutants we treated transfected U2OS cells with MG132, a proteasome inhibitor, before collecting extracts. In these cells SOCS1 levels are greatly enhanced compared to that of untreated cells (Figure 6B). This indicates that SOCS1 lacking functional BC box and Cul box domains are targeted for proteasomal degradation whereas it appears SOCS1 constructs lacking the entire SOCS box domain are not (Figure 6A & B). *SOCS1 ΔBC* and *SOCS1 ΔCUL* mutants still retain parts of the SOCS box and possibly still able to present epitopes or potential binding sites for other E3 ligases (such as other SOCS molecules) to recognize them and mark them for degradation (Figure 7A). The *SOCS1 Δbox* mutant lacks the entire SOCS box and may present no epitope and could be therefore protected from degradation (Figure 7B).

It had previously been demonstrated in our lab that SOCS1 induces the p53 pathway in fibroblasts and that SOCS1 senescence was p53 dependent (Malette et. al

Unpublished). We wanted to see if SOCS1 regulated p53 in a similar manner in U2OS and to see if the SOCS box mutants had any effect on p53 activity. As was shown by other members of our lab in primary cells, *SOCS1* expression does lead to an induction of p53 (Figure 6A). As well SOCS1 expression lead to an increase in the level of phosphorylation at Serine 15 on p53 and an increase in the levels of p21 (Figure 6A). These results show activation of the p53 signaling pathway by SOCS1 in U2OS. The activation of p53 by SOCS1 seems to be a general part of its activity in inducing growth arrest. The Rb pathway showed no activation by SOCS1 indicating that *SOCS1* growth arrest though p53 dependant, does not seem to engage the Rb pathway (Figure 6A).

The transcription factor p53 is regulated by many E3 ligases, including MDM2, COP1 and Pirh2 [126]. One possible mechanism by which p53 levels could be stabilized and increased is by degradation or inhibition of MDM2, a negative regulator of p53. SOCS1 does not lead to any changes in MDM2 levels meaning there must be another mechanism by which *SOCS1* expression leads to increases in p53 levels (Figure 6A). The effects of SOCS1 on COP1 and Pirh2, other E3 ligases that regulate p53 were not tested.

The *SOCS1* mutants did not show any increases in p53, p53 ser15 or p21 (Figure 6A). However as mentioned before the *SOCS1 ΔBC* and *SOCS1 ΔCUL* mutants are poorly expressed thus we cannot interpret anything from their results (Figure 6A). The *SOCS1 ΔBOX* is relatively stable however its failure to activate the p53 pathway suggests the SOCS box or subdomains within the SOCS box could be required for p53 activation, though not necessarily in a manner dependent on E3 ligase activity.

Since large domain mutations of SOCS1 lead to instability of SOCS1 we tried a different approach to determine the role of the SOCS box in SOCS1 induced growth

arrest. Kamura et al. had previously shown that SOCS1 shares a unique sequence in its Cul box that allows to it to bind to Cul2/Rbx1 whereas all other SOCS family members bind to Cul5/Rbx2 complex (Figure 8). Specifically SOCS1 has an Asparagine residue at position 198, whereas all other SOCS proteins have a proline. Using site directed mutagenesis we created two more SOCS1 mutants, *SOCS1 N198P* and *SOCS1 ΔN198*, containing specific mutations to this sequence. These mutants change the IPLN sequence of SOCS1 to IPLP, which resembles the LPxP consensus site for Cul5 binding. Therefore these mutants should allow SOCS1 to bind to Cul52/Rbx2 like all other members of the SOCS family, thus altering its usual E3 ligase activity.

We again used the colony assay in U2OS to study the effects of these mutants on cell growth. As seen in Figure 9 both mutants abolished the usual growth inhibition of SOCS1 (Figure 9A). While cells transfected with both of the Cul box mutants resembled those transfected with the control vector, the SOCS1 ΔN198 construct had the largest effect with a relative growth of 1.56 times that of the control (Figure 9B). The SOCS1 N198P mutant showed slightly less growth relative to the control (0.76) but much more than SOCS1 (Figure 9B).

Figure 1 SOCS1-induced senescence in the fibroblast line IMR90. **(a)** Cells were infected with a control vector pLPC, pLPC SOCS1, or two positive controls pBABE STAT5A1*6 and pBABE RAS. Cells were fixed and stained 6 days post selection for senescence associated β-galactosidase, (SA-β-gal). Differences in morphology of cells can be seen. Percentages of cells that stained positive after 2 counts of 100 cells in 2 separate plates are labeled. **(b)** Immunodetection of PML bodies: cells were infected with a control vector, pLPC, pLPC SOCS1 or the positive control pBABE STAT5A1*6. Cells were plated to a density of $2.5x10^4$ cells / plate in a 6 well plate at 6 days post selection and fixed the day after. PML was detected using an anti-PML antibody manufactured in our lab. Nuclei were counterstained with DAPI.

Figure 1

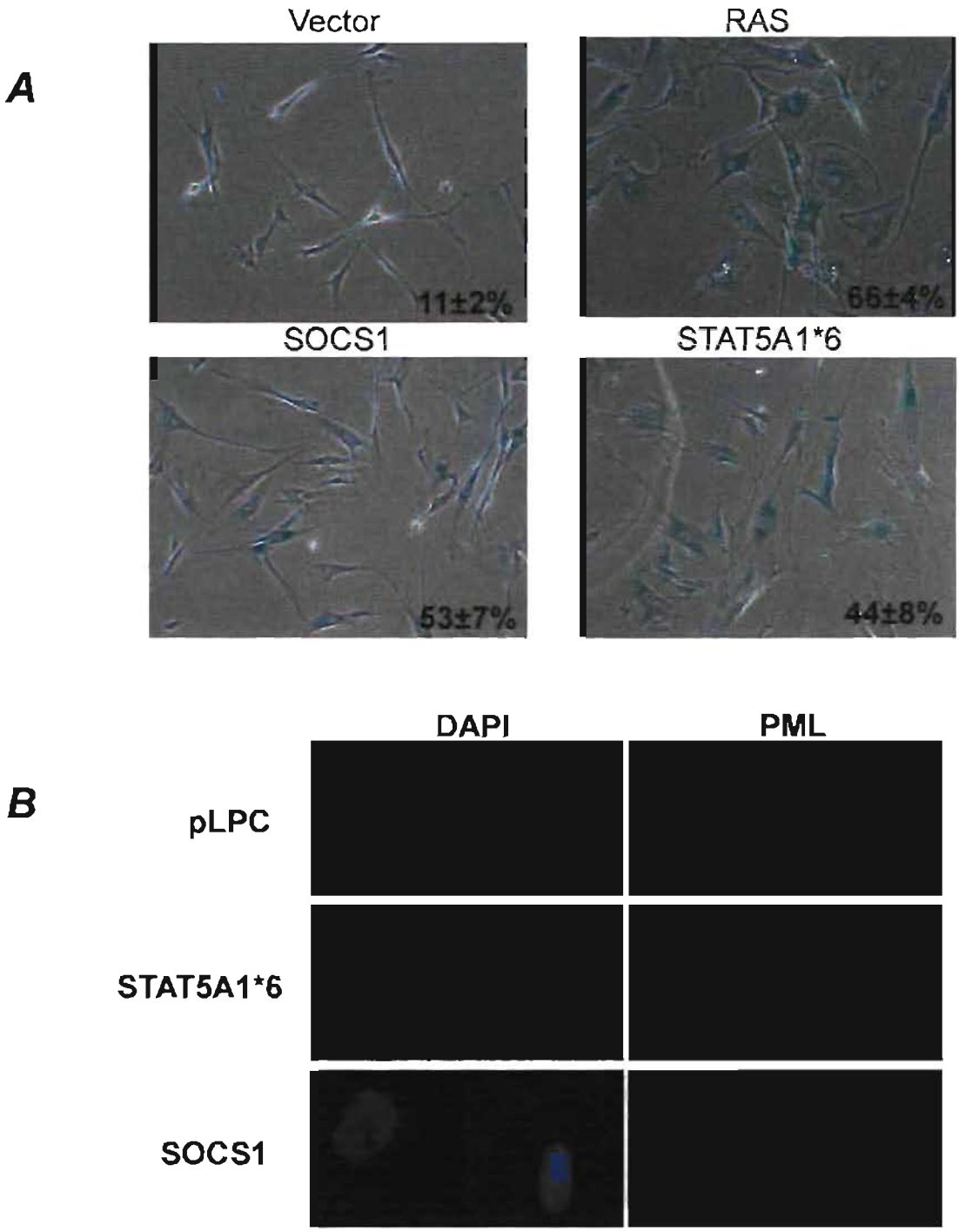
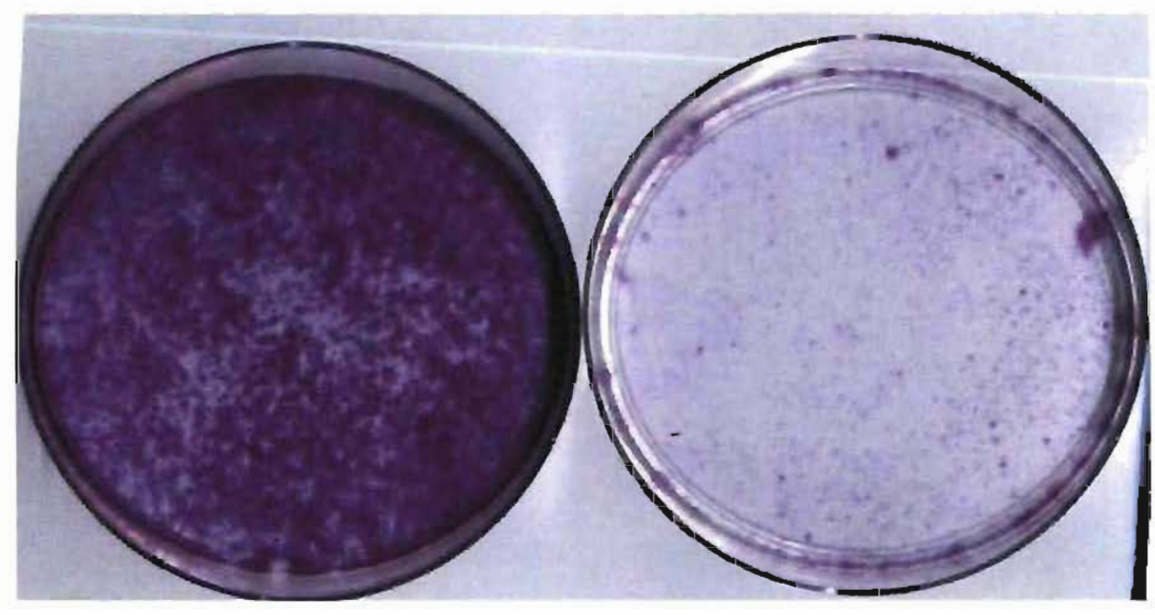

