Understanding SOCS protein specificity

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Key words: cytokine, SOCS, JAK, STAT, SH2, signaling

Abstract

The development and activity of our immune system is largely controlled by the action of pleiotropic cytokines and growth factors, small secreted proteins which bind to receptors on the surface of immune cells to initiate an appropriate physiological response. Cytokine signalling is predominantly executed by intracellular proteins known as the Janus Kinases (JAKs) and the Signal Transducers and Activators of Transcription (STATs). Whilst the 'nuts and bolts' of cytokine activated pathways have been well established, the nuanced way in which distinct cellular outcomes are achieved and the precise molecular details of the proteins which regulate these pathways are still being elucidated. This is highlighted by the intricate role of the Suppressor Of Cytokine Signalling (SOCS) proteins. The SOCS proteins act as negative feedback inhibitors, dampening specific cytokine signals to prevent excessive cellular responses and returning the cell to a homeostatic state. A great deal of study has demonstrated their ability to inhibit these pathways at the receptor complex, either through direct inhibition of JAK activity or by targeting the receptor complex for proteasomal degradation. Detailed analysis of individual SOCS proteins is slowly revealing the complex and highly controlled manner by which they can achieve specificity for distinct substrates. However, for many of the SOCS, a level of detail is still lacking, including confident identification of the full suite of tyrosine phosphorylated targets of their SH2 domain. This review will highlight the general mechanisms which govern SOCS specificity of action and discuss the similarities and differences between selected SOCS proteins, focusing on CIS, SOCS1 and SOCS3. Due to the functional and sequence similarities within the SOCS family, we will also discuss the evidence for functional redundancy.

Introduction

The cellular response to cytokines and growth factors is predominantly driven by the activity of protein tyrosine kinases. They are either an intrinsic part of the receptor cytoplasmic domain (receptor tyrosine kinases, RTKs) or are found associated with the receptor cytoplasmic domain (Janus Kinases, JAKs). Upon ligand binding the kinases become activated, resulting in the phosphorylation of tyrosine residues within the receptor intracellular region, as well as phosphorylation of other signalling proteins which are recruited to the receptor complex. The most critical of these are the Signal Transducers and Activators of Transcription (STATs). Once phosphorylated, they translocate to the nucleus, initiating a transcriptional response to translate the initial cytokine "message" into the correct cellular outcome. There are over 30 cytokines which signal through approximately 40 receptors, leading to the activation of one or

more of the four JAKs and subsequently one or more of the seven STATs (Kiu and Nicholson 2012). Whilst the components of these pathways have been identified and the hierarchy of activation is understood to occur in a somewhat linear fashion (the effector transcription factor is activated at the cell surface receptor), the complexity and specificity of responses initiated by these highly-related pathways is still not fully understood. The pleiotropic nature of many cytokines hints that there is more going on 'under the hood' than initially appreciated (O'Shea and Murray 2008; Delgoffe, Murray, and Vignali 2011). For example, interleukin (IL)-4 activates distinct transcriptional profiles in T cells versus macrophages, despite utilising essentially the same core signalling molecules, JAK1/3 and STAT6, in both cell types (Murray 2007). This illustrates that additional levels of regulation and cell type-specific mechanisms exist to ensure the correct cellular interpretation of the external signal.

This concept is also pertinent to the Suppressor Of Cytokine Signalling (SOCS) proteins, an important family of negative regulators. The eight SOCS family members found in mammals (CIS and SOCS1-7) share a conserved domain architecture consisting of an N-terminal region of varying length and sequence, a central Src Homology 2 (SH2) domain and a C-terminal SOCS box motif (Figure 1) (Hilton et al. 1998). CIS and SOCS1-3 are further distinguished by a short N-terminal region (33-69 residues) (Feng et al. 2012) and their rapid induction in response to cytokine stimulation (Starr et al. 1997). In comparison, SOCS4-7 have much longer N-termini (270-385 residues) (Feng et al. 2012) and are often constitutively expressed (Hilton et al. 1998). The SOCS box motif recruits an E3 ubiquitin ligase complex consisting of Elongins B and C, Rbx2 and Cullin5 (Kamura et al. 1998; Zhang et al. 1999) (Cullin-RING ubiquitin ligases; CRL), and the SOCS proteins therefore inhibit signalling through the binding, ubiquitination and degradation of intracellular proteins, commonly at the receptor complex (Linossi and Nicholson 2012). In addition, SOCS1 and SOCS3 can directly inhibit JAK kinase activity (Babon et al. 2012; Liau et al. 2018).

SOCS activity is intimately linked to the specificity of the SOCS-SH2 domain, as this dictates the signalling molecules, and therefore pathways, they regulate. Despite this simple observation, for many family members the precise physiological targets and cellular context in which they act are still being elucidated. A key example is CIS or Cytokine Inducible SH2-Containing protein (encoded by the *Cish* gene and the first SOCS family member to be discovered (Yoshimura et al. 1995)). Preliminary analysis of CIS-deficient animals indicated no obvious phenotype in the steady state; comment from Marine et al. (1999) indicated "CIS-

deficient mice have no detectable phenotype including alterations in embryonic or adult erythropoiesis (unpublished data)". This was at a time when the SOCS1, SOCS2 and SOCS3-deficient mice displayed dramatic and lethal phenotypes that were highly specific for the cytokine or growth factor pathway they regulated (Starr et al. 1998; Alexander et al. 1999; Marine et al. 1999; Metcalf et al. 2000). More recent analyses of CIS-deficient mice have revealed a range of immunomodulatory roles for CIS including a key role in limiting the anti-tumour response of CD8+ T cells and Natural Killer (NK) cells (Yang et al. 2013; Palmer et al. 2015; Delconte et al. 2016; Putz et al. 2017).

Many SOCS proteins inhibit overlapping pathways when exogenously expressed, despite their apparent specificity in vivo and this raises interesting questions as to what regulates the activity of different SOCS under discrete cellular contexts and whether there is any functional redundancy? Whilst the general mechanisms utilised by the SOCS are well established, the nuances that govern their specificity as well as the full suite of molecules they target, are still being investigated.

