Title:

Kinase inhibition, competitive binding and proteasomal degradation; resolving the molecular function of the Suppressor Of Cytokine Signaling (SOCS) proteins

Edmond M Linossi[1,2] & Sandra E Nicholson[1,2]

[1]*Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia;* [2]*The University of Melbourne, Parkville, Victoria, Australia*

*To whom correspondence should be addressed:
Sandra E Nicholson
Phone: +61 3 9345 2555
Fax: +61 3 9347 0852
Email: snicholson@wehi.edu.au

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Running title: Mechanisms of SOCS protein action

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Summary

The Suppressor of Cytokine Signaling (SOCS) family of proteins are key negative regulators of cytokine and growth factor signaling. They act at the receptor complex to modulate the intracellular signaling cascade, preventing excessive signaling and restoring homeostasis. This regulation is critical to the normal cessation of signaling, highlighted by the complex inflammatory phenotypes exhibited by mice deficient in SOCS1 or SOCS3. These two SOCS proteins remain the best characterized of the eight family members (CIS, SOCS1-7) and in particular, we now possess a sound understanding of the mechanism of action for SOCS3. Here we review the mechanistic role of the SOCS proteins and identify examples where clear, definitive data has been generated, and discuss areas where the information is less clear. From this functional viewpoint, we will discuss how the SOCS proteins achieve exquisite and specific regulation of cytokine signaling and highlight outstanding questions regarding the function of the less well-studied SOCS family members.

Introduction – The SOCS proteins are key negative regulators of cytokine and growth factor signaling

Cytokines and growth factors are soluble extracellular messengers that serve to communicate specific messages to cells. These signals are transmitted through the spatial rearrangement of receptor subunits to the associated intracellular Janus Kinases (JAK). This leads to the activation of the JAKs, which then propagate the intracellular signal via an intricate and complex signaling cascade to achieve the correct transcriptional profile, often as a result of Signal Transducer and Activator of Transcription (STAT) protein activation(1). The subsequent cellular response is key to the normal physiology of the cell and includes cellular proliferation, differentiation and survival. Aberrant signaling at many levels of these cascades has now been unequivocally identified as an important contributor to specific diseases. For example, activating mutations in JAK2 are particularly prevalent in hematopoietic malignancies, amongst others, and JAK inhibitors are now employed to treat autoimmune diseases such as rheumatoid arthritis(2,3).

The key negative regulators of cytokine and growth factor signaling are the Suppressor Of Cytokine Signaling (SOCS) family of proteins. Since their discovery in the late 1990's(4-7), these small intracellular proteins have been shown to play critical roles in orchestrating the cellular response to many cytokines and growth factors. There are eight SOCS family members, Cytokine-Inducible SH2-containing protein (CIS) and SOCS1-7, that are

characterized by a highly conserved C-terminal SOCS box motif(8) that is responsible for forming an E3 ubiquitin ligase complex(9,10). The SOCS proteins also contain a central Src Homology 2 (SH2) domain and an adjacent alpha-helical extension, termed the Extended SH2-Subdomain (ESS) that collectively bind tyrosine phosphorylated motifs on target proteins(11,12). The SOCS proteins also harbor an N-terminal region that varies in both sequence and length across the family, which for SOCS1 and SOCS3 encompasses their unique Kinase Inhibitory Region (KIR). SOCS4-7 contain an extensive N-terminal region that distinguishes them from SOCS1-3 and CIS (*Fig. 1*). From an evolutionary perspective, the SOCS family appear to have expanded to help deal with an increasingly complex JAK/STAT system, which increases from a single JAK/receptor and STAT in insects to four JAKs, over 40 receptors, and 7 STATs in *Homo sapiens*(13,14).

~ Figure 1 here ~

Exogenous expression of SOCS1 and SOCS3 leads to potent inhibition of JAK/STAT signaling from most cytokine and growth factor receptor complexes. However, mice genetically engineered to lack SOCS1 or SOCS3 exhibit dramatic inflammatory phenotypes related to excessive signaling from only a few cytokine receptors, namely Interferon (IFN)-γ(15) and Leukemia Inhibitory Factor (LIF)/Interleukin (IL)-6 family cytokines(16,17), respectively. These experiments highlighted both the physiological importance of these genes and provided important clues as to their specific biological roles. The absence of a SOCS protein does not generally lead to an increase in the total quantity of the signal (for

example STAT3 phosphorylation downstream of IL-6), rather it results in a prolonged activation of the signaling pathway. This subtle modulation of signaling has often proven difficult to detect at an endogenous level. This is particularly true for SOCS4-7, as mice lacking these *Socs* genes don't display the dramatic phenotypes associated with *Socs1, 2* or *3* in the steady state, and thus provide fewer clues as to their function. It is becoming increasingly clear that the action of a SOCS protein is often highly context dependent. Defining the physiological function of a SOCS protein requires identification of the inducing stimuli, the relevant cell type, the SOCS substrate/s and the biological context where SOCS regulation is critical.