A
Vector
RAS
11±2%
66±4%
SOCS1
STAT5A1*6
53±7%
44±8%
B
DAPI
PML
pLPC
STAT5A1*6
SOCS1

Figure 2 SOCS1 growth arrest in U2OS cell line. Cells were transfected with a control vector pLPC or its derivative pLPC SOCS1. After transfection cells were selected for 6 days with puromycin followed by a 2 day recovery period for colonies to grow. Colonies were stained with 0.5% Crystal violet solution.

Figure 2

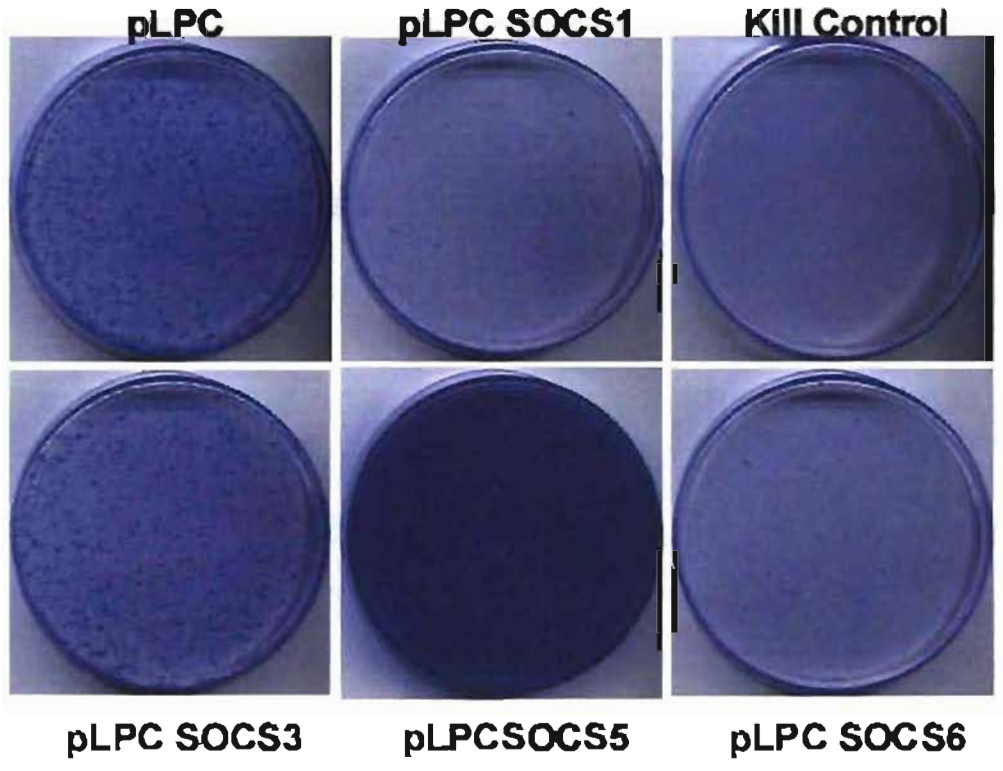


pLPC pLPCSOCS-1

Figure 3 SOCS1 growth arrest in U2OS cell line. **(a)** Cells were transfected with a control vector pLPC or derivatives containing SOCS family members. After transfection, cells were selected for 6 days with puromycin followed by a 2 day recovery period for colonies to grow. Colonies were stained with 0.5% Crystal violet solution. Untransfected cells are demonstrated as a negative killing control. **(b)** Relative growth of cells when normalized to control vector, pLPC. Cells stained with crystal violet were destained with 10% glacial acetic acid. 2 100 ul volumes from each plate were transferred to a 96 well plate and measured for absorbance at 600 nm. Averages are obtained from 2 counts per plate from two plates of each condition.

Figure 3

A


pLPC
pLPC SOCS1
Kill Control
pLPC SOCS3
pLPCSOCS5
pLPC SOCS6

B

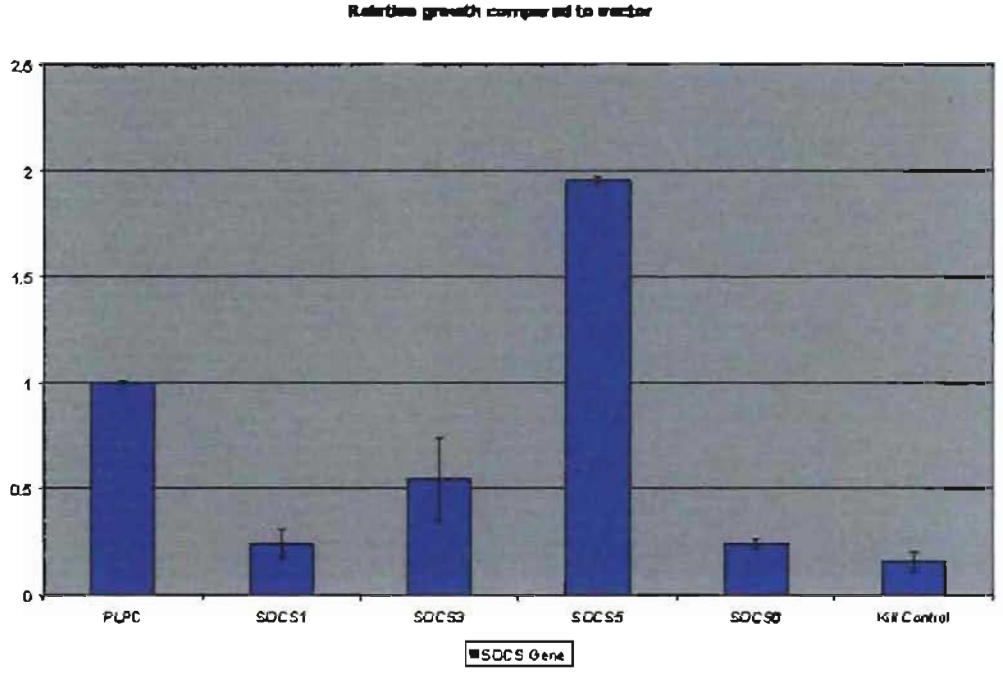

2.5
2
1.5
1
0.5
0
pLPC
SOCS1
SOCS3
SOCS5
SOCS6
Kill Control
SOCS Gene

Figure 4 SOCS6 fails to induce senescence in the fibroblast line IMR90. Cells were infected with a control vector pLPC, pLPC SOCS1, pLPC SOCS6 or the positive control pBABE RAS. Cells were fixed and stained 6 days post selection for senescence associated β-galactosidase, (SA-β-gal). Differences in morphology of cells can be seen. Percentages of cells that stained positive after 2 counts of 100 cells in 2 separate plates are labeled.