Figure 1 here

Understanding SOCS specificity

The function of a SOCS protein is predominantly determined by two key factors. The first, and seemingly obvious one, is that they can only regulate a pathway or target molecule if they are expressed. The induction and turnover of the SOCS proteins, particularly CIS, SOCS1, SOCS2 and SOCS3, is tightly regulated. The SOCS proteins are commonly induced by the signal they then act to regulate, acting in a classic negative feedback loop. Paradoxically, the SOCS can also be induced in response to different cytokines or stimuli that they do not directly regulate (Palmer and Restifo 2009), and there are several examples where SOCS induction by one pathway can be linked to the suppression of opposing or parallel signalling pathways. For instance, IL-6 induces SOCS1 expression in CD4+ T helper (Th) cells which negatively regulates the Th1 cytokine IFNγ and the Th2 cytokine IL-4, promoting Th17 differentiation. Similarly, SOCS3 expression in Th cells blocks the activation of the Th1 and Th17 stimuli, IL-12 and IL-6, to promote Th2 differentiation (reviewed in (Yoshimura et al. 2012)). In addition, SOCS3 is induced by IL-10 (which it does not directly regulate) to limit IL-6-driven STAT3

responses and augment the anti-inflammatory roles of IL-10(Yasukawa et al. 2003; Lang et al. 2003). Indeed, pre-treatment of bone marrow macrophages with a range of SOCS3-inducing stimuli (IFNγ, LPS, IL-6 and IL-10) reduces IL-6-mediated STAT3 activation (Lang et al. 2003). Cells are often exposed to a myriad of cytokines or stimuli in vivo, and the induction of multiple SOCS at varying levels no doubt helps fine-tune the message to achieve the correct response. In addition, the rapid induction of multiple SOCS may cast a wider net to limit excessive signalling, for example, in response to an episode of inflammation and the complex milieu it creates. It should be noted that robust reagents for the confident detection of some of the endogenous SOCS proteins are still lacking, thus the correlation between mRNA expression and protein levels is often not clear, nor is the relative expression of different SOCS at the protein level. Thus, caution is required when ascribing function to a SOCS protein based purely on its mRNA induction.

The second major determinant of SOCS function is the specificity of their SH2 domain for distinct phosphotyrosine containing sequences in target proteins. This adds an intrinsic level of regulation to SOCS function, as their target substrate must be phosphorylated and this often equates with pathway activation (Hunter 2014). Thus, the SOCS simultaneously engage the target and the components of the E3 ubiquitin ligase machinery in a highly dynamic but controlled manner, and can therefore be classed as substrate recognition modules, targeting bound molecules for degradation via the proteasome. Interestingly, the SOCS-SH2 domain also contains several unique features, which suggests it has evolved to perform more tasks than simply distinguishing phosphorylated substrates.

The phosphotyrosine-binding SH2 domain is the prototypical ‘modular’ protein-protein interaction domain and is found in over 100 unique mammalian proteins, with many studies aimed at globally characterising the structural and biochemical similarities and differences of this domain (Sadowski, Stone, and Pawson 1986; Songyang et al. 1993; Songyang et al. 1994; Liu et al. 2006; Huang et al. 2008). Structurally, the SH2 domain is composed of a central anti-parallel beta (β) sheet that is flanked by two alpha (α) helices to create two major binding sites (Waksman et al. 1992; Waksman et al. 1993) (*see* v-Src; Figure 2A). The first pocket accommodates the negatively charged phosphate group, which is predominantly coordinated by an invariant arginine located on the βB strand. The so called ‘specificity pocket’ sits on the opposing side of the central β-sheet and is generally formed by residues from the loop regions

(denoted DE, EF and BG) and βD and βE strands (Waksman et al. 1993; Liu et al. 2006; Kaneko et al. 2010) (Figure 2A). The positioning of the various loops and their amino acid composition make major contributions to the recognition of distinct phosphotyrosine sites (Kaneko et al. 2010; Liu, Engelmann, and Nash 2012). These loop regions help determine which amino acids are preferred or accommodated in target sequences. For most SH2 domains, selective binding to the target sequence is determined by residues C-terminal to the phosphotyrosine residue which engage the specificity pocket. Consensus binding motifs for individual SH2 domains have been derived from the analysis of short linear phosphopeptides (Songyang et al. 1993; Huang et al. 2008), although most experimental approaches have only identified residues which are permissive for binding, and it is now clear that non-permissive residues, even those distal to permissive residues, can impact on peptide binding(Liu et al. 2010).

The SOCS-SH2 domains have largely escaped systematic analysis in these studies, mostly because they were difficult to express and purify as recombinant proteins. This explains at least in part, why the field currently lacks the same level of detail available for other SH2 domains. However, dedicated studies have established conditions for the production of recombinant SOCS proteins and helped elucidate key biochemical and structural features of the SOCS-SH2 domain (Nicholson et al. 2000; De Souza et al. 2002; Krebs et al. 2002; Babon et al. 2005; Babon et al. 2006; Bergamin, Wu, and Hubbard 2006; Bullock et al. 2006; Bullock et al. 2007; Zadjali et al. 2011; Babon et al. 2012; Kershaw et al. 2013; Liau and Babon 2018; Liau et al. 2018). To date, various structures of five family members have been published (PDB IDs: SOCS1 6C5X, 6C7Y, SOCS2 4JGH, 5B04, 2C9W, SOCS3 2BBU, 2HMH, 4GL9, 2JZ3, SOCS4 2IZV, SOCS5 2N34, SOCS6 2VIF), with the structures of SOCS3 (gp130 pY757) and SOCS6 (c-Kit pY568) solved bound to phosphorylated peptides.

Structural distinctions of the SOCS SH2 domains

The first point of difference from the canonical SH2 domain structure is the presence of an additional α-helix immediately N-terminal to the SOCS-SH2 domain, termed the extended SH2-subdomain (ESS) (Yasukawa et al. 1999) (Figure 2B-D). This forms part of the 'modular' SOCS-SH2 domain, making direct contact with the BG loop and other residues that form the phosphopeptide binding pocket (Babon et al. 2006). The ESS also contributes to a second unique binding surface present in SOCS1 and SOCS3 (discussed below) and is thought to provide some stability between the hydrophobic interfaces of the SOCS box and SH2 domain

(Bullock et al. 2006; Bullock et al. 2007). A number of SH2 domain proteins contain adjacent domains whose proximity or interaction with the SH2 domain regulates protein function, contributing to substrate specificity, affinity or intermolecular interactions (Liu, Engelmann, and Nash 2012). Notably, the SH2 domains of STAT1b, STAT3 and Cbl also contain an additional α-helix (as part of adjoining domains or linkers), although in each of these the α-helix is positioned differently to the ESS of the SOCS (Babon et al. 2006; Bullock et al. 2007).

It is clear from the existing structures and alignment of key structural features, that the SOCS-SH2 domains exhibit significant diversity in their loop length and sequence (Zadjali et al. 2011; Bullock et al. 2007). For example, SOCS1 contains an extremely short EF and BG loop compared to the other SOCS (Figure 2) (Liau et al. 2018). The sequence of the loop regions thought to determine binding to the pY+4 position also vary across the SOCS family (Kaneko et al. 2010). Additional structures of SOCS-SH2 domains bound to phosphorylated peptides will further delineate the contribution of various structural features to binding specificity.