One of the key questions arising since the discovery of the SOCS is: how do they inhibit specific cytokine/receptor complexes *in* vivo? This question has been carefully addressed both *in vivo* and *in vitro* and we now understand that the exquisite and specific regulation of JAK/STAT signaling occurs through multiple mechanisms. These include the tight regulation of *Socs* gene expression (SOCS1-3 and CIS), which imparts temporal control to the system, the specificity of both phosphotyrosine-dependent SH2-binding (all SOCS), which relies on an active signal to provide targets, and non-canonical SH2 binding to JAKs (shown for SOCS3). SOCS1 and SOCS3 are also able to directly inhibit JAK1, 2 and TYK2 via their KIR, whilst all SOCS proteins form a SOCS box-mediated E3 ligase complex, resulting in the ubiquitination and proteasomal degradation of their target proteins (*Fig. 2*). These different mechanisms act in concert to orchestrate control of JAK/STAT signaling. However, many questions remain about the precise physiological function and *bona fide* targets of

the SOCS proteins, and we still lack complete detail as to how they act mechanistically to inhibit signaling. There is much to learn about this important family of negative regulators, providing exciting opportunities to contribute to our understanding of cytokine and growth factor signaling. Defining the physiological role of the SOCS proteins, combined with biochemical and structural data demonstrating how they regulate their targets, will identify niche opportunities for the development of novel therapeutics. In the present review, we discuss the molecular mechanisms by which the SOCS proteins exert stunning specificity *in vivo*, and address areas where key data are missing, in particular regarding the lesser-known SOCS4-7. We have not attempted to survey the entire field, but have described the mechanisms of SOCS protein function based on key examples from the literature and our own personal insights.

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SOCS3: the quintessential SOCS protein

As mentioned, SOCS3 is highly specific for several key cytokines. SOCS3 deficient animals die at embryonic day 10-13 due to excessive LIF signaling which disrupts normal placental development(16,18). Subsequent analysis of adult mice with restricted tissue deletion of *Socs3* demonstrated a non-redundant ability to inhibit signaling from Leptin, IL-6 family cytokines and G-CSF, reviewed in (19). Mechanistically, SOCS1 and SOCS3 contain a unique KIR region upstream of their ESS/SH2 domain that facilitates direct, non-competitive

inhibition of JAK proteins(20,21). More specifically, the SOCS3 interaction with the JAK/receptor complex requires a phosphorylation-dependent interaction between the SH2 domain and the gp130 receptor cytoplasmic domain, and a second interaction between the ESS/SH2 domain of SOCS3 and the kinase domain of JAK. The inhibition of JAK kinase activity together with the ubiquitination of bound substrates makes SOCS3 a potent negative regulator of JAK/STAT signaling. Detailed biochemical and structural information has been generated which describes the function of SOCS3 and we will use this as a template to describe SOCS function and to raise specific questions about how the other SOCS proteins might act.

Temporal control of SOCS protein function: the when

The first, and very simple way in which the SOCS proteins regulate the correct signal is by being present at the right time, and they are most often rapidly induced in response to STAT activation. This places the SOCS under the control of the signaling cascade/s that they then act to inhibit, forming a classical negative feedback loop. However, as discussed below, this is not always strictly the case.

SOCS1-3 and CIS are expressed at very low levels in most cells of the body in the steady state. They are rapidly induced by key cytokines, often within 60 minutes and sometimes to over 100 times their basal rate at a message level. For example, injection of mice with IFN-γ leads to increased SOCS1 mRNA and protein in the spleen within 60 minutes (22). SOCS3 is

also transcriptionally regulated by other stimuli, including bacterially-derived lipopolysaccharide (LPS). In bone marrow-derived macrophages, we observe expression of *Socs3* within 30 minutes of LPS addition, to levels above or comparable to that seen with IL-6 or GM-CSF induction at later time points. IL-10 induces SOCS3 through the activation of STAT3, however SOCS3 does not inhibit IL-10 signaling, but rather counteracts the pro-inflammatory action of IL-6, thus promoting the anti-inflammatory actions of IL-10(23,24). This example also hints at the complexity of these signaling cascades and some of the obvious challenges in untangling them at a signaling level; both IL-10 and IL-6 activate STAT3, whereas SOCS3 only inhibits signaling from the IL-6 complex. SOCS function outside of their roles as classic negative feedback inhibitors requires further attention, and has the potential to reveal regulation of new non-canonical targets or pathways.

In contrast, SOCS4-7 are generally expressed constitutively, albeit in specific tissues/cells and at low levels. Where they are induced, it is usually not as rapid and to a lesser extent than that observed for SOCS1-3 and CIS. SOCS5 for example, is almost ubiquitously expressed(8,25). SOCS5 expression can also be induced by IL-4 (Nicholson, unpublished) and forced expression can block IL-4-induced STAT6 transcription(26); however there remains no definitive evidence for SOCS5 as a physiological regulator of IL-4 signaling(25). As yet, it is unclear why SOCS4-7 are expressed more broadly. We hypothesis that these SOCS proteins may be primed to regulate their respective signaling cascades in unstimulated cells, a process that is likely to be linked to their extensive N-terminal

regions. Further understanding of their subcellular localization and the mechanisms that regulate protein levels in the steady state will be required to define their specific roles.