Figure 4

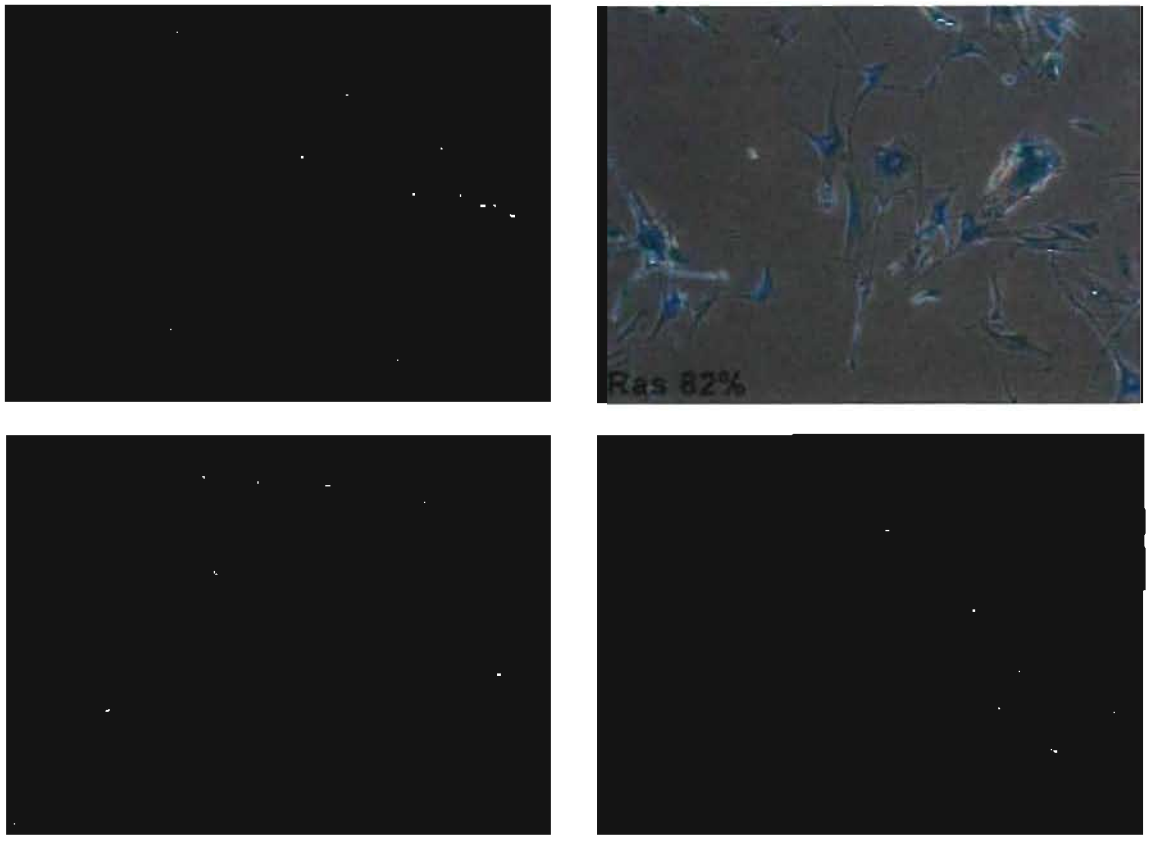


Figure 5 SOCS1 domain mutant affects on growth arrest in U2OS cell line. Cells were transfected with a control vector pLPC or derivatives containing wild-type SOCS1 or SOCS1 domain mutants. After transfection cells were selected for 6 days with puromycin followed by a 2 day recovery period for colonies to grow. Colonies were stained with 0.5% Crystal violet solution.

Figure 5

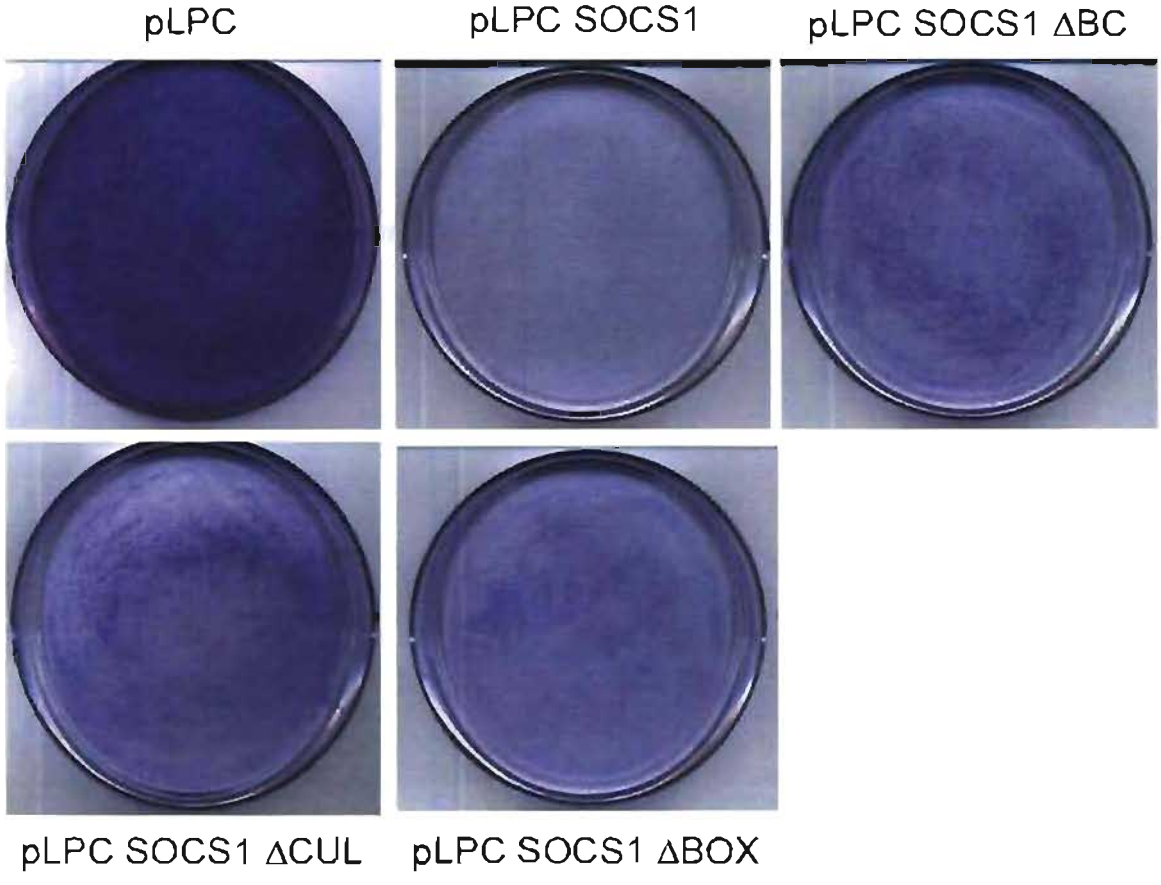

pLPC
pLPC SOCS1
pLPC SOCS1 ΔBC
pLPC SOCS1 ΔCUL
pLPC SOCS1 ΔBOX

Figure 6 SOCS1 domain mutant affects on cell cycle regulators. **(a)** U2OS cells were transfected with a control vector pLPC or derivatives containing wild-type SOCS1 or SOCS1 domain mutants. Cell extracts were prepared from cells collected a day after transfection. 20μg of protein from total cell lysate was used for SDS-PAGE gels and individual proteins were detected with immunoblotting using specific antibodies. **(b)** SOCS1 domain mutants have low stability due to protein degradation. U2OS cells were transfected as in (a) but treated with MG132 (+) or untreated (-) for 8 hrs before being collected for lysate preparation. Levels of SOCS1 proteins were detected after immunoblotting with the 4H1 anti-SOCS1 antibody (Upstate). A loading error occurred during preparation of the ΔCul box lane so that the treated sample (+) was loaded before the untreated (-). In all other samples, treated samples follow untreated controls.

Figure 6

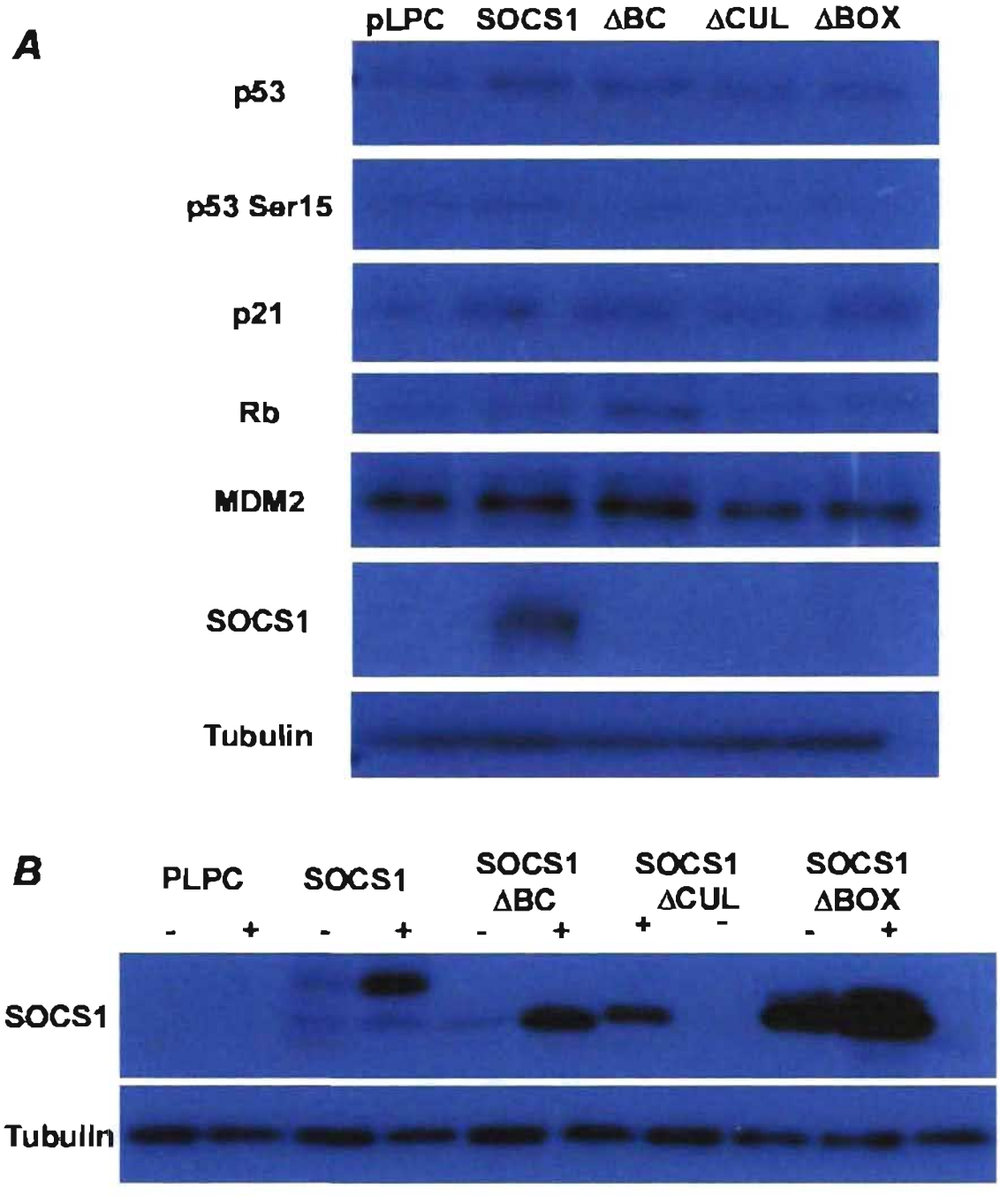

A
pLPC
SOCS1
ΔBC
ΔCUL
ΔBOX
p53
p53 Ser15
p21
Rb
MDM2
SOCS1
Tubulin
B
PLPC
SOCS1
SOCS1 ΔBC
SOCS1 ΔCUL
SOCS1 ΔBOX
- + - + - + + - - +
SOCS1
Tubulin