Another unusual feature of the CIS and SOCS3 SH2 domains is the presence of an unstructured loop, which is inserted between the αB helix and the BG loop (Babon et al. 2005) (Figure 2D). This region has no apparent bearing on phosphopeptide binding and has been designated as a putative Proline, Glutamine, Serine and Threonine (PEST) motif (Babon et al. 2005; Babon et al. 2006); a sequence commonly involved in the regulation of protein stability (Rogers, Wells, and Rechsteiner 1986). The exact function of the PEST motif in these two SOCS proteins is unclear, but it doesn't appear to alter phosphopeptide binding in vitro (CIS; unpublished) and for SOCS3 appears to regulate its stability in cells (Babon et al. 2005; Babon et al. 2006) (Figure 2D). A recent report suggests that Cavin-1 binding to the SOCS3 PEST motif is important for SOCS3 localisation to the plasma membrane (Williams et al. 2018). Cavin-1$^{-/-}$ fibroblasts displayed enhanced STAT3 phosphorylation in response to IL-6, leukemia inhibitory factor (LIF) and oncostatin M (OSM), whilst the proportion of endogenous SOCS3 present in the plasma membrane fraction of these cells was reduced (Williams et al. 2018). Cavin-1 is clearly not required for all SOCS3-dependent functions, as Cavin-1-deficient mice don't phenocopy Socs3 null mice, which die prematurely from excessive LIF signalling (Robb et al. 2005; Liu et al. 2008); implicating a cell-type specific interplay between cavin-1, SOCS3 and IL-6 signalling. Nonetheless, this study identifies the first interacting protein for the SOCS3-PEST motif and may predict that additional proteins, either in distinct cell types or for the other SOCS, may regulate their sub-cellular localisation.

The kinase inhibitory region or KIR of SOCS1 and SOCS3 represents another distinctive feature related to the SOCS-SH2 domain (Figure 2B). Whilst this region sits upstream of the modular SH2 domain, its binding to the substrate pocket of the JAK1, JAK2 and TYK2 kinase domains relies on an approximately 1,000Å surface that consists of the KIR, BG loop and the ESS (Kershaw et al. 2013; Liau et al. 2018) (Figure 3C & D). This phosphotyrosine-independent interface on the SH2 domain of SOCS1 and SOCS3 allows them to position their KIR in the substrate binding groove of the JAK kinase domain, potently inhibiting JAK activity (Babon et al. 2012; Liau et al. 2018). Interestingly, despite the proximity of this binding surface to the phosphotyrosine binding pocket (Figure 3D), phosphopeptide binding has no impact on the inhibition of JAK and conversely, the KIR interaction with JAK doesn't alter phosphopeptide affinity. For SOCS3, the two binding events are complimentary, creating a high affinity and specific complex between JAK, receptor and SOCS (Babon et al. 2012; Kershaw et al. 2013; Liau et al. 2018).

The other SOCS family members do not contain a functional KIR in this position, and it remains to be determined whether the region upstream of their SH2 domain contributes to binding or regulation of target substrates. We have previously identified a semi-structured motif in the N-terminus of SOCS4 and SOCS5 which can bind to the kinase domain of the JAKs (Feng et al. 2012; Linossi et al. 2013; Chandrashekaran et al. 2015) (Figure 1), and SOCS6 binds to the tyrosine kinase Lck via an extended region in its extended N-terminus (between amino acids 47-218) (Choi et al. 2010). Whilst the full relevance of these interactions requires investigation, it remains plausible that additional regulatory features and determinants of specificity will be found in the SOCS-N-terminal regions, in addition to the presence of non-canonical binding sites on their SH2 domains.

Figure 2 here

Phosphotyrosine binding and specificity

Many candidate binding proteins for the SOCS-SH2 domains have been identified and have predominantly been interrogated using overexpression studies. SH2 domains can bind to non-physiological targets when expressed at high levels and the SOCS are no exception, as

evidenced by their promiscuous inhibition of multiple pathways under such conditions (Croker, Kiu, and Nicholson 2008). The full suite of physiological protein targets for individual SOCS is yet to be defined, including those that lie outside the JAK-STAT pathways.

Most SH2 domains have a preference for residues C-terminal to the phosphotyrosyl residue and this commonly extends to pY+4 residues (Waksman et al. 1993; Huang et al. 2008), resulting in binding affinities in the low micromolar to high nanomolar range for physiologically relevant targets (Ladbury et al. 1995). A number of studies have suggested that SH2 domains also show specificity for residues N-terminal to the tyrosine and this appears to be the case for SOCS3, SOCS4, SOCS5 and SOCS6 (Nicholson et al. 2000; Krebs et al. 2002; De Souza et al. 2002; Bullock et al. 2006; Bullock et al. 2007; Zadjali et al. 2011; Linossi et al. 2013). Although equivalent information is not available for the remaining family members, this may be a characteristic of the SOCS-SH2 family.

The SH2 domain of SOCS3 binds with high affinity (Kd: 50 nM) to a single key tyrosine from the shared IL-6 signaling receptor (pY757 of the mouse gp130 protein) (Nicholson et al. 2000; Babon et al. 2005); SOCS3 also has binding sites in the granulocyte colony stimulating receptor (G-CSFR), leptin (LepR) and erythropoietin receptors (EpoR), reviewed in (Babon and Nicola 2012). The high affinity is achieved through an extended interaction which relies on residues both N- and C-terminal to the key phosphotyrosine in gp130 (position pY-2 Val and pY+3 and +4 Val) (Figure 3C). A similar extended interface was observed in the crystal structure of the SOCS6-SH2 domain bound to a phosphorylated peptide from the c-Kit receptor (pY568, Kd 300 nM), which displays extensive contacts between the SH2 domain and peptide residues spanning the pY-1 Asn to the pY+6 Thr (Zadjali et al. 2011) (Figure 3B). As with SOCS3, the BG and EF loops envelope the phosphopeptide providing extensive contacts with the C-terminal peptide tail (Figure 3D). SOCS1 shows high affinity to the activation loop tyrosines of the different JAKs (as linear peptides; Kd 100-600 nM) (Liau et al. 2018) and it was suggested by Liau and colleagues that in this instance, the short EF and BG loops of the SOCS1-SH2 domain may help accommodate the restricted activation loop region of JAK that is wedged between the N- and C-lobes of the kinase domain. As discussed below however, whether the activation loop tyrosines are the physiological targets of the SOCS1-SH2 domain remains unresolved.