Identification and characterization of biochemical targets: the who

As with many signaling proteins, the function of any particular SOCS protein is intrinsically linked to the proteins that it interacts with and the complexes it forms; the SOCS interactome. Identification of SOCS binding partners and the subsequent characterization of both how the proteins interact (structural) and the mechanistic outcome of that interaction (function) is paramount to defining the role of that SOCS protein in the correct biological context. It should be noted that this family of proteins have been notoriously difficult to produce as recombinant proteins and this has hampered efforts to perform detailed structure/function analyses. Despite these difficulties, informative structural data has been generated. The Extended SH2 Subdomain (ESS)(27,28) makes significant contact with a hydrophobic patch underneath the phosphotyrosine binding pocket and aids in stabilizing the SH2 domain(12). In addition, the SOCS box and SH2 domain stabilize each other, and in some instances this contributes to SH2-mediated phosphotyrosine binding(11,29). The SOCS box itself is only semi-structured in the absence of the adapter proteins ElonginB and C(30), and this trimeric complex is thought to be constitutively present in cells. The inclusion of the ESS and SOCS box sequences in SH2 constructs, together with co-expression of ElonginB and C has formed the basis of a successful strategy for the production of recombinant protein and has resulted in crystal structures for SOCS2

and SOCS4(11,29). SOCS3 and CIS also contain a Proline, Glutamic acid, Serine, Threonine rich region or PEST motif inserted into their SH2 domains and deletion of this region from SOCS3 further enhances its stability in cells and the yields of recombinant protein(12,31). Finally, the N-terminal region of the SOCS proteins is predicted to be largely unstructured. Despite these difficulties, the targets of many of the SOCS-SH2 domains have been identified and characterized, predominantly using cell lines and overexpression studies. The following section discusses in more detail how the SOCS proteins interact with their targets and how this facilitates their functional capacity to inhibit signaling, either through ubiquitination, competitive binding or kinase inhibition.

SH2 domain and phosphotyrosine dependent binding

Tyrosine phosphorylation is one of the key events required to propagate signaling downstream of the JAK/receptor complex. Accordingly, many signaling proteins in these cascades contain phosphotyrosine binding domains, such as an SH2 domain, allowing them to 'dock' to this hub and carry out their function. SH2 domains bind to linear, tyrosine phosphorylated motifs and display varying preferences for the residues that flank the tyrosine, most commonly those amino acids C-terminal to the tyrosine(32). The SOCS-SH2 domains also demonstrate specificity for residues upstream of the tyrosine, creating an extended binding interface that results in higher binding affinities for their phosphorylated targets (11,12,29,33). The SOCS-SH2 domain is only functionally relevant if the correct phosphorylated target is present, and thus the SOCS rely on an active signal, adding a

further intrinsic level of regulation to the system. Phosphotyrosine-dependent binding of the SOCS-SH2 domain to its cognate target contributes to its ability to regulate signaling in two ways; firstly, localization to the correct target/receptor complex, which allows for ubiquitination/inhibition of bound targets, and secondly in some cases by competition with other signaling molecules for the same phosphorylated site. SOCS3 utilizes its SH2 domain to achieve both of these. The regulation of IL-6 family cytokines, Leptin and G-CSF signaling relies on the preference of the SOCS3-SH2 domain for tyrosine residues in these receptors. For example, SOCS3 binds the IL-6 receptor subunit gp130 pY757 with 110 nM affinity *in vitro*(34), directing SOCS3 to the correct receptor and additionally bringing it into close proximity to the JAKs. From here, SOCS3 can directly inhibit JAK activity via its KIR, ubiquitinate components in the receptor complex via its SOCS box and block access of the signaling molecule SHP2 to the gp130 receptor.

The identification of an SH2 domain target is greatly strengthened by the definition of the key tyrosine that mediates the binding event. Determining the kinetics of phosphorylation can also inform when and how the SOCS protein may be acting on that target. Using biophysical assays to determine binding affinities with recombinant protein and phosphorylated peptides, allows for a comparative analysis to identify physiologically relevant binding constants. Where possible, *in vitro* binding data should be functionally validated in cells through mutational analysis of candidate tyrosines either through immunoprecipitation and Western blotting of SOCS/target complexes and/or functional analysis of downstream signaling events. This sort of type has been invaluable in building a

complete picture of SOCS action. However, for some of the SOCS proteins the SH2 domain target is not yet known or the biological context for the interaction is unclear.

