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TARGETING SOCS PROTEINS TO CONTROL JAK-STAT SIGNALLING IN DISEASE

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Abstract:	Defective regulation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway in cancers, haematological diseases and chronic inflammatory conditions highlights its clinical significance. While several biologic and small molecule therapeutics targeting this pathway have been developed, these have several limitations. Therefore there is a need to identify new targets for intervention. Suppressor of cytokine signalling (SOCS) proteins are a family of inducible inhibitors of cytokine receptors that activate the JAK-STAT pathway. Here we propose that newly identified mechanisms controlling SOCS function could be exploited to develop molecularly targeted drugs with unique modes of action to inhibit JAK-STAT signalling in disease.

HIGHLIGHTS

The JAK-STAT signalling system utilised by many cytokines and growth factors has recently been exploited to develop a range of new therapies for chronic inflammatory disease, autoimmune conditions and cancers. However there have several limitations, emphasising the need for more therapeutic options.

JAK-STAT signalling is controlled by induction of SOCS proteins which act as part of a negative feedback loop to prevent sustained activation.

Identification of new regulatory mechanisms and protein-protein interactions has revealed new targets for generating more selective drugs for the range of conditions resulting from hyperactivation of the JAK-STAT pathway.

TARGETING SOCS PROTEINS TO CONTROL JAK-STAT SIGNALLING IN DISEASE

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Abstract: Defective regulation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway in cancers, haematological diseases and chronic inflammatory conditions highlights its clinical significance. While several biologic and small molecule therapeutics targeting this pathway have been developed, these have several limitations. Therefore there is a need to identify new targets for intervention. Suppressor of cytokine signalling (SOCS) proteins are a family of inducible inhibitors of cytokine receptors that activate the JAK-STAT pathway. Here we propose that newly identified mechanisms controlling SOCS function could be exploited to develop molecularly targeted drugs with unique modes of action to inhibit JAK-STAT signalling in disease.

JAKs: significance, structure and signalling

Cytokines and cytokine-activated signalling pathways are central players in the development of **autoimmune disorders** (see **Glossary**), such as **rheumatoid arthritis (RA)**, chronic inflammatory diseases and cancers [1,2]. A particularly important group of cytokines are those which activate the **Janus kinase-signal transducer and activator of transcription (JAK-STAT)** pathway. The importance of the JAK-STAT pathway in immunity is neatly demonstrated by primary immunodeficiencies caused by inactivating mutations in specific JAK and STAT genes; these include **severe combined immunodeficiency**, due to inactivation of JAK3, and **autosomal dominant IgE syndrome** caused by **dominant-negative** mutations in STAT3 [1]. Therefore, therapeutic approaches to block JAK-STAT signalling using inhibitors of cytokine-receptor interactions have proved invaluable treatment options for patients with autoimmune disorders such as RA and **psoriasis**, while oral small molecule JAK inhibitors, which block signalling downstream of cytokine receptors, have been developed for management of RA, **myeloproliferative neoplasms (MPNs)**, psoriatic arthritis and ulcerative colitis [2,3]. Here, we focus on recently discovered mechanisms involved in the regulation of JAK-STAT signalling and discuss how these could be exploited to develop new therapeutics to inhibit signalling in indications with enhanced JAK-STAT activation.

Janus kinases (JAKs) comprise a family of four tyrosine (Tyr) kinases (JAK1-3, and Tyk2). JAKs interact with type I and type II cytokine receptors, which include receptors for interleukins**, interferons (IFNs)** and hormones such as leptin. As these receptors have no intrinsic enzymatic activity, preferential interaction with distinct JAK isoforms is required for downstream signalling. For example gp130 (the signal transducing component of the IL-6 receptor complex) associates with JAK1-2 and Tyk2, while IL-2 receptor complexes specifically associate with JAK1 and JAK3 [2]. Each JAK protein comprises seven JAK-homology (JH) domains (JH1, kinase domain; JH2, **pseudokinase (PK) domain**, JH3-5, **SH2 domain** (although it cannot bind phospho-Tyr residues), and JH6-7, **N-terminal 4.1, ezrin, radixin, moesin (FERM) domain**) (Figure 1A). While the FERM and SH2 domains mediate JAK interaction with membrane-proximal box1/2 regions of cytokine receptors, the PK domain restrains Tyr kinase activity by binding the kinase domain [4,5].