Figure 3 here

Our analysis of SOCS-SH2 binding preferences in vitro suggests that there is some overlap within the family for different phosphopeptides. For example, CIS and SOCS1 binding to known phosphorylation sites within the IL-2 receptor complex demonstrates both the similarities and differences between these two family members in binding specific ligands (Table 1); both proteins bind comparably to IL2Rβ pY355 and pY392, whereas respectively, CIS and SOCS1 bind exclusively to pY365 and pY510. How pertinent these sites are to CIS or SOCS1 mediated regulation of IL-2 signalling is currently unclear. However, the ability of both proteins to regulate this pathway raises some interesting questions regarding specificity and redundancy (discussed below).

Table 1. Comparative analysis of CIS and SOCS1 SH2 domain binding to phosphopeptides derived from the IL-2 receptor complex.

		Peptide sequence tested											Affinity (Kd, μM)	
Receptor	**pY site**	**-3**	**-2**	**-1**	**pY**	**+1**	**+2**	**+3**	**+4**	**+5**	**+6**	**+7**	**+8**	**CIS**	**SOCS1**
IL-2Rβ	338	N	G	Q	Y	F	F	F	H	L	P	D	A	7.00	0.96
	355	C	Q	V	Y	F	T	Y	D	P	Y	S	E	0.94	0.61
	358	Y	F	T	Y	D	P	Y	S	E	E	D	P	-	-
	361	Y	D	P	Y	S	E	E	D	P	D	E	G	1.50	-
	392	D	D	A	Y	C	T	F	P	S	R	D	D	1.80	1.00
	510	T	D	A	Y	L	S	L	Q	E	L	Q	G	-	0.21
IL-2Rγ	303	V	T	E	Y	Q	G	N	F	S	A			-	-
	325	Q	P	D	Y	S	E	R	F	C	H			-	-
	357	H	S	P	Y	W	P	P	P	C	Y			-	-
	363	P	P	C	Y	S	L	K	P	E	A			-	-

[1]pY indicates phosphotyrosine, dashes indicate no detectable binding. Values were derived by Isothermal Titration Calorimetry and all data is from (Delconte et al. 2016; Liau et al. 2018).

Specificity and redundancy

It has long been assumed that because the SOCS can regulate overlapping targets and pathways when overexpressed, essentially using the same mechanisms of action, that in vivo the loss of one SOCS could be accommodated for by other family members. However, evidence to support this notion across the family is fundamentally lacking.

Molecular comparisons of SOCS1 and SOCS3

SOCS1 and SOCS3 are most related to each other; sharing 37% amino acid identity between their SH2 domains (including their ESS) and uniquely amongst the family, the KIR motif. Despite this architectural similarity and the ability of both SOCS to inhibit JAK enzymatic activity, they have distinct and non-overlapping functions in vivo (*see also* discussion on functional redundancy below).

The SOCS3-SH2 domain tethers it to the phosphorylated cytokine receptors, bringing it into close proximity to the JAK molecules and enabling SOCS3 to directly inhibit JAK activity via its KIR. The high-affinity binding site within the receptor and inhibition of JAK activity results in exquisite regulation of IL-6 family cytokines which utilize the gp130 receptor (Babon, Varghese, and Nicola 2014). This is illustrated by the lethality of SOCS3-deficient mice which suffer from placental defects due to dysregulated LIF signaling (Roberts et al. 2001; Takahashi et al. 2003; Robb et al. 2005), and through the conditional deletion of SOCS3, which established it as a negative regulator in vivo for other IL-6 family cytokines, as well as a physiologically important regulator of G-CSF, ciliary neurotrophic factor (CTNF) and Leptin (Croker et al. 2003; Croker et al. 2004; Mori et al. 2004; Kievit et al. 2006; Smith et al. 2009).

Definitively identifying the individual tyrosine residues required for SOCS-SH2-dependent regulation of particular cytokine receptors in vivo is challenging as these sites often bind multiple different proteins. In the case of SOCS3, the gp130 pY757 site also mediates a high affinity interaction with the tyrosine phosphatase SHP2, leading to activation of MAPK signaling. Various studies have tried to unpick the relative contribution of SOCS3 versus SHP2 binding to gp130 pY757 and their relative importance for the inhibition, activation and regulation of the STAT3 and the RAS-MAPK pathways (Ernst and Jenkins 2004). Detailed kinetic analysis of IL-6 signaling in $Socs3^{-/-}$ macrophages demonstrated enhanced SHP2 activation, but no corresponding increase in downstream Erk1/2 phosphorylation, indicating that whilst both SHP2 and SOCS3 bind the same site, they don't compete for pathway activation (Lang et al. 2003). It is possible that the later induction of SOCS3 and its regulation of JAK1 activity has minimal impact on the early binding of SHP2 to gp130 pY757 in response to IL-6. Conversely, in $Socs3^{-/-}$ embryonic stem (ES) cells, there was enhanced and prolonged phosphorylation of both SHP2 and Erk1/2 in response to LIF (Forrai et al. 2006). In this study, MAPK pathway inhibitors rescued the aberrant LIF-induced differentiation of $Socs3^{-/-}$ ES cells, indicating that SOCS3 normally acts to dampen both STAT3 and SHP2 signaling from the

receptor. Further, it is somewhat curious that mice with a germline mutation of gp130 Tyr757 ($gp130^{Y757F}$) do not mimic the early LIF-dependent lethality of the $Socs3^{-/-}$ mice (Tebbutt et al. 2002). This may be due to the accompanying decrease in SHP2-mediated MAPK signaling, and/or some potential binding of SOCS3 to the phosphorylated LIFR (Y974 shows some sequence overlap with gp130 Y757, not shown). Alternatively, it is possible that receptor independent regulation of JAKs by SOCS3 is marginally sufficient to prevent lethal signaling by LIF, but not other pathways which lead to disease in the $gp130^{Y757F}$ mice (Tebbutt et al. 2002).

These studies highlight the context-dependent nature of related signaling pathways and equally, the complex roles of the SOCS in regulating these pathways. In cells, SOCS3 overexpression does not reduce JAK phosphorylation without a relevant receptor being present (Nicholson et al. 1999). In contrast, SOCS1 potently reduces JAK phosphorylation independent of receptor expression (Nicholson et al. 1999). This may be due to either the greater inhibition of JAK activity by SOCS1 (10-fold more than SOCS3 in vitro), the ability of SOCS1 to bind to unphosphorylated JAK as evidenced from the recent crystal structure, or the capacity of SOCS1 to bind directly to the JAK activation loop (Yasukawa et al. 1999; Babon et al. 2012; Kershaw et al. 2013; Liau et al. 2018). Despite the ability of SOCS1 to regulate JAK independently of receptors, it appears to have a strict requirement for an intact SH2 domain.