One such example is SOCS4. The SOCS4-SH2 domain binds with high affinity (0.5 μM) to a phosphopeptide corresponding to tyrosine 1096 within the Epidermal Growth Factor receptor (EGFR) cytoplasmic domain (29), and overexpression of the related SOCS5 protein (92% sequence homology across the SH2 domain) can lead to degradation of the EGFR(35,36). Expression of SOCS4 and SOCS5 is also negatively correlated with EGFR expression in patients with aggressive hepatocellular carcinoma(37). However, SOCS4 mutant mice, which harbor a mutation that introduces a stop codon at amino acid 108 of the N-terminal region and likely produces no functional protein, have no apparent defects in EGFR-mediated development or signaling (38)(Nicholson, unpublished observations). Thus it is unclear whether SOCS4 is a physiological regulator of EGFR signaling and if so, in what niche biological context this regulation may be important. Conversely, the regulation of c-KIT and Flt3 by SOCS6 has been nicely demonstrated both *in vitro* and in cells(33,39,40). SOCS6 binds to phosphorylated Tyr (pY)568 of c-KIT (0.3 μM), the receptor for stem cell factor, and pY591 and pY919 of the Flt3 receptor, and can functionally ubiquitinate and degrade these receptors. In cells, overexpression of SOCS6 reduces ligand-induced MAPK signaling and subsequently reduces proliferation. Its expression is also negatively correlated with patients with acute myeloid leukemia (AML) who harbor activating Internal Tandem Duplications (ITDs) of the Flt3 receptor. It will be of interest to

investigate whether the SOCS6 deficient animals demonstrate any c-KIT or Flt3 mediated pathologies, specifically in models of AML.

Competitive binding

Early analysis of the phosphotyrosine sites recognized by the SOCS-SH2 domains, revealed an overlap with binding sites for other SH2-containing signaling molecules, leading to the hypothesis that SOCS proteins would compete via their SH2 domain with proteins such as the STATs, to inhibit downstream signaling. For SOCS proteins that do not contain a functional KIR region, namely SOCS2 and CIS, it has been proposed that this is the main mechanism of inhibition, outside of ubiquitination(41-43) The strongest experimental evidence for competitive binding however, belongs to studies of SOCS3 in the gp130 receptor complex. The gp130 receptor subunit contains multiple tyrosine sites that are phosphorylated by the JAKs upon IL-6, LIF or IL-11-induced receptor oligomerization. The phosphatase SHP2 binds to phosphorylated Tyr757 within gp130, leading to the activation of MAPK and promoting mitogenic signaling(44). As this is also the docking site used by SOCS3, SOCS3 simultaneously inhibits JAK-mediated STAT3 activation and competes with SHP2 to block MAPK signaling(45,46). Mice containing a mutation of gp130 Tyr757 can no longer be regulated by either SOCS3 or SHP2, and show enhanced STAT and reduced MAPK activation following receptor engagement(46). This also raises the question as to whether SOCS3 inhibition of SHP2/MAPK activity has purposefully evolved, or whether this is simply a bystander effect of SOCS3 recruitment to the receptor complex. Addressing this

question has clear implications for the design of SOCS3-based therapeutics that either enhance or block its activity.

SOCS3-SH2 domain and phosphotyrosine independent binding

The canonical binding between a SOCS-SH2 domain and a specific tyrosine phosphorylated motif contributes to key aspects of SOCS function. However, greater insight into the function of the SOCS3-SH2 domain was revealed by the co-crystal structure of SOCS3 simultaneously bound to the JAK2 kinase domain, and a phosphopeptide corresponding to gp130 Y757. This structure demonstrated two interfaces on the SH2 domain; the canonical phosphopeptide interaction and on the opposing side of the SH2 domain, an extended interaction with the JAK2 kinase domain. This non-canonical interaction is mediated predominantly by the BC loop of the SH2 domain, ESS and KIR of SOCS3 and a hydrophobic patch centered on the 'GQM' motif of the JAK2 kinase domain C-lobe(21). These interactions allow the formation of a trimeric complex between SOCS3, JAK2 and the receptor, resulting in a high affinity and highly specific interaction module. Whilst the KIR is a unique feature of SOCS1 and SOCS3, these data raise the interesting possibility that other SOCS proteins may also form reciprocal, non-canonical interactions via their ESS/SH2 regions. The crystal structures of the SOCS4 and SOCS6-SH2 domains revealed that their ESS is positioned differently to that of SOCS2 and SOCS3(29,33) and it is hypothesized that this alternative packing of the ESS acts to support the longer N-terminal extension of SOCS4-7(29). With the exception of the SOCS3 KIR, we currently lack

structural data on the immediate N-terminal extensions of the SOCS proteins, and hence more data is required to validate these hypotheses.

KIR and the inhibition of kinases

Early observations indicated that SOCS1 and SOCS3 could directly inhibit JAK activation, and this function was attributed to a 12 amino acid stretch upstream of the SH2 domain that shared some sequence homology with the JAK activation loop(47,48). The so called Kinase Inhibitory Region (KIR) is critical for the inhibitory role of these SOCS and the most recent structural and biochemical evidence now provides a rational for these early observations(20,21). Through the non-canonical SH2 domain interaction with the JAK kinase domain, the KIR is positioned in the substrate-binding pocket of JAK, where it can block access of incoming JAK substrates. The *Drosophila* JAK homologue lacks the 'GQM' motif of mammalian JAK1, JAK2 and TYK2 and thus the KIR of SOCS1 and SOCS3 appears to have co-evolved to directly inhibit the expanded JAK repertoire in higher organisms(20)

Apart from the JAKs, SOCS proteins have also been shown to target a small number of alternate kinases. SOCS1 and SOCS3 can regulate Focal Adhesion Kinase (FAK)1 via an SH2 mediated interaction with a phosphotyrosine in the FERM domain of FAK1, and SOCS3 has been shown to directly regulate its enzymatic activity (49). Importantly, these observations have been validated in *Socs3* knockout mice, where SOCS3 expression during B cell development is critical for the negative regulation of chemokine-induced FAK activation