Figure 7 Depiction of SOCS1 marked for degradation by polyubiquitin chains **(a)** Both SOCS1 ΔBC and SOCS1 ΔCul present epitopes allowing them to be marked by other E3 ligases, possibly other SOCS molecules. **(b)** SOCS1 ΔBox presents no epiptopes and is protected from degradation.

Figure 7

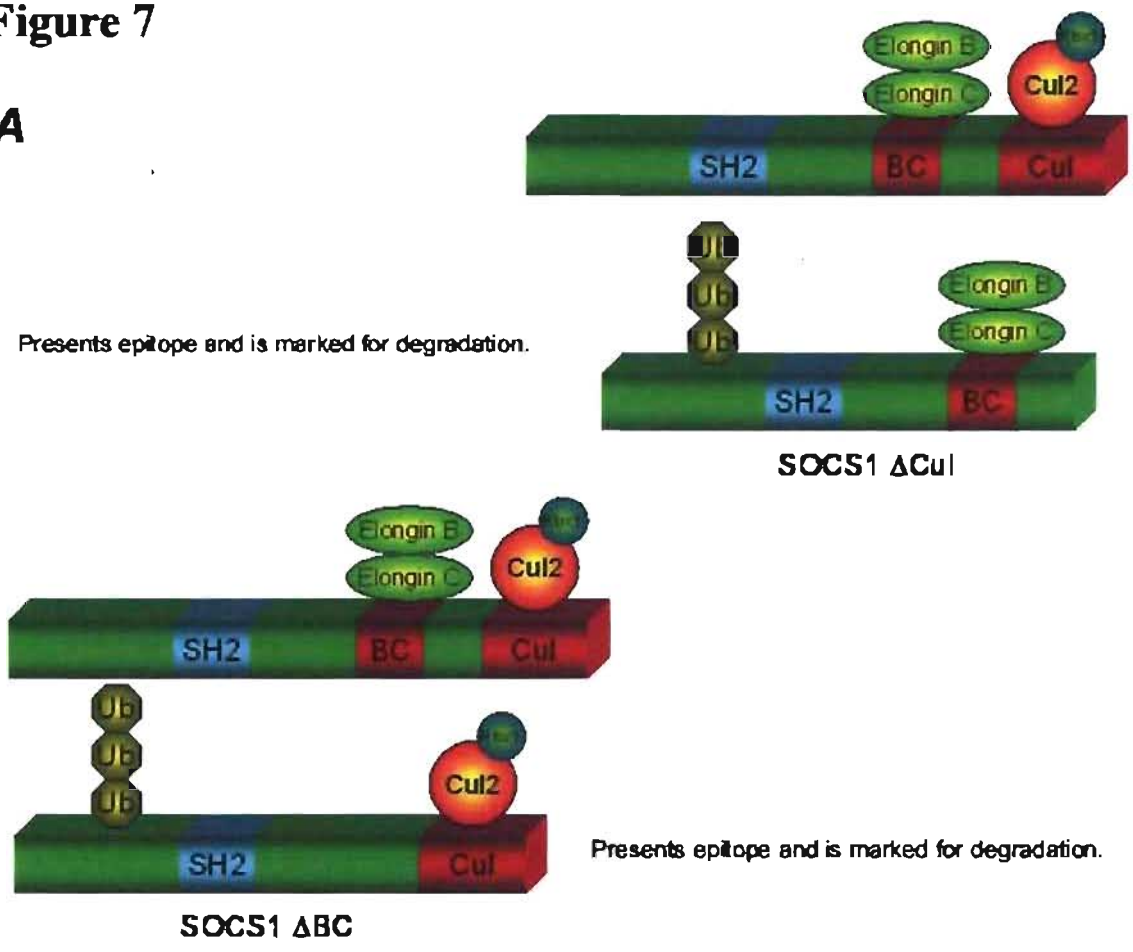

A
Elongin B
Elongin C
Cul2
SH2
BC
Cul
Ub
Ub
Ub
Presents epitope and is marked for degradation.
SOCS1 ΔCul
SOCS1 ΔBC
Presents epitope and is marked for degradation.
B
Lacks epitope and is not marked for degradation.
SOCS1 ΔBox

Figure 8 Clustal W alignment of SOCS1 protein sequences. **(a)** Alignment of sequences from the SOCS Box domains of the SOCS family members. Cul box binding sequences are highlighted in yellow. **(b)** Alignment demonstrating changes in Cul box binding in SOCS1 ΔN198 and SOCS1 N198P mutants.

Figure 8

A

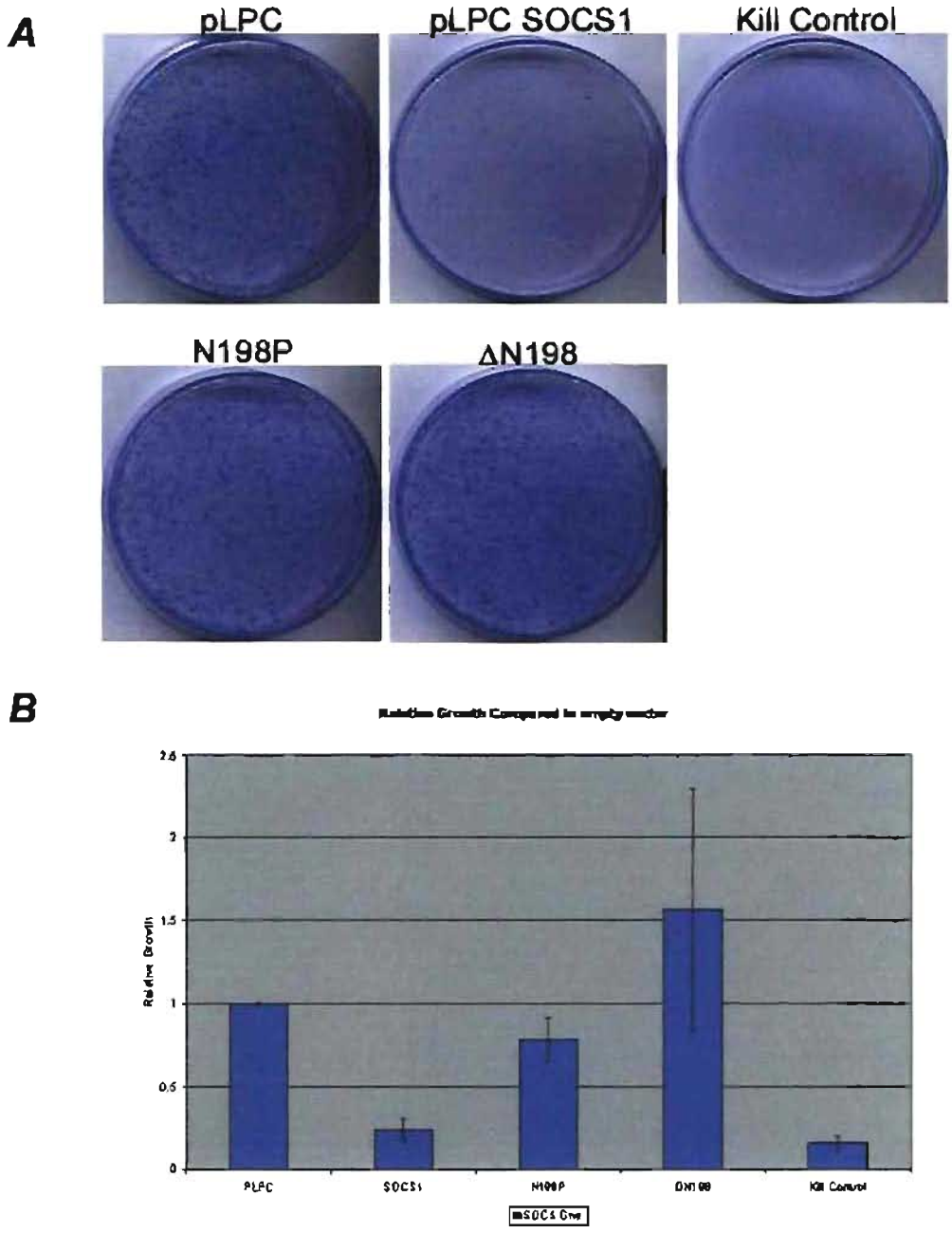

SOCS1
CIS
SOCS2
SOCS3
SOCS4
SOCS5
SOCS6
SOCS7

B


SOCS1
SOCS1 ΔN198
SOCS1 N198P
SOCS3
SOCS4
SOCS5
SOCS6
SOCS7

Figure 9 Effects of Cul box binding mutants on growth arrest in U2OS cells. **(a)** Cells were transfected with a control vector pLPC or SOCS1 derivatives including wild-type SOCS1, SOCS1 ΔN198, SOCS1 N198P and a SH2 domain mutant, SOCS1 R105E. After transfection cells were selected for 6 days with puromycin followed by a 2 day recovery period for colonies to grow. Colonies were stained with 0.5% Crystal violet solution. Untransfected cells are demonstrated as a negative killing control. **(b)** Relative growth of cells when normalized to control vector, pLPC. Cells stained with crystal violet were destained with 10% glacial acetic acid. 2 100 ul volumes from each plate were transferred to a 96 well plate and measured for absorbance at 600 nm. Averages are obtained from 2 counts per plate from two plates of each condition.