In early overexpression studies the SOCS1-SH2 domain was shown to be crucial to its activity, with mutation of the invariant arginine (R105) ablating its inhibition of LIF, IL-6 and EPOR signaling (Narazaki et al. 1998; Nicholson et al. 1999; Yasukawa et al. 1999), in addition to preventing its interaction with the JAK activation loop tyrosines (Narazaki et al. 1998; Yasukawa et al. 1999). Whilst these early studies indicated the importance of the SOCS1-SH2 domain, the definitive target/s of its SH2 domain and by extension, how SOCS1 specifically inhibits distinct cytokine pathways has remained obscure. Tyrosine 441 in the IFN gamma receptor 1(IFNGR1) was initially proposed to be important for SOCS1-mediated inhibition of signaling (Qing et al. 2005; Starr et al. 2009). However, mice with a "knock-in" mutation of Tyr441 ($IFNGR1^{Y441F}$) display an extremely mild phenotype, indicating that either this residue is not required for SOCS1 regulation of IFNγ signaling or that it only contributes in part to SOCS1 function. Biophysical analysis of SOCS1 binding to peptides derived from the IFNγ receptors also didn't detect binding to any of the tyrosines from this receptor (Liau et al. 2018).

Given the stark phenotypic differences between the IFNGR^{Y441F} mouse and a Socs1$^{-/-}$ mouse, and no detectable binding to this site *in vitro*, it is likely that Tyr441 is not the key site for SOCS1-mediated regulation of IFNγ signaling. Similarly, while SOCS1 regulation of IFNα/β occurs via the IFN alpha and beta receptor subunit 1 (IFNAR1) (Fenner et al. 2006), mutation of the receptor tyrosines did not alter SOCS1-dependent regulation, with SOCS1 instead found to bind to the IFNAR1-associated Tyk2(Piganis et al. 2011).

SOCS1 has been shown to regulate a range of cytokine pathways in vivo; Type I and II IFN(Alexander et al. 1999; Fenner et al. 2006), IL-2 family cytokines(Davey et al. 2005), IL-12/23(Eyles et al. 2002) and IL-4/13(Naka et al. 2001). A comprehensive analysis of phosphotyrosine sites from the IFNγ, IFNα/β and the IL-2 receptor subunits failed to identify a high affinity site/s that could explain the specific regulation of those pathways by SOCS1; for example, a high affinity tyrosine in the IL-2Rγ chain would link SOCS1 regulation to all IL-2 cytokines. Instead, as previously mentioned, the SOCS1-SH2 domain bound with high affinity to the activation loop tyrosines of all JAK family members (Liau et al. 2018), consistent with the earlier observations by Yasukawa et al. (1999). There is some contention as to whether an SH2 domain could comfortably bind this site, as unlike other SH2 binding sites, this one is spatially restricted, being sandwiched between the N- and C-lobes of the JAK kinase domain. If JAKs are indeed the physiological SOCS1-SH2 target, this also does not satisfactorily explain its specificity for distinct receptor complexes, as all cytokine receptors utilize pairs of JAKs, where at least one of the pair can be inhibited by SOCS1. It remains plausible that SOCS1 is localized to key receptors via alternative SH2 domain interactions at the receptor complex or via other mechanisms, such as that suggested by cavin-1-mediated localization of SOCS3 (Williams et al. 2018).

Functional comparison of SOCS1 and SOCS3

The SOCS3 requirement for specific receptor tyrosine sites (along with the specificity of its SH2 domain for those tyrosines) appears to distinguish it from SOCS1. Consistent with this, SOCS1 and SOCS3 have a range of non-redundant biological functions. However, very few studies have addressed whether these two SOCS proteins can functionally regulate the same pathway in the same cell. One example is in T cells, where deletion of Socs1 (but not Socs3) from bone marrow progenitor cells results in a delay in T cell development in vitro at the double negative (DN)3:DN4 transition, and compound deletion of Socs1 and Socs3 results in an earlier

block at DN2 (Croom et al. 2008). Although this suggests a level of functional redundancy, it is still unclear from this study whether the absence of SOCS1 leads to the increased production of cytokines that are susceptible to Socs3 deletion, or whether SOCS3 directly compensates for SOCS1 loss.

To further interrogate the potential functional overlap of SOCS1 and SOCS3 in the haematopoietic compartment, Ushiki et al. (2016) derived mice with reconstituted bone marrow lacking SOCS1, SOCS3 or both (on an IFNγ null background). Similar to the observations in T cell development, the compound loss of SOCS3 exacerbated the inflammatory disease observed in SOCS1-deficient mice (Ushiki et al. 2016). However, this could not be linked to further enhancement of SOCS1-regulated pathways and instead stemmed from the global effects of multiple dis-regulated pathways (Ushiki et al. 2016). These studies are intrinsically complicated by the early lethality observed in both $Socs1^{-/-}$ and $Socs3^{-/-}$ animals. Thus, while definitive proof of functional redundancy between SOCS1 and SOCS3 is lacking (and unlikely), it is clear that the SOCS proteins have evolved to co-operatively suppress excessive responses to many cytokines, as illustrated by the exacerbated phenotype of mice lacking both SOCS1 and SOCS3.

SOCS1, CIS, and the selective regulation of IL-2 signalling

SOCS1 has defined roles in the regulation of IL-2 signalling, primarily in T cells; regulating responses to cytokines which signal through the common IL-2 receptor γ (IL-2Rγ) subunit, such as IL-2, IL-4, IL-7 and IL-15(Fujimoto et al. 2002; Cornish, Davey, et al. 2003; Chong et al. 2003; Cornish, Chong, et al. 2003; Davey et al. 2005; Ramanathan et al. 2006). The role of CIS in the regulation of IL-2 signalling is less clear but appears to be highly specific. CIS has been shown to interact with the IL-2Rβ subunit (Aman et al. 1999) and mice with exogenous expression of CIS, show a modest reduction in IL-2-mediated STAT5 phosphorylation in CD4+ T cells (Matsumoto et al. 1999; Li et al. 2000). Consistent with this, CIS-deficient mice show enhanced CD4+ activation in response to IL-2 and IL-4 (Yang et al. 2013). In contrast, an independent study found no enhanced STAT5 activation by IL-2 in $Cish^{-/-}$ CD8+ T cells and instead suggested that CIS inhibited T cell receptor (TCR) signalling (discussed further below) (Palmer et al. 2015).