(50). SOCS3 also binds Breast tumor kinase (Brk) (also called Protein tyrosine kinase 6) via its SH2 domain and can inhibit Brk-mediated STAT3 activation via its KIR(51,52). SOCS2 binds active Proline-rich tyrosine kinase 2 (Pyk2) via pY402 and ubiquitinates it in an IL-15-dependent manner in Natural Killer (NK) cells(53), and SOCS6 is able to regulate the active form of the T cell specific kinase p56Lck via the proteasome(54). Complimentary biochemical analysis of these putative targets will help establish whether these interactions are direct and if there are additional receptors/adaptor proteins required. There is however, a precedent for SOCS proteins interacting with kinases independently of a receptor interaction. The regulation of type I IFN signaling by SOCS1 was shown to not require any of the phosphorylation sites in the IFNAR1 receptor, but was a direct, SH2 and KIR-dependent interaction with Tyk2(55).

The N-terminal region – An intrinsically disordered understanding

Although the N-terminal region of the SOCS proteins is highly variable across the family, for any particular SOCS protein it is well conserved across species(56) indicating a potential conservation of function. The SOCS N-termini are predicted to be largely unstructured and to date, share no sequence homology with known protein domains(8,56). This in part, has resulted in little functional data or characterization of these regions. The first 20 amino acids of SOCS3 do not appear to contribute to SH2 domain binding, inhibition of JAK or ubiquitination of substrates, and have been routinely omitted from recombinant protein constructs. It is currently unclear whether CIS, and SOCS1-2 have a functional N-terminal

region. This point is worth exploring as CIS and SOCS1, in particular, contain ~40 amino acids in addition to their ESS/KIR region (*Fig. 3*).

~ Figure 3 here ~

SOCS4-7 are distinguished from the other four family members by long N-terminal extensions. The N-termini of these SOCS proteins constitute over 50% of the protein sequence (368 of 536 amino acids for murine SOCS5) and are unlikely to have evaded any evolutionary culling without retention of function. Indeed, over the past two decades it has become increasingly clear that proteins containing regions of disorder have important roles in cell signaling, transcription, and transportation. Strikingly, approximately 30% of all Eukaryotic proteins contain sequences over 30 amino acids that are predicted to be intrinsically unstructured(57). Intrinsically disordered proteins (IDP) are commonly well conserved at the amino acid level and show distinct functional capacity compared to globular domains(58,59). They can provide larger interacting surfaces, afford conformational flexibility and often contain multiple short linear peptide motifs. In addition, a number of intrinsically disordered proteins fold upon binding, a feature that allows interaction with multiple different proteins, often with low affinity but with very high specificity. We hypothesis that the N-terminal region of the SOCS proteins will act as scaffolds, mediating multiple protein interactions that are likely to be regulated by post-

translational modification, and will therefore aid in protein localization and recruitment of substrates for ubiquitination (*Fig. 1*).

Only one region of amino acid homology has been identified within the SOCS N-termini and this is shared between SOCS4 and SOCS5 (~70 residues), and is highly conserved from mammals to lower vertebrates(56). Although the majority of this region appears to be disordered, this conserved region contains segments predicted to adopt short alpha helices, an observation that was validated by NMR using a recombinant fragment corresponding to the mouse SOCS4 fragment(56). Somewhat surprisingly, the equivalent region of SOCS5 binds directly to the JAK kinase domain (K_D 0.5 μM binding to JAK1). Overexpression of full-length SOCS5 in cells inhibits JAK1 and JAK2 activity, a function that relies on a region in the N-terminus, which includes this conserved fragment(60). It is now of interest to determine how a predominantly intrinsically unstructured region is able to interact with the JAK kinase domain, and how the surrounding sequences of the SOCS5 N-terminus may contribute to inhibition of JAK activity. SOCS6 also contains an apparently unrelated region in its N-terminus (amino acids 47-218) that mediates binding to the kinase domain of p56Lck(54), suggesting a common mechanism within the family.

Of the three *Drosophila* SOCS homologues, SOCS36E has been the most extensively characterized and is closely related to the mammalian *Socs5* gene (78% amino acid homology across the SH2 domain)(61). SOCS36E has been elegantly demonstrated to negatively regulate both JAK/STAT and EGFR signaling in normal development and

tumorigenesis(61). Most recently, the SOCS36E SOCS box, SH2 domain and N-terminal region have been shown to have distinct and overlapping functions in the regulation of Dome receptor interaction, degradation, and inhibition of receptor phosphorylation(62), suggesting that this SOCS5 homolog acts to inhibit signaling by utilizing its domains in a combinatorial manner. Interestingly, these effects were not via inhibition or interaction with Hopscotch(62), and thus the mechanism appears to differ both from the JAK interaction observed for mammalian SOCS5(60) and from the ability of SOCS1 and SOCS3 to directly regulate JAK activity(20).