Figure 9


A
pLPC
pLPC SOCS1
Kill Control
N198P
ΔN198
B
2.5
2
1.5
1
0.5
0
PLPC
SOCS1
N198P
ΔN198
Kill Control

Discussion:

Senescence is a permanent cell cycle arrest that protects cells against oncogenic transformation and has been linked to aging [116-118]. Senescence usually signals through the p53 or Rb pathway or both. The p53 protein is a transcription factor which regulates apoptosis and many of the genes involved in senescence [127]. It is mutated in over half of all cancers and has been linked to aging. Retinoblastoma (Rb) protein is also highly mutated in a large number of cancers and exerts control over E2F responsive genes [128]. Premature senescence was first described in response to oncogenic RAS [129]. Since then other stimuli including DNA damage, over expression of PML, oncogenes such as STAT5 and prolonged IFN-β exposure have been shown to induce the senescence program [119, 120, 130]. SOCS1 is induced both by IFN-β and STAT5 hinting at its involvement in the senescence program [131].

Here we show that overexpression of SOCS1 in primary cells is able to induce a permanent cell cycle arrest showing several senescent markers including accumulation of PML bodies and a high number of cells that stain positive for senescence associated β-galactosidase. PML bodies are foci that form in the nucleus of senescent cells and help recruit p53 to nuclear bodies [132, 133]. Furthermore SOCS1 is able to directly inhibit the growth of cancer cell lines such as U2OS. Inhibition of growth in U2OS cells is accompanied by an induction in p53 and phosphorylation at serine 15 as well as an induction in its downstream target p21, indicating activation of the p53 pathway. This coincides with earlier reports in which SOCS1 has been previously described as having tumor suppressor activity, as well as the numerous studies showing that SOCS is methylated in a variety of cancers [97, 106]. Previously the tumor suppressor activity of

SOCS1 has been credited by blocking transformation through inhibition of the JAK/STAT pathways. The involvement of SOCS1 in directly inducing growth arrest and cellular senescence independently of inhibition of JAK/Stats, perhaps by acting directly on the p53 pathway is a novel concept explored in our lab.

Despite the fact that all SOCS molecules can inhibit JAK/STAT signaling, only SOCS1 was shown to be able to induce both growth arrest in cancer cell lines and cellular senescence in primary cells in our limited study. SOCS6 was able to induce growth arrest but failed to induce premature senescence. Even SOCS3, the most similar of SOCS family members to SOCS1 (and often methylated in many cancers as well), lacked this ability in our study. This is a limited study however as SOCS2, and SOCS7 and CIS were unavailable and therefore not studied. However, it is worth nothing that not all SOCS proteins are known to have negative effects on their targets. SOCS2 for example, though known to negatively regulate GH in low levels, can actually lead to increases in GH signaling when overexpressed [134]. It was also shown that SOCS2 (as well as SOCS6) can antagonize SOCS1 signaling and lead to the degradation of SOCS1, so it would seem unlikely that SOCS2 overexpression would induce growth arrest [121]. The roles of SOCS7 and CIS in growth arrest are unknown and their interactions with other SOCS molecules less clear. It is known that SOCS7 can interact with all SOCS molecules though it has yet to be shown that SOCS7 negatively regulates SOCS1. Why only SOCS1 is capable of inducing a permanent cell cycle arrest is unknown at this point. Although it shares a close similarity with SOCS3, there are still differences, among them a different sequence in the Cul box of SOCS1 that allows it differential Cullin binding [89]. We have shown that mutating this sequence to resemble those of other SOCS molecules can

prevent SOCS1 from inducing growth arrest in U2OS cells. Perhaps this unique Cul box sequence allows SOCS1 to bind to other proteins involved in growth arrest pathways that other SOCS family proteins cannot, or perhaps the binding of Cul2 gives SOCS1 a different group of substrate targets for degradation compared to other SOCS molecules which bind Cul5. It is also possible that other sequences in or near the SH2 domain of SOCS1 allow it to target unique molecules. Or alternatively perhaps one or more of the specific cytokines, JAK/Stat pathways or other target proteins under control of SOCS1 happen to be the only ones under SOCS control that are involved in growth arrest pathways as well.

Interestingly enough, some SOCS family members such as SOCS5 seem to actually promote cell growth. The mechanisms for this are unknown though it may be possible that SOCS5 acts as an oncogene. If SOCS5 targets proteins that inhibit growth, or shares a dual function as does SOCS2 with GH, it could be possible that SOCS5 overexpression would promote growth, as we have demonstrated. Another explanation could lie in interactions between SOCS proteins. As mentioned above SOCS family members have been previously shown to have overlapping functions which may interfere with one another [121]. SOCS2 expression in HEK293-T cells abolished SOCS1 and SOCS3 inhibition of GH signaling pathway. SOCS2 mutants lacking a functional SOCS box failed to do so. The role and mechanisms by which SOCS molecules interact on one another is not clear, though the SOCS box and ability to form E3 ligases seems to be heavily involved. In low concentrations SOCS2 is able to reduce β-IFN levels in HEK293-T cells, a molecule typically inhibited by SOCS1, in higher concentrations SOCS2 leads to increases in β-IFN levels. This dual effect clouds the mechanisms of

SOCS2 interactions with SOCS1 and SOCS3. SOCS6 has been shown to directly lead to proteasomal degradation of SOCS1, and its inhibition of SOCS1 is interrupted by proteasomal inhibitors like cycloheximide. Furthermore it requires the recruitment of the Elongin BC complex to SOCS6. In this model SOCS family members are able to recognize the SOCS box domains of other SOCS molecules and regulate them via targeting them for proteasomal degradation. Degradation of SOCS1 by other SOCS family members could erase the growth arrest program initiated by SOCS1. If the net effect of SOCS5 over expression favors SOCS1 degradation or reduction of SOCS signaling, preventing the induction of growth arrest, than it may actually promote growth and proliferation, much in the same way SOCS2 overexpression does in the GH signaling pathway [121]. More study is required to determine if SOCS5 and SOCS1 interact, and if SOCS5 negatively regulates the other SOCS molecules. As well further investigations into the roles of all SOCS molecules on growth arrest need to be explored further. At this time the exact outcome of interacting SOCS molecules and the effects on growth arrest are not well known, hence overexpression of one SOCS molecule at a time may not be the best way to elucidate their function. For this study we chose to focus solely on the involvement of SOCS1 in growth arrest.

We attempted to show dependency of SOCS1 activity on the BC and Cul boxes, subdomains of the SOCS Box, since many functions of SOCS1 require the SOCS box domain. There is currently much debate as to whether or not SOCS1 binding to the Elongin BC complex actually leads to protein stabilization or to proteasomal degradation of SOCS1 [76, 77, 123, 124]. The results of this study offer no clear conclusion. When overexpressed the protein levels of ΔBC and ΔCul mutants are lower than wild-type

SOCS1 though this is not seen with the ΔBox mutants. Perhaps the formation of an incomplete E3 ligase through binding to only the Elongin BC complex or the Cul/Rbx complex leads to degradation. The SOCS1 ΔBox mutant without any SOCS box perhaps has no recognition site for other SOCS molecules to bind and target it for proteasomal whereas epitopes may remain within the ΔBC box and ΔCul box mutants to allow normal recognition and subsequent degradation.

SOCS1 has been shown to act as an adaptor protein linking the modules of an E3 ligase together. This E3 ligase activity has been attributed to its negative regulation of the JAK/Stat signaling cascades. The results of this study show that SOCS1 not only induces activity of the tumor suppressor p53 as well as its phosphorylation but also its downstream target p21. Direct degradation of p53 would not lead to the growth arrest accompanied with over expression of SOCS1, so SOCS1 must either target a negative regulator of p53 for degradation or act upon p53 in a way previously undescribed that leads to its activation. MDM2 levels remain unchanged in SOCS1 transfected U2OS cells, though it is possible SOCS1 targets other E3 ligases regulating p53 such as COP1 and Pirh2, this possibility needs to be explored before ruling out the obvious E3 ligase activity of SOCS1 in leading to p53 activation. Cullin based E3 ligases are also capable of many other post transductional modifications such as neddylation and sumoylation [135-138]. Another Cullin based E3 ligase, FBXO11, was recently shown to directly neddylate p53, leading to an increase in its transcriptional activity [139]. As well the role of SUMO upon p53 has begun to come under more intense study [140]. The possibility of SOCS1 based E3 ligases regulating p53 through direct post transductional modification has not been explored in this study. Yet more and more examples of neddylation and

sumoylation appearing to play important roles in regulating proteins, and important pathways, even the cell cycle, have begun to appear in recent literature [141, 142] . This dictates that the roles of these modifications and the enzymes that produce them require more study.