IL-15 is uniquely trans-presented along with its α-chain by neighbouring cells to the IL-2 receptor complex on Natural Killer (NK) and T cells, and hence triggers the same signalling cascade as IL-2(Lin and Leonard 2017). CIS is a critical regulator of IL-15 signalling in NK cells and a number of tyrosines which bind with high affinity to the CIS-SH2 domain have been identified in the JAK kinases and the IL-2 receptor beta (IL-2Rβ) subunit (Table 1) (Delconte et al. 2016). Curiously, despite IL-15 inducing Socs1 mRNA in NK cells, and the role of SOCS1 in regulating IL-15 in T cells (Davey et al. 2005; Ramanathan et al. 2006), CIS appears to be the predominant regulator of this pathway in NK cells (Delconte et al. 2016). This raises an interesting question - if both SOCS1 and CIS can bind to the IL-2Rβ and JAKs via similar sites, why do they appear to have non-overlapping roles in the regulation of IL-2 signalling? Hypothetically, and with equivalent expression levels, SOCS1 should dominate as, at least in vitro, it binds tyrosines in the IL-2Rβ (Table 1) and JAK activation loop tyrosines more tightly than CIS, and in addition can directly inhibit JAK via its KIR (Delconte et al. 2016; Liau et al. 2018).

Interestingly, CIS appears to play a cytokine-independent role in the regulation of TCR signalling in CD8+ cells and has been suggested to negatively regulate Plcγ1 downstream of the TCR to dampen T cell activation (Palmer et al. 2015). Conversely, exogenous expression of CIS in CD4+ T cells augmented TCR signalling through increased PKCΘ and MAPK activation, leading to enhanced survival (Li et al. 2000). Whilst these phenotypes have not been directly compared in the same cell type, this dichotomous relationship with a signalling pathway is reminiscent of SOCS2 regulation of growth hormone (GH) signalling; Socs2-deficient mice exhibit gigantism due enhanced GH signalling and mice with a Socs2 transgene show the same phenotype (Metcalf et al. 2000; Greenhalgh, Metcalf, et al. 2002; Greenhalgh, Bertolino, et al. 2002). What factors differentiate CIS and SOCS1 in the regulation of IL-2 signalling? Why does CIS appear to have specific roles in regulating different targets in two related cell types? Whilst more detailed work is required to validate the targets regulated by CIS in these cells, it is clear that we have more to learn about how different SOCS achieve specificity in discrete cellular contexts.

Redundancy between other SOCS family members; SOCS4, 5, 6 and 7

SOCS4 and SOCS5 are the most highly related family members, sharing 88% amino acid identity across their SH2 domains and predicting that in the right context, they would be

indistinguishable in their recognition of phosphorylated target proteins. Neither SOCS4 nor SOCS5 deficient mice exhibit pronounced defects in the steady state, but both strains are more susceptible to influenza infection compared to control mice, showing enhanced viral load and increased cytokine production in the lungs (Brender et al. 2004; Kedzierski et al. 2014; Kedzierski et al. 2015; Kedzierski et al. 2017). Despite this, SOCS4 and SOCS5 appear to modulate distinct aspects of the anti-viral response; SOCS4-deficient mice show defective homing of influenza-specific CD8+ T cells to the lungs and decreased T cell receptor-mediated activation, whereas SOCS5 restricts viral infection of human and mouse lung epithelial cells via negative regulation of EGFR and PI3K signalling.

SOCS6 and SOCS7 share the second highest amino acid identity across their SH2 domain (54%). Mice lacking SOCS6 exhibit reduced body weight compared to wild-type mice, whereas SOCS7 deficient mice develop hydrocephalus or survive to reveal an increased sensitivity to glucose, depending on the genetic background of the mice (Krebs et al. 2002; Krebs et al. 2004; Banks et al. 2005). Both SOCS6 and SOCS7 can bind to IRS and the PI3K p85 subunit and transgenic SOCS6 mice show perturbed insulin sensitivity (Krebs et al. 2002; Li et al. 2004). Multiple SOCS proteins (including SOCS1 and SOCS3) have been implicated in the regulation of insulin signalling and glucose homeostasis (Howard and Flier 2006). Whether mice lacking individual SOCS genes do not show defects in insulin signalling as a result of functional redundancy remains possible, and more careful analysis of compound SOCS knockouts is required to definitively address this possibility.

The most definitive evidence for functional redundancy comes from the compound deletion of Socs6 and Socs7, which surprisingly leads to an early lethality, despite deletion of the individual genes having no impact on viability (Lawrenson et al. 2017). The lethality is attributed to deregulated cortical neuron migration as a consequence of enhanced Dab1 levels and phosphorylation downstream of Reelin activation (Lawrenson et al. 2017). The loss of either SOCS6 or SOCS7 alone resulted in milder defects in cortical neuron layering (Simo and Cooper 2013; Lawrenson et al. 2017). Thus, SOCS6 and SOCS7 appear to converge on Dab1 during neuronal development to regulate Reelin signalling (Lawrenson et al. 2017).

Conclusions

Cytokines drive the development, maintenance and effector functions of multiple immune cells and these messages must be carefully interpreted and tightly regulated. The SOCS proteins act

to negatively regulate these pathways to prevent excessive signaling and to help modulate the response. Central to their function is the specificity of their SH2 domain for phosphorylated motifs in target proteins which links the SOCS box-associated E3 ligase complex to specific substrates. Detailed studies of SOCS3 have illustrated the importance of its SH2 domain in determining which pathways it targets, in addition to precisely locating SOCS3 so that it can directly inhibit JAK activity. In contrast, despite being one of the most studied SOCS proteins, the mechanism by which SOCS1 achieves selectivity for its target pathways is still not clear. The activation loop tyrosines of the JAKs present as compelling targets of the SOCS1-SH2 domain. However, if these are the true targets of the SOCS1-SH2 domain, additional mechanisms must exist that contribute to the selective SOCS1 inhibition of cytokine signaling.

Apart from SOCS6 and SOCS7, the SOCS proteins do not appear share a great deal of redundancy, suggesting that different family members have evolved to account for the diversity in cytokine and growth factor pathways and to distinguish between similar cellular targets. More broadly, the SH2-binding preferences of many SOCS family members have not been comprehensively explored and going forward this would help delineate the precise targets. Additional structures of SOCS protein bound to phosphorylated peptides will also provide further insight into how the SOCS select their targets and aid in understanding both the similarities and differences between related family members. Structural and biochemical analysis of the SOCS and their substrates may also highlight new regulatory regions, particularly in their N-termini, that contribute to their function. Understanding how the SOCS proteins achieve specificity remains an important area of research and will provide insight into how the SOCS fine-tune cytokine signaling in distinct cellular and disease contexts.