It is clear that a functional understanding of the SOCS N-terminal regions has proven intrinsically difficult to define, but the N-termini are likely to make subtle but important contributions to the function of CIS, SOCS1 and SOCS2, and to have a critical role in the function of SOCS4-7.

The SOCS box and ubiquitination of substrates

Post-translational modification of proteins by the covalent attachment of ubiquitin and the related ubiquitin-like proteins (UBLs) can influence protein function in a variety of ways, including subcellular localization, protein-protein interactions and the formation of larger complexes, degradation, as well as regulation of enzymatic activity(63). E3 ubiquitin ligases mediate the attachment of ubiquitin to a lysine residue on the target substrate. Ubiquitin itself has seven lysine residues upon which additional ubiquitin molecules can be added to

form either linear or branched polyubiquitin chains. Of these, K48 linear ubiquitination remains the most well-characterized and commonly results in degradation of the tagged protein by the 26S proteasome(64).

The SOCS proteins form part of a larger family of E3 ubiquitin ligases termed the Cullin-Ring E3 Ligases (CRLs)(9,10,30,65). The E3 ligase is the final step in an enzymatic cascade that requires an E1 activating and E2 conjugating enzyme and results in the covalent transfer of a ubiquitin molecule to a SOCS-bound substrates(66). The SOCS box motif consists of two conserved motifs important for binding to ElonginC (BC box) and Cullin5 (Cul box)(30). The heterodimer of ElonginB and C stabilizes the SOCS box, and this trimeric complex binds to the N-terminal domain of the scaffolding protein Cullin5. Cullin5 also binds the RING box protein Rbx2 on its opposing C-terminal region. Together this multi-protein complex forms an active E3 ligase. The specific E2 conjugating enzyme/s that serve the SOCS box CRLs are not yet defined, although *in vitro*, UbcH5a, 5b, 5c and UbcH3 facilitate this reaction(67). The SOCS proteins primarily add K48-linked ubiquitin chains and many targets have been identified(68). The majority of information has been achieved through the use of proteasome inhibitors (such as MG132), the analysis of substrates using overexpression of ubiquitin, and with ubiquitin linkage-specific antibodies. SOCS2, CIS and SOCS4-7 display a higher affinity for Cullin5 when compared to SOCS1 and SOCS3(65), and in general rely on their SOCS box to regulate substrates(33,35,36,40,43,69-71). However, SOCS1 and SOCS3 also utilize their SOCS box to efficiently inhibit signaling, as evidenced by the ameliorated phenotypes of mice genetically engineered to lack only the SOCS box

sequence(72-74). In an *in vitro* ubiquitination assay, an active SOCS3-E3 ligase complex was able to ubiquitinate both the gp130 receptor and the JAK kinase domain, albeit with differing kinetics(67). Multiple ubiquitin linkages were detected on a number of different lysine residues on these substrates, and whilst these assays do not recapitulate the complex cellular environment in which these reactions normally occur, they indicate that there may be flexibility in this system(67). Identification of the modified lysines on target proteins would allow for a temporal analysis of the construction of the ubiquitin chains and would build a clearer picture of when the SOCS box is called into(*Fig. 2*).

Spatial control of SOCS protein function: the where

In general, the SOCS proteins reside in the cytoplasm and are recruited to the receptor/membrane region in response to an activating signal (phosphorylation of target sequence). However, an in-depth analysis of SOCS protein localization under various conditions has not been extensively undertaken. What happens to the SOCS protein after it has engaged and inhibited/ubiquitinated its targets is currently not well resolved. Tracking the localization and movement of these transiently induced and often short-lived proteins with detailed microscopy studies would enhance our understanding of how these proteins function. Fluorescent tags such as GFP have been successfully used to define the movement of dynamic intracellular proteins, including the STATs(75). The increased use and development of the CRISPR/Cas9 technology(76,77) may also facilitate the incorporation

of fluorescent or other tractable tags into *Socs* loci to enable analysis of the endogenous proteins.

Outside of their established roles as cytoplasmic proteins, there is experimental evidence showing that SOCS1 can localize to the nucleus and degrade NF-κβ(78), and a Nuclear Localization Signal (NLS) has been mapped to a loop region located between the SH2 domain and SOCS box(79). SOCS1 has also been shown to localize to the nuclei of bronchial epithelial cells of asthmatic patients, where it suppresses rhinovirus-induced interferon production. However, this effect was independent of the SOCS1-SOCS box and NF-κβ degradation and hence the precise mechanism of action in this system is unclear(80). Mice in which only the SOCS box of SOCS1 has been deleted, demonstrate a reduced capacity to regulate IFN-γ signaling, highlighting the ancillary role of the SOCS box relative to the inhibitory function of the KIR/SH2 domain (74). This deletion also encompass half of the putative NLS(81), and hence it is possible that some aspects of the phenotype in these SOCS box deficient mice may also be due to perturbed nuclear localization.

Based on a Eukaryotic Linear Motif (ELM) analysis of their amino acid sequence, SOCS4-7 also contain putative nuclear localization signals in their N-termini(56), although these still need to be validated experimentally. Supporting evidence is available for SOCS6 and SOCS7, which have been detected in the nucleus, where they are suggested to regulate STAT3(82) and NCK(83), respectively.