While we were unable to directly demonstrate the dependency of the BC or Cul box domains for SOCS1 induced growth arrest, we have identified a necessary residue within the Cul box domain of SOCS1 for this activity. Kumara et al., originally identified a stretch of amino acids within the Cul box of SOCS1 that differs from a conserved sequence found in all other SOCS family members [89]. Specifically deletion or mutation to the N198 residue alters the IPLNP sequence in the Cul box of SOCS1 to resemble the LPxP sequence found in all other SOCS family proteins. As well, both these modifications abolish the ability of SOCS1 to induce growth arrest in the U2OS cell line we studied. The Cul box of SOCS1 resembles that of the VHL tumor suppressor, and both have been shown to bind to a Cul 2/Rbx1 complex. [74, 89]. Other SOCS members bind to Cul 5/ Rbx 2 complex. VHL has also been shown to bind directly to p53 and stabilize p53 levels upon activation by both blocking MDM2 induced degradation and recruiting p53 modifying proteins [143]. Other students in our lab have demonstrated that SOCS1 can form direct interactions with p53 (Calabresse et al. unpublished). Also, VHL can induce growth arrest in different cell lines [143]. Both SOCS1 and VHL share a similar Cul box, unique from that of other SOCS1 members, alteration of the Cul box of SOCS1 to that of other SOCS proteins through mutations to N198 abolishes growth arrest. Hence, SOCS1 can be acting in a manner very similar to VHL and can be acting as an adaptor protein to recruit proteins to modify p53. This could explain the

phosphorylation of p53 at serine 15 seen in U2OS cells overexpressing SOCS1. The binding site for p53 is unknown but it could be likely that SOCS1 can act as an adaptor protein for many complexes aside from an E3 ligase.

It is possible however that other proteins that protect p53 from ubiquitination or activate it through modifications bind to this region. In this model, the components of the E3 ligase could be in competition for binding sites within SOCS1 with p53 and p53 modifying proteins. However, the binding of p53 to SOCS1 must be further explored. Another model would be SOCS1 binding to Cul5/Rbx2 allows it to degrade different targets than other SOCS molecules binding to Cul2/Rbx1. This would give SOCS1 a unique advantage and possibly allow it to degrade proteins which promote growth or proteins that act as a negative regulator of p53, inducing a growth arrest. Switching the Cul box sequence in theory would alter SOCS1 so that it binds to Cul2/Rbx1, not Cul5/Rbx2, preventing normal SOCS1 function. We were unable to show Cul2 binding with SOCS1 or the SOCS1 mutants with the antibodies available to us, therefore we cannot assume that this is indeed the case with the SOCS1 ΔN198 or SOCS1 N198P mutants.

Conclusion:

We have shown that SOCS1 appears to be the only member of the SOCS family of proteins capable of inducing a growth arrest phenotype. In primary cells, the growth arrest is manifested as premature senescence, this implicates SOCS1 in a powerful anti-tumor mechanism, coinciding with its role as a tumor suppressor. Other members of the SOCS family do not share this ability and some appear to have roles as an oncogene.

Furthermore, we have shown SOCS1 can activate the p53 pathway. The exact mechanisms of this are not known and must be further explored. As well, we have shown a residue within SOCS1 that is necessary for the growth arrest phenotype of SOCS1. Since others in our lab have shown that SOCS1 can bind p53 directly, we suggest that alternative binding to Cul5/Rbx2 or a differential binding domain within SOCS1 allows SOCS1 to bind to p53 and stabilize it directly. Possibly, p53 binds to SOCS1 at one site and other proteins are recruited to SOCS1 to modify and stabilize p53 through another site, which possibly contains the N198 residue. These results, although only preliminary, demonstrate the role of SOCS1 in senescence as a possible role of SOCS1 as a tumor suppressor which could act independent of its role in regulating JAK/Stat pathways. The activation of p53 makes SOCS1 an important protein of interest. Its widespread methylation in a variety of cancers further illustrates its importance. We suggest the full function of SOCS1 remains a mystery and that it may have other roles and functions independent of its E3 ligase activity. We conclude that further studies on the mechanisms of p53 activation by SOCS1, and on the role played by the Cul box and the N198 residue on growth arrest, are required.

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References:

1. Ihle, J.N., *Cytokine receptor signalling.* Nature, 1995. **377**(6550): p. 591-4.
2. Smith, K.A., et al., *The functional relationship of the interleukins.* J Exp Med, 1980. **151**(6): p. 1551-6.
3. Nagano, Y., Y. Kojima, and Y. Sawai, *[Immunity and interference in vaccinia; inhibition of skin infection by inactivated virus.].* C R Seances Soc Biol Fil, 1954. **148**(7-8): p. 750-2.
4. Robb, R.J. and K.A. Smith, *Heterogeneity of human T-cell growth factor(s) due to variable glycosylation.* Mol Immunol, 1981. **18**(12): p. 1087-94.
5. Smith, K.A., *Interleukin-2: inception, impact, and implications.* Science, 1988. **240**(4856): p. 1169-76.
6. Mosmann, T.R., *Role of a new cytokine, interleukin-10, in the cross-regulation of T helper cells.* Ann N Y Acad Sci, 1991. **628**: p. 337-44.
7. Watanabe, Y., *Fifty years of interference.* Nat Immunol, 2004. **5**(12): p. 1193.
8. Dinarello, C.A., *Interleukin-1.* Adv Pharmacol, 1994. **25**: p. 21-51.
9. Dinarello, C.A., *The interleukin-1 family: 10 years of discovery.* Faseb J, 1994. **8**(15): p. 1314-25.
10. Goodwin, R.G. and A.E. Namen, *The cloning and characterization of interleukin-7.* Year Immunol, 1989. **6**: p. 127-39.
11. Fernandez, E.J. and E. Lolis, *Structure, function, and inhibition of chemokines.* Annu Rev Pharmacol Toxicol, 2002. **42**: p. 469-99.
12. Ziemiecki, A., A.G. Harpur, and A.F. Wilks, *JAK protein tyrosine kinases: their role in cytokine signalling.* Trends Cell Biol, 1994. **4**(6): p. 207-12.
13. Ihle, J.N., *The Janus protein tyrosine kinase family and its role in cytokine signaling.* Adv Immunol, 1995. **60**: p. 1-35.
14. Ihle, J.N. and I.M. Kerr, *Jaks and Stats in signaling by the cytokine receptor superfamily.* Trends Genet, 1995. **11**(2): p. 69-74.
15. Yasukawa, H., A. Sasaki, and A. Yoshimura, *Negative regulation of cytokine signaling pathways.* Annu Rev Immunol, 2000. **18**: p. 143-64.
16. Miura, O., et al., *Erythropoietin induces association of the JAK2 protein tyrosine kinase with the erythropoietin receptor in vivo.* Blood, 1994. **84**(5): p. 1501-7.
17. Quelle, F.W., et al., *JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region.* Mol Cell Biol, 1994. **14**(7): p. 4335-41.
18. Miyajima, A., *Molecular structure of the IL-3, GM-CSF and IL-5 receptors.* Int J Cell Cloning, 1992. **10**(3): p. 126-34.
19. Miyajima, A., et al., *Cytokine receptors and signal transduction.* Annu Rev Immunol, 1992. **10**: p. 295-331.
20. Taniguchi, T., *Cytokine signaling through nonreceptor protein tyrosine kinases.* Science, 1995. **268**(5208): p. 251-5.

21. Colamonici, O., et al., *Direct binding to and tyrosine phosphorylation of the alpha subunit of the type I interferon receptor by p135tyk2 tyrosine kinase.* Mol Cell Biol, 1994. **14**(12): p. 8133-42.
22. Yan, H., et al., *Molecular characterization of an alpha interferon receptor 1 subunit (IFNaR1) domain required for TYK2 binding and signal transduction.* Mol Cell Biol, 1996. **16**(5): p. 2074-82.
23. Igarashi, K., et al., *Interferon-gamma induces tyrosine phosphorylation of interferon-gamma receptor and regulated association of protein tyrosine kinases, Jak1 and Jak2, with its receptor.* J Biol Chem, 1994. **269**(20): p. 14333-6.
24. Novick, D., B. Cohen, and M. Rubinstein, *The human interferon alpha/beta receptor: characterization and molecular cloning.* Cell, 1994. **77**(3): p. 391-400.
25. Darnell, J.E., Jr., I.M. Kerr, and G.R. Stark, *Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins.* Science, 1994. **264**(5164): p. 1415-21.
26. Shuai, K., *Interferon-activated signal transduction to the nucleus.* Curr Opin Cell Biol, 1994. **6**(2): p. 253-9.
27. Horvath, C.M., Z. Wen, and J.E. Darnell, Jr., *A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain.* Genes Dev, 1995. **9**(8): p. 984-94.
28. Zhang, X., et al., *Requirement of serine phosphorylation for formation of STAT-promoter complexes.* Science, 1995. **267**(5206): p. 1990-4.
29. De Vos, J., et al., *JAK2 tyrosine kinase inhibitor tyrphostin AG490 downregulates the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways and induces apoptosis in myeloma cells.* Br J Haematol, 2000. **109**(4): p. 823-8.
30. Inamura, K., et al., *Rapid inhibition of MAPK signaling and anti-proliferation effect via JAK/STAT signaling by interferon-alpha in hepatocellular carcinoma cell lines.* Biochim Biophys Acta, 2005. **1745**(3): p. 401-10.
31. Lutticken, C., et al., *Interleukin-6-induced serine phosphorylation of transcription factor APRF: evidence for a role in interleukin-6 target gene induction.* FEBS Lett, 1995. **360**(2): p. 137-43.
32. Wang, L.H., et al., *JAK3, STAT, and MAPK signaling pathways as novel molecular targets for the tyrphostin AG-490 regulation of IL-2-mediated T cell response.* J Immunol, 1999. **162**(7): p. 3897-904.
33. Starr, R., et al., *A family of cytokine-inducible inhibitors of signalling.* Nature, 1997. **387**(6636): p. 917-21.
34. Endo, T.A., et al., *A new protein containing an SH2 domain that inhibits JAK kinases.* Nature, 1997. **387**(6636): p. 921-4.
35. Yoshimura, A., et al., *A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors.* Embo J, 1995. **14**(12): p. 2816-26.
36. Nicholson, S.E. and D.J. Hilton, *The SOCS proteins: a new family of negative regulators of signal transduction.* J Leukoc Biol, 1998. **63**(6): p. 665-8.
37. Naka, T., et al., *Structure and function of a new STAT-induced STAT inhibitor.* Nature, 1997. **387**(6636): p. 924-9.