We apologize to those colleagues whose work we have not been able to include due to scope and space restraints.

Acknowledgements: We would like to thank Tracy Putoczki, Jeffrey Babon and Nadia Kershaw for helpful discussions. The authors were supported in part by an NHMRC IRIISS grant and a Victorian State Government Operational Infrastructure Scheme grant. SEN is supported by a Cancer Research Institute CLIP Grant.

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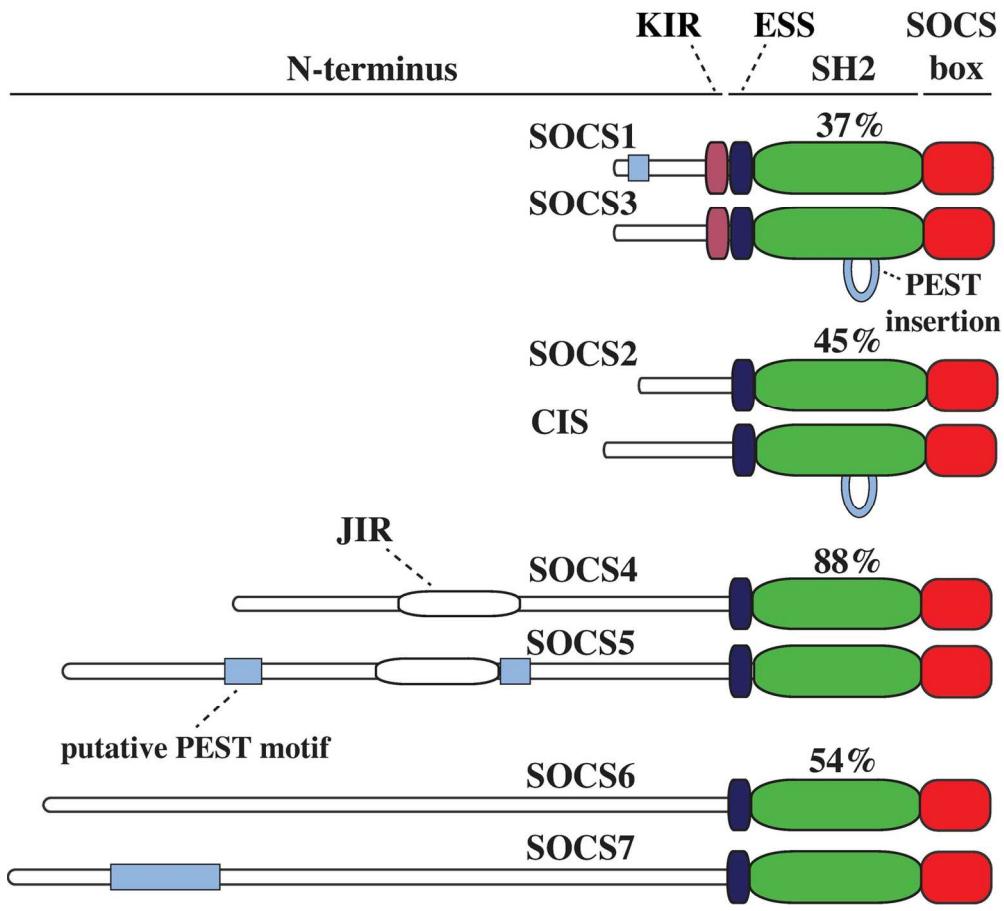

Figure 1: Domain architecture of the SOCS family. The eight SOCS proteins contain a central SH2 domain (green) flanked by a variable N-terminal region and a C-terminal SOCS box (red). The SOCS are arranged as pairs based on the relative amino acid identity between their ESS and SH2 domains (shown as a percentage). Light blue indicates the location of putative pest motifs identified in (Babon et al. 2005); light blue loops in the SH2 domain of SOCS3 and CIS indicate the PEST insertion in this modular domain (see also Figure 2D). KIR: Kinase Inhibitory Region, dark blue, ESS: Extended SH2-subdomain, mauve, JIR: JAK interaction region.

144x129mm (300 x 300 DPI)