A note on redundancy

A fundamental question regarding the SOCS proteins is whether any functional redundancy exists within the family. This is due to the degree of sequence homology within the SH2 domains, as well as the number of family members that act on related pathways, bind like targets and use similar mechanisms to inhibit signaling. In particular, SOCS6 and SOCS7 appear to share similar expression patterns in the brain and both can bind Insulin Receptor Substrate (IRS) proteins(84,85). Compound SOCS6/7 knockout mice will be required to address their relative contribution and redundancy in the development and function of neurons and in particular to the regulation of insulin signaling. Apart from SOCS6 and SOCS7 however, we are not aware of any definitive data that suggests functional redundancy between SOCS family members. Whilst the SOCS proteins often target similar pathways, their specific function *in vivo* appears to be unique. This is likely due in part to either the magnitude or timing of induction, or differential expression in specific cell types. There may also be different affinities for targets or differences in subcellular localization that preclude redundancy. For example, whilst the SH2 domain and conserved N-terminal regions of SOCS4 and SOCS5 can bind the same targets *in vitro* and in cells(29,60), exogenous SOCS5, but not SOCS4, can inhibit IL-4-induced STAT6 transcription, presumably because only SOCS5 is recruited to the IL-4 receptor complex through its N-terminus (Nicholson, unpublished). We would predict that any redundancy within the SOCS family will be highly context dependent and will require careful analysis of compound

knockout animals. Addressing these questions will be particularly important in the context of any SOCS-derived therapeutics.

Therapeutic intervention or, how to target a negative regulator

One of the underlying drives to study and understand this family of small, intracellular negative regulators of cytokine signaling, is the idea that understanding their function, interacting proteins and their physiological role will help identify novel therapeutic opportunities to treat diseases that are driven by excessive cytokine responses. Conceptually, it is easier to inhibit the function of a protein, either through blocking a binding event or negating enzymatic function, than it is to enhance the activity of a negative regulator. These challenges will require innovative approaches that utilize our knowledge of SOCS function. One such approach would rely on the design of small molecules that mimic the physiological action of the KIR/ESS/SH2 of SOCS1 and SOCS3, and takes advantage of their exquisite specificity in targeting JAK1, JAK2 and TYK2.

To date, effort has been mostly aimed at increasing the expression of SOCS proteins in target cells during pathological inflammatory signaling, such as rheumatoid arthritis and in solid tumors where growth is driven by STAT activation. Forced expression of a SOCS protein may be an effective treatment option where the culprit cytokine is well defined. Delivery of SOCS1 and SOCS3 constructs through the use of oncolytic adenovirus or as recombinant, cell-permeable proteins has been shown to efficiently control aberrant

signaling in hepatocellular carcinoma(86,87) and in LPS and IFN-γ driven inflammation(88,89), respectively.

Concluding remarks

The discovery of the SOCS proteins has helped to define how cells control normal cytokine signaling. The dramatic phenotypes of the SOCS1 and SOCS3-deficient mice demonstrated the fine specificity as well as the global physiological importance of these proteins. However, it has since become clear that SOCS proteins often provide a subtle modulation of signaling cascades, and that loss of the SOCS protein does not always result in profound pathological phenotypes. Despite some of these more subtle effects, understanding SOCS function will provide us with key insights on how to modulate and potentially intervene in pathologies that are driven by too much, even just a little too much, cytokine input.

Figure legends

Figure 1 Domain comparison between SOCS3 and SOCS5. These proteins are archetypal for the two different groups of SOCS proteins. Both SOCS3 and SOCS5 can ubiquitinate substrates via their SOCS box and bind tyrosine phosphorylated proteins via their SH2 domains. Whilst SOCS3 can bind and inhibit JAK via its SH2/ESS/KIR interface, SOCS5 contains a distal region in its N-terminus that can bind to JAK. Note that the KIR is a unique feature of SOCS1 and SOCS3. pY: phosphotyrosine, NTR: N-terminal region, PEST: Proline Glutamic acid Serine Threonine rich region, SH2: Src Homolgy 2, ESS: Extended SH2 sub domain, SB: SOCS box, SBE: SOCS box extension.

Figure 2 Conservation of the CIS and SOCS1 N-terminal extensions. Sequences preceding the SH2 domain from the listed species were collected from Uniprot for SOCS1 and CIS and aligned in with ClustalX. A red star indicates complete sequence homology. The N-terminal extension is longer for SOCS1 and CIS compared to SOCS2 and SOCS3 and may contribute to their function.

Figure 3 Schematic of temporal SOCS protein function. SOCS induction closely follows pathway activation. Pathway activation provides phosphorylated substrates for SOCS binding. Increasing SOCS expression then allows for competitive binding and inhibition of JAK kinases. Ubiquitination of substrates requires additional components and in the case of SOCS1 and SOCS3 provides an additional layer of regulation, possibly by eliminating remaining active signals and returning the cell to homeostasis.