38. Yoshimura, A., *Negative regulation of cytokine signaling.* Clin Rev Allergy Immunol, 2005. **28**(3): p. 205-20.
39. Hilton, D.J., et al., *Twenty proteins containing a C-terminal SOCS box form five structural classes.* Proc Natl Acad Sci U S A, 1998. **95**(1): p. 114-9.
40. Kawahara, A., et al., *Critical role of the interleukin 2 (IL-2) receptor gamma-chain-associated Jak3 in the IL-2-induced c-fos and c-myc, but not bcl-2, gene induction.* Proc Natl Acad Sci U S A, 1995. **92**(19): p. 8724-8.
41. Starr, R., et al., *Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1.* Proc Natl Acad Sci U S A, 1998. **95**(24): p. 14395-9.
42. Naka, T., et al., *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**(26): p. 15577-82.
43. Marine, J.C., et al., *SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality.* Cell, 1999. **98**(5): p. 609-16.
44. Sakamoto, H., et al., *A Janus kinase inhibitor, JAB, is an interferon-gamma-inducible gene and confers resistance to interferons.* Blood, 1998. **92**(5): p. 1668-76.
45. Cornish, A.L., et al., *Suppressor of cytokine signaling-1 regulates signaling in response to interleukin-2 and other gamma c-dependent cytokines in peripheral T cells.* J Biol Chem, 2003. **278**(25): p. 22755-61.
46. Alexander, W.S., et al., *Suppressors of cytokine signaling (SOCS): negative regulators of signal transduction.* J Leukoc Biol, 1999. **66**(4): p. 588-92.
47. Chong, M.M., et al., *Suppressor of cytokine signaling-1 is a critical regulator of interleukin-7-dependent CD8+ T cell differentiation.* Immunity, 2003. **18**(4): p. 475-87.
48. Trop, S., et al., *Overexpression of suppressor of cytokine signaling-1 impairs pre-T-cell receptor-induced proliferation but not differentiation of immature thymocytes.* Blood, 2001. **97**(8): p. 2269-77.
49. Catlett, I.M. and S.M. Hedrick, *Suppressor of cytokine signaling 1 is required for the differentiation of CD4+ T cells.* Nat Immunol, 2005. **6**(7): p. 715-21.
50. Ilangumaran, S., et al., *Suppressor of cytokine signaling 1 attenuates IL-15 receptor signaling in CD8+ thymocytes.* Blood, 2003. **102**(12): p. 4115-22.
51. Brugnera, E., et al., *Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells.* Immunity, 2000. **13**(1): p. 59-71.
52. Cornish, A.L., et al., *Suppressor of cytokine signaling-1 has IFN-gamma-independent actions in T cell homeostasis.* J Immunol, 2003. **170**(2): p. 878-86.
53. Yu, C.R., et al., *Suppressor of cytokine signaling 3 regulates proliferation and activation of T-helper cells.* J Biol Chem, 2003. **278**(32): p. 29752-9.
54. Fox, S.W., et al., *The possible role of TGF-beta-induced suppressors of cytokine signaling expression in osteoclast/macrophage lineage commitment in vitro.* J Immunol, 2003. **170**(7): p. 3679-87.
55. Hanada, T., et al., *Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity.* Immunity, 2003. **19**(3): p. 437-50.

56. Evel-Kabler, K., et al., *SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling.* J Clin Invest, 2006. **116**(1): p. 90-100.
57. Zimmerer, J.M., et al., *IFN-alpha-induced signal transduction, gene expression, and antitumor activity of immune effector cells are negatively regulated by suppressor of cytokine signaling proteins.* J Immunol, 2007. **178**(8): p. 4832-45.
58. Mostecki, J., B.M. Showalter, and P.B. Rothman, *Early growth response-1 regulates lipopolysaccharide-induced suppressor of cytokine signaling-1 transcription.* J Biol Chem, 2005. **280**(4): p. 2596-605.
59. Kamio, M., et al., *SOCS1 [corrected] inhibits HPV-E7-mediated transformation by inducing degradation of E7 protein.* Oncogene, 2004. **23**(17): p. 3107-15.
60. Nakagawa, R., et al., *SOCS-1 participates in negative regulation of LPS responses.* Immunity, 2002. **17**(5): p. 677-87.
61. Kimura, A., et al., *SOCS-1 suppresses TNF-alpha-induced apoptosis through the regulation of Jak activation.* Int Immunol, 2004. **16**(7): p. 991-9.
62. Larsen, L. and C. Ropke, *Suppressors of cytokine signalling: SOCS.* Apmis, 2002. **110**(12): p. 833-44.
63. Yasukawa, H., et al., *The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop.* Embo J, 1999. **18**(5): p. 1309-20.
64. Feng, J., et al., *Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop.* Mol Cell Biol, 1997. **17**(5): p. 2497-501.
65. Chen, X., et al., *Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA.* Cell, 1998. **93**(5): p. 827-39.
66. Sasaki, A., et al., *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**(6): p. 339-51.
67. Flowers, L.O., et al., *Characterization of a peptide inhibitor of Janus kinase 2 that mimics suppressor of cytokine signaling 1 function.* J Immunol, 2004. **172**(12): p. 7510-8.
68. Waiboci, L.W., et al., *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**(8): p. 5058-68.
69. Flowers, L.O., P.S. Subramaniam, and H.M. Johnson, *A SOCS-1 peptide mimetic inhibits both constitutive and IL-6 induced activation of STAT3 in prostate cancer cells.* Oncogene, 2005. **24**(12): p. 2114-20.
70. Mujtaba, M.G., et al., *Treatment of mice with the suppressor of cytokine signaling-1 mimetic peptide, tyrosine kinase inhibitor peptide, prevents development of the acute form of experimental allergic encephalomyelitis and induces stable remission in the chronic relapsing/remitting form.* J Immunol, 2005. **175**(8): p. 5077-86.
71. Ciechanover, A., *The ubiquitin-proteasome pathway: on protein death and cell life.* Embo J, 1998. **17**(24): p. 7151-60.
72. Giordanetto, F. and R.T. Kroemer, *A three-dimensional model of Suppressor Of Cytokine Signalling 1 (SOCS-1).* Protein Eng, 2003. **16**(2): p. 115-24.

73. Kile, B.T., et al., *The SOCS box: a tale of destruction and degradation.* Trends Biochem Sci, 2002. **27**(5): p. 235-41.
74. Kamizono, S., et al., *The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2.* J Biol Chem, 2001. **276**(16): p. 12530-8.
75. Bullock, A.N., et al., *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**(20): p. 7637-42.
76. Zhang, J.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**(5): p. 2071-6.
77. Kamura, T., et al., *The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families.* Genes Dev, 1998. **12**(24): p. 3872-81.
78. Lonergan, K.M., et al., *Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2.* Mol Cell Biol, 1998. **18**(2): p. 732-41.
79. Pause, A., et al., *The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins.* Proc Natl Acad Sci U S A, 1997. **94**(6): p. 2156-61.
80. Bradsher, J.N., et al., *RNA polymerase II transcription factor SIII. I. Identification, purification, and properties.* J Biol Chem, 1993. **268**(34): p. 25587-93.
81. Duan, D.R., et al., *Inhibition of transcription elongation by the VHL tumor suppressor protein.* Science, 1995. **269**(5229): p. 1402-6.
82. Duan, D.R., et al., *Characterization of the VHL tumor suppressor gene product: localization, complex formation, and the effect of natural inactivating mutations.* Proc Natl Acad Sci U S A, 1995. **92**(14): p. 6459-63.
83. Kibel, A., et al., *Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C.* Science, 1995. **269**(5229): p. 1444-6.
84. Iwai, K., et al., *Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex.* Proc Natl Acad Sci U S A, 1999. **96**(22): p. 12436-41.
85. Ohta, T., et al., *ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity.* Mol Cell, 1999. **3**(4): p. 535-41.
86. Seol, J.H., et al., *Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34.* Genes Dev, 1999. **13**(12): p. 1614-26.
87. Kipreos, E.T., et al., *cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family.* Cell, 1996. **85**(6): p. 829-39.
88. Kamura, T., et al., *Mufl, 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**(32): p. 29748-53.
89. Kamura, T., et al., *VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases.* Genes Dev, 2004. **18**(24): p. 3055-65.