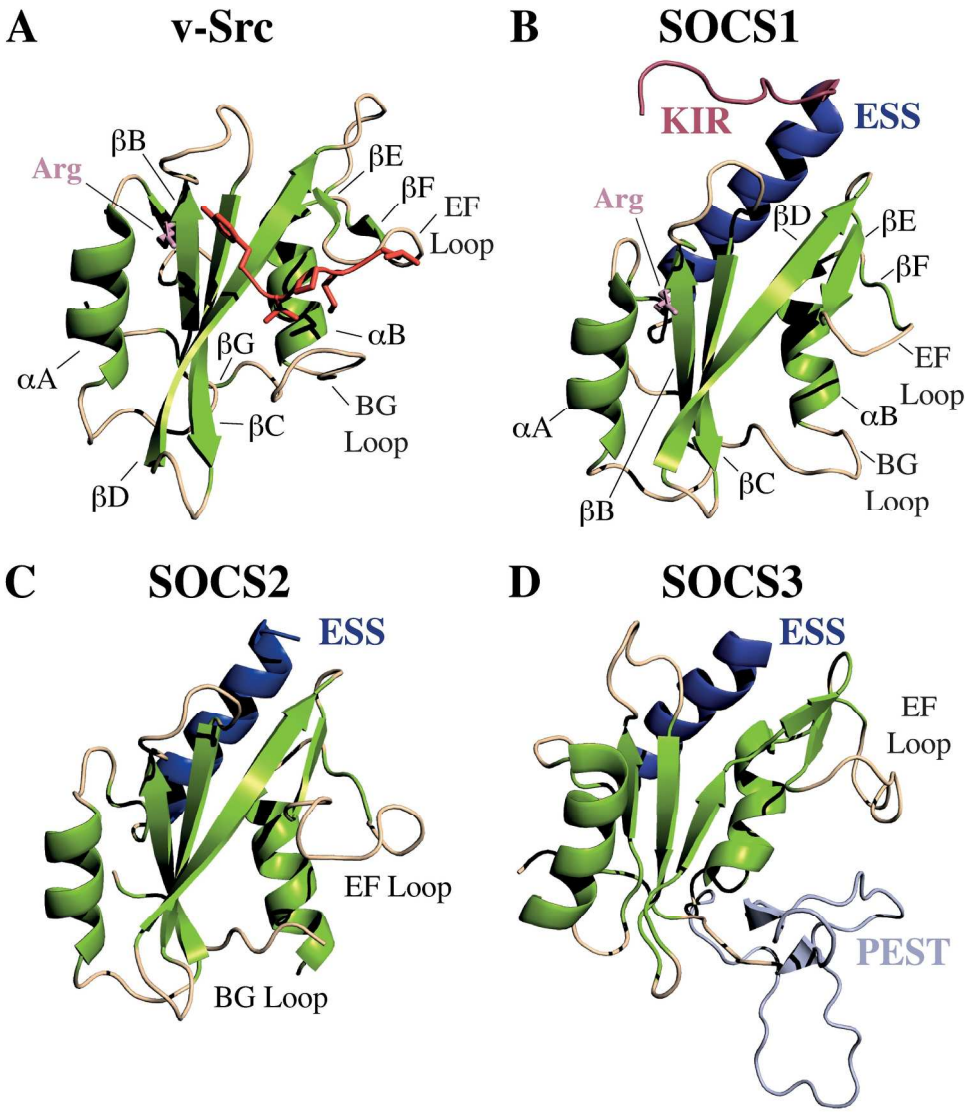

Figure 2: Distinctive structural features of SOCS-SH2 domains. Cartoon representations of the crystal structures for (A) v-Src (PDB:1SHA), (B) SOCS1 (PDB:6C7Y) and (C) SOCS2 (PDB:2C9W) and (D) the solution structure of SOCS3 (PDB:2BBU). (A) v-Src is included for comparison as a canonical SH2 domain structure. The SH2 domain contains three central, antiparallel β-sheets (βC, βD, βG) flanked by two α-helices (αA, αB) (green). All loop regions are shown in wheat. Distinctive SOCS-SH2 features are highlighted; KIR (mauve), ESS (dark blue) and PEST motif (light blue). The phosphopeptide bound to v-Src is shown in red (A) and the invariant arginine that coordinates the phosphate ion is shown in pink (A & B).

206x230mm (300 x 300 DPI)

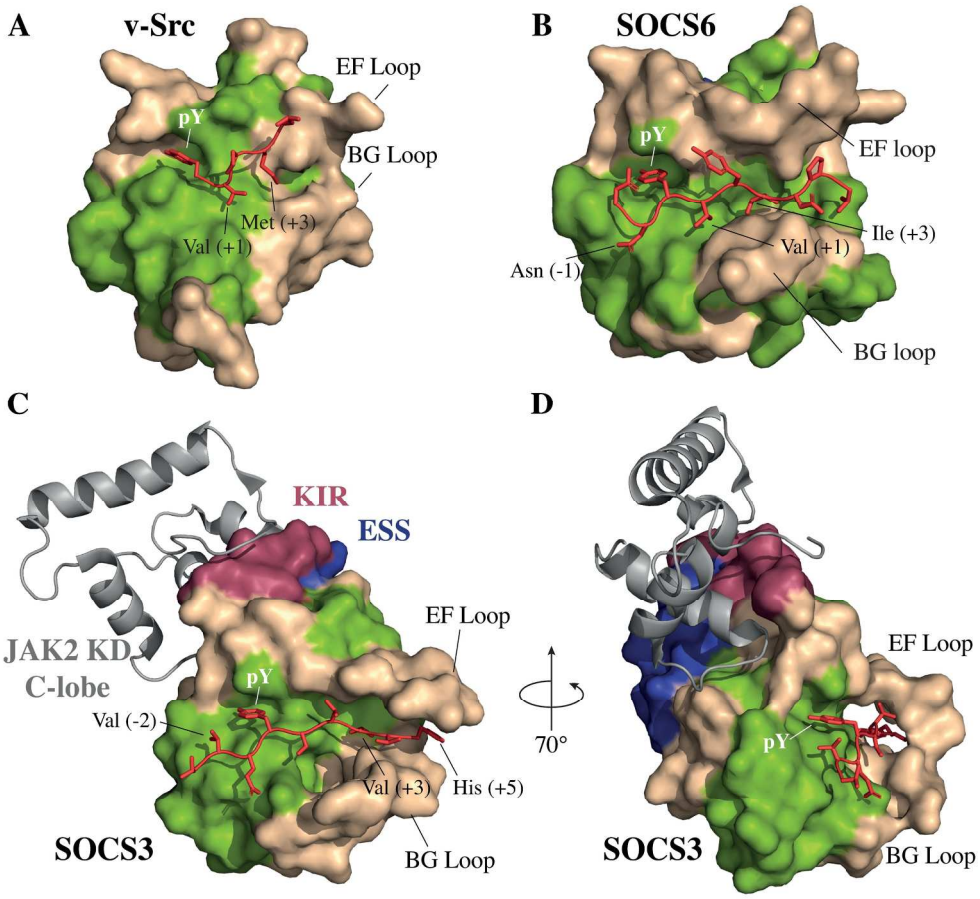

Figure 3: Binding interfaces on the SOCS-SH2 domain. Surface representation of the crystal structures for (A) v-Src-SH2 (PDB:1SHA), (B) SOCS6-SH2 (PDB:2VIF) and (C & D) SOCS3-SH2 in complex with the JAK2 kinase domain (KD) (PDB:4GL9). Phosphorylated peptides are shown in red as cartoon representations with side chains and specificity-determining residues are highlighted. (B & C) Parts of the EG and BG loops for SOCS6 and SOCS3 have been removed for clarity. (C & D) The SOCS3:JAK2 binding interface is formed by residues of the ESS, KIR and BC loop of the SOCS3-SH2 domain. Only the parts of JAK2 KD C-lobe involved in the interaction are shown here in cartoon representation (grey). (D) shows a 70° rotation of (C) to highlight the two binding interfaces. Colouring of secondary structural features and loops are as in Figure 1.

209x187mm (300 x 300 DPI)

		Peptide sequence tested												Affinity (Kd, μM)	
Receptor	pY site	-3	-2	-1	pY	+1	+2	+3	+4	+5	+6	+7	+8	CIS	SOCS1
IL-2Rβ	338	N	G	Q	Y	F	F	F	H	L	P	D	A	7.00	0.96
	355	C	Q	V	Y	F	T	Y	D	P	Y	S	E	0.94	0.61
	358	Y	F	T	Y	D	P	Y	S	E	E	D	P	-	-
	361	Y	D	P	Y	S	E	E	D	P	D	E	G	1.50	-
	392	D	D	A	Y	C	T	F	P	S	R	D	D	1.80	1.00
	510	T	D	A	Y	L	S	L	Q	E	L	Q	G	-	0.21
IL-2Rγ	303	V	T	E	Y	Q	G	N	F	S	A			-	-
	325	Q	P	D	Y	S	E	P	F	G	H			-	-
	357	H	S	P	Y	W	P	P	P	C	Y			-	-
	363	P	P	C	Y	S	L	K	P	E	A			-	-