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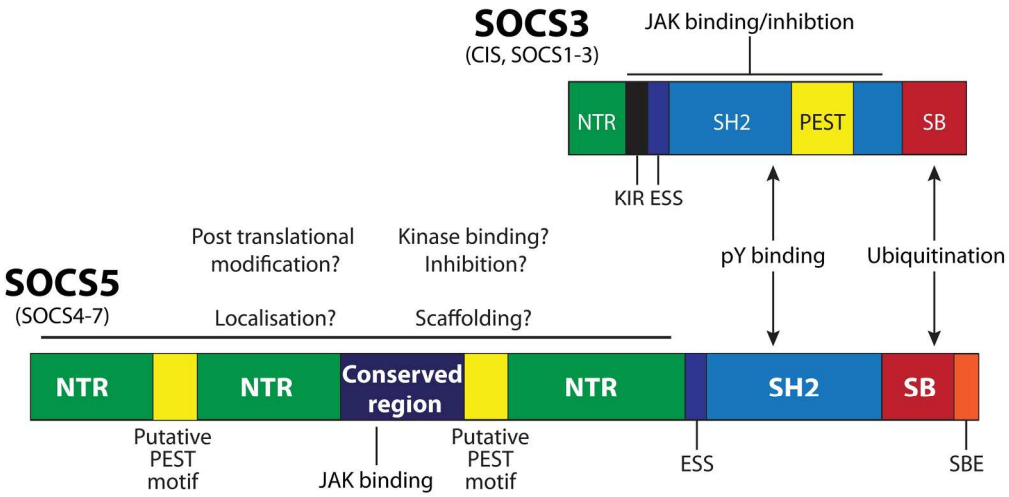

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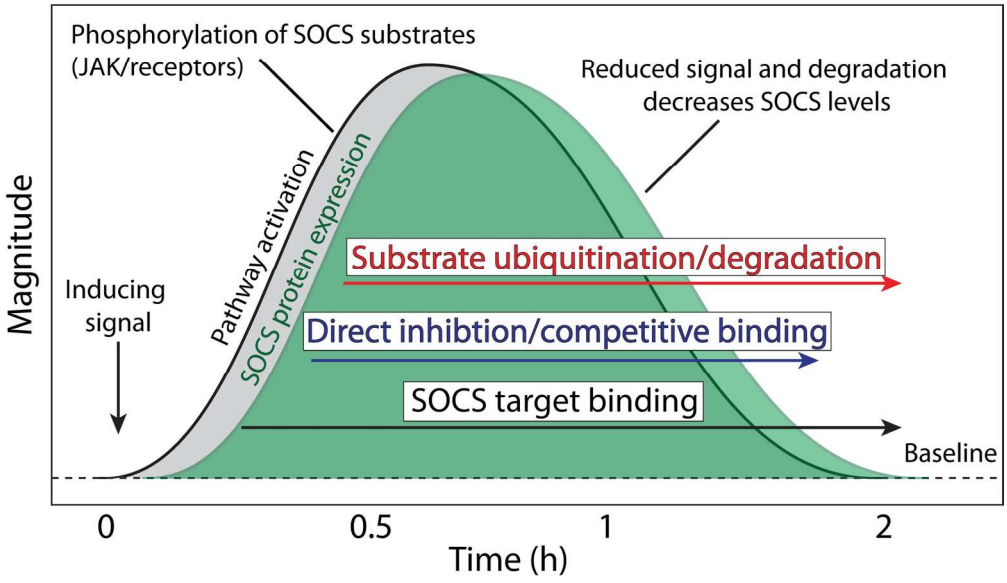

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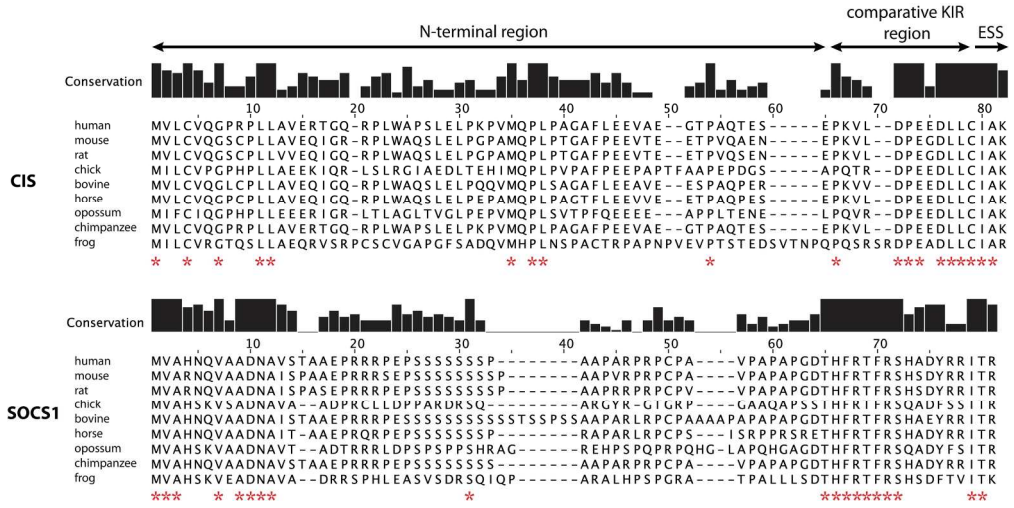

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