90. Vuong, B.Q., et al., *SOCS-1 localizes to the microtubule organizing complex-associated 20S proteasome.* Mol Cell Biol, 2004. **24**(20): p. 9092-101.
91. De Sepulveda, P., S. Ilangumaran, and R. Rottapel, *Suppressor of cytokine signaling-1 inhibits VAV function through protein degradation.* J Biol Chem, 2000. **275**(19): p. 14005-8.
92. Frantsve, J., et al., *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**(10): p. 3547-57.
93. Rui, L., et al., *SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2.* J Biol Chem, 2002. **277**(44): p. 42394-8.
94. van de Geijn, G.J., J. Gits, and I.P. Touw, *Distinct activities of suppressor of cytokine signaling (SOCS) proteins and involvement of the SOCS box in controlling G-CSF signaling.* J Leukoc Biol, 2004. **76**(1): p. 237-44.
95. He, Y., et al., *SOCS1 inhibits tumor necrosis factor-induced activation of ASK1-JNK inflammatory signaling by mediating ASK1 degradation.* J Biol Chem, 2006. **281**(9): p. 5559-66.
96. Mansell, A., et al., *Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation.* Nat Immunol, 2006. **7**(2): p. 148-55.
97. Rottapel, R., et al., *The tumor suppressor activity of SOCS-1.* Oncogene, 2002. **21**(28): p. 4351-62.
98. Yoshikawa, H., et al., *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**(1): p. 29-35.
99. Miyoshi, H., et al., *Methylation status of suppressor of cytokine signaling-1 gene in hepatocellular carcinoma.* J Gastroenterol, 2004. **39**(6): p. 563-9.
100. Nagai, H., et al., *Combined hypermethylation and chromosome loss associated with inactivation of SSI-1/SOCS-1/JAB gene in human hepatocellular carcinomas.* Cancer Lett, 2002. **186**(1): p. 59-65.
101. Okochi, O., et al., *Methylation-mediated silencing of SOCS-1 gene in hepatocellular carcinoma derived from cirrhosis.* Clin Cancer Res, 2003. **9**(14): p. 5295-8.
102. Tannapfel, A., et al., *Identification of novel proteins associated with hepatocellular carcinomas using protein microarrays.* J Pathol, 2003. **201**(2): p. 238-49.
103. Chen, C.Y., et al., *SOCS1 methylation in patients with newly diagnosed acute myeloid leukemia.* Genes Chromosomes Cancer, 2003. **37**(3): p. 300-5.
104. Galm, O., et al., *SOCS-1, a negative regulator of cytokine signaling, is frequently silenced by methylation in multiple myeloma.* Blood, 2003. **101**(7): p. 2784-8.
105. Komazaki, T., et al., *Hypermethylation-associated inactivation of the SOCS-1 gene, a JAK/STAT inhibitor, in human pancreatic cancers.* Jpn J Clin Oncol, 2004. **34**(4): p. 191-4.
106. Sutherland, K.D., et al., *Differential hypermethylation of SOCS genes in ovarian and breast carcinomas.* Oncogene, 2004. **23**(46): p. 7726-33.
107. Fujitake, S., et al., *Aberrant methylation of SOCS-1 was observed in younger colorectal cancer patients.* J Gastroenterol, 2004. **39**(2): p. 120-4.

108. Hanada, T., et al., *IFNgamma-dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice.* J Exp Med, 2006. **203**(6): p. 1391-7.
109. Fukushima, N., et al., *Aberrant methylation of suppressor of cytokine signalling-1 (SOCS-1) gene in pancreatic ductal neoplasms.* Br J Cancer, 2003. **89**(2): p. 338-43.
110. Yoshida, T., et al., *SOCS1 is a suppressor of liver fibrosis and hepatitis-induced carcinogenesis.* J Exp Med, 2004. **199**(12): p. 1701-7.
111. Bowman, T., et al., *STATs in oncogenesis.* Oncogene, 2000. **19**(21): p. 2474-88.
112. Chim, C.S., et al., *SOCS1 and SHP1 hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway.* Blood, 2004. **103**(12): p. 4630-5.
113. Weniger, M.A., et al., *Mutations of the tumor suppressor gene SOCS-1 in classical Hodgkin lymphoma are frequent and associated with nuclear phospho-STAT5 accumulation.* Oncogene, 2006. **25**(18): p. 2679-84.
114. Melzner, I., et al., *Biallelic mutation of SOCS-1 impairs JAK2 degradation and sustains phospho-JAK2 action in the MedB-1 mediastinal lymphoma line.* Blood, 2005. **105**(6): p. 2535-42.
115. Melzner, I., et al., *Biallelic deletion within 16p13.13 including SOCS-1 in Karpas1106P mediastinal B-cell lymphoma line is associated with delayed degradation of JAK2 protein.* Int J Cancer, 2006. **118**(8): p. 1941-4.
116. Campisi, J., *Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors.* Cell, 2005. **120**(4): p. 513-22.
117. Mathon, N.F. and A.C. Lloyd, *Cell senescence and cancer.* Nat Rev Cancer, 2001. **1**(3): p. 203-13.
118. Serrano, M. and M.A. Blasco, *Putting the stress on senescence.* Curr Opin Cell Biol, 2001. **13**(6): p. 748-53.
119. Mallette, F.A., M.F. Gaumont-Leclerc, and G. Ferbeyre, *The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence.* Genes Dev, 2007. **21**(1): p. 43-8.
120. Moiseeva, O., et al., *DNA damage signaling and p53-dependent senescence after prolonged beta-interferon stimulation.* Mol Biol Cell, 2006. **17**(4): p. 1583-92.
121. Piessevaux, J., et al., *Functional cross-modulation between SOCS proteins can stimulate cytokine signaling.* J Biol Chem, 2006. **281**(44): p. 32953-66.
122. Chen, X.P., et al., *Pim serine/threonine kinases regulate the stability of Socs-1 protein.* Proc Natl Acad Sci U S A, 2002. **99**(4): p. 2175-80.
123. Narazaki, M., et al., *Three distinct domains of SSI-1/SOCS-1/JAB protein are required for its suppression of interleukin 6 signaling.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13130-4.
124. Haan, S., et al., *Tyrosine phosphorylation disrupts elongin interaction and accelerates SOCS3 degradation.* J Biol Chem, 2003. **278**(34): p. 31972-9.
125. Ferbeyre, G., et al., *Oncogenic ras and p53 cooperate to induce cellular senescence.* Mol Cell Biol, 2002. **22**(10): p. 3497-508.
126. Brooks, C.L. and W. Gu, *p53 ubiquitination: Mdm2 and beyond.* Mol Cell, 2006. **21**(3): p. 307-15.

127. Liebermann, D.A., B. Hoffman, and D. Vesely, *p53 induced growth arrest versus apoptosis and its modulation by survival cytokines.* Cell Cycle, 2007. **6**(2): p. 166-70.
128. Korenjak, M. and A. Brehm, *E2F-Rb complexes regulating transcription of genes important for differentiation and development.* Curr Opin Genet Dev, 2005. **15**(5): p. 520-7.
129. Serrano, M., et al., *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.* Cell, 1997. **88**(5): p. 593-602.
130. Ferbeyre, G., et al., *PML is induced by oncogenic ras and promotes premature senescence.* Genes Dev, 2000. **14**(16): p. 2015-27.
131. Qin, H., et al., *IFN-beta-induced SOCS-1 negatively regulates CD40 gene expression in macrophages and microglia.* Faseb J, 2006. **20**(7): p. 985-7.
132. de Stanchina, E., et al., *PML is a direct p53 target that modulates p53 effector functions.* Mol Cell, 2004. **13**(4): p. 523-35.
133. Chelbi-Alix, M.K., et al., *Induction of the PML protein by interferons in normal and APL cells.* Leukemia, 1995. **9**(12): p. 2027-33.
134. Favre, H., et al., *Dual effects of suppressor of cytokine signaling (SOCS-2) on growth hormone signal transduction.* FEBS Lett, 1999. **453**(1-2): p. 63-6.
135. Chew, E.H. and T. Hagen, *Substrate-mediated regulation of cullin neddylation.* J Biol Chem, 2007. **282**(23): p. 17032-40.
136. Fuchs, O. and R. Neuwirtova, *[Ubiquitins, proteasomes, sumoylation and application today and in future for cancer and other diseases therapy II. Sumoylation and neddylation as posttranslational modifications of proteins and their ubiquitinylation and its significance].* Vnitr Lek, 2006. **52**(6): p. 619-27.
137. Fuchs, O. and R. Neuwirtova, *[Ubiquitins, proteasomes, sumoylation and therapeutic application today and in future for cancer and other diseases. I. Ubiquitin-proteasome system and the transcription factor NF-kappaB].* Vnitr Lek, 2006. **52**(4): p. 371-8.
138. Watts, F.Z., *SUMO modification of proteins other than transcription factors.* Semin Cell Dev Biol, 2004. **15**(2): p. 211-20.
139. Abida, W.M., et al., *FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity.* J Biol Chem, 2007. **282**(3): p. 1797-804.
140. Melchior, F. and L. Hengst, *SUMO-1 and p53.* Cell Cycle, 2002. **1**(4): p. 245-9.
141. Di Bacco, A. and G. Gill, *SUMO-specific proteases and the cell cycle. An essential role for SENP5 in cell proliferation.* Cell Cycle, 2006. **5**(20): p. 2310-3.
142. Oved, S., et al., *Conjugation to Nedd8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases.* J Biol Chem, 2006. **281**(31): p. 21640-51.
143. Roe, J.S. and H.D. Youn, *The positive regulation of p53 by the tumor suppressor VHL.* Cell Cycle, 2006. **5**(18): p. 2054-6.