Cytokine-receptor interaction triggers phosphorylation of receptor-associated JAKs on specific Tyr residues required to turn on kinase activity. Structural studies suggest cytokine-receptor ligation re-orientates JAK-receptor complexes so that JAKs become sufficiently close to allow trans-phosphorylation of **activation loops** present in their kinase domains (Figure 1A). Activated JAKs then Tyr phosphorylate bound cytokine receptor on specific residues to generate docking sites for SH2 domain-containing STAT proteins. Receptor-bound STATs are then phosphorylated on conserved Tyr residues within their C-terminal domains prior to dissociation from the receptor and formation of homo- or heterodimers. These translocate to the nucleus, bind target gene promoters and stimulate target gene transcription (Figure 1B). STATs comprise a family of seven proteins (STAT1-4, STAT5A, STAT5B, STAT6) and cytokine receptors preferentially activate distinct STATs. For example, gp130-utilizing cytokines preferentially activate STAT3 via phosphorylation on Tyr705, while IFNγ receptors activate STAT1 through Tyr701 phosphorylation (reviewed in [1]).

JAK-STAT therapeutics and the need for new targets

Hyperactivation of the JAK-STAT signalling pathway in haematological and solid tumour malignancies, MPNs and autoimmune disorders has driven the development of therapeutics targeting this pathway. **Biologics** that block IL-6 signalling include siltuximab (anti-IL-6 antibody), which is licenced for **multicentric Castleman's disease**, and tocilizumab (anti-IL-6 receptor [IL-6R] antibody) which is approved for treatment of RA, juvenile idiopathic arthritis, **giant cell arteritis** and chimeric antigen receptor (**CAR) T cell-induced cytokine release syndrome** [6]. Biologics blocking other JAK-STAT-activating cytokines include IL-17 blocker secukinumab for management of psoriasis, psoriatic arthritis and **ankylosing spondylitis** [7], and anti-IL-5 blocker mepolizumab for severe eosinophilic asthma and vasculitis [8].

In addition, three orally available small molecule first generation JAK inhibitors or "JAKinibs" (tofacitinib, baricitinib, and ruxolitinib) are licensed in Europe and the USA. Ruxolitinib is approved for management of intermediate or high-risk myelofibrosis (MF) and polycythaemia vera (PV) in patients intolerant of or resistant to cytoreductive therapy with hydroxycarbamide. While baricitinib is approved for RA, tofacitinib is licensed for treatment of RA, active psoriatic arthritis and, most recently, moderate to

severely active ulcerative colitis [9]. Each drug targets the ATP-binding site in the kinase domain [9].

While effective, current JAK-STAT-targeted therapies have several drawbacks. For example, tociluzimab is associated with adverse effects such as anaemia, headaches and increased risk of infection. Due to conservation of primary sequences between kinase domain ATP-binding sites, first generation JAKinibs are non-selective inhibitors of multiple JAK isoforms. Therefore, they are associated with increased susceptibility to infections, particularly by **herpes zoster**, due to suppression of IFN-stimulated anti-viral signalling [10]. JAKinibs can also cause **thrombocytopenia** and **neutropenia** from inhibition of JAK2 [3]. Another potential risk of immunosuppressive JAKinibs is increased risk of malignancies, although studies in RA patients have not shown increased risk of haematological cancers or solid tumours after long-term treatment with ruxolitinib [11].

While on-going development of JAKinibs with greater isoform-selectivity, such as JAK1-selective inhibitor filgotinib [12], could mitigate some of these issues , increasing selectivity may come at a price of reduced efficacy as autoimmune and chronic inflammatory diseases typically display imbalances in multiple cytokine pathways. Therefore, there is a need for new strategies acting *via* alternative mechanisms to target JAK-STAT signalling while minimising adverse reactions.

SOCS proteins: inducible inhibitors of cytokine signalling

One mechanism that has evolved to limit JAK-STAT signalling involves suppressor of cytokine signalling (SOCS) proteins, inducible gene products that inhibit JAK-STAT signalling as part of a negative-feedback loop. There are eight SOCS family members (SOCS1-7 and cytokine-inducible SH2-containing protein (CIS)). SOCS1 and SOCS3 are the most extensively characterised members. Each comprises an N-terminal domain, which includes a **kinase inhibitory region (KIR)**, a central SH2 domain and a C-terminal SOCS box domain (Figure 2A) [13]. SOCS proteins use a range of mechanisms to inhibit cytokine signalling. Thus while the KIR and SH2 domain of SOCS1 can directly bind the JAK kinase domain to inhibit phosphorylation (Figure 2B) [14], the CIS SH2 domain binds directly to JAK-phosphorylated cytokine receptors to block STAT recruitment [13]. In contrast, SOCS3 targets cytokine receptor complexes,

including those for IL-6 family cytokines containing the signalling receptor gp130, to inhibit JAK-STAT signalling. Upon binding phosphorylated Tyr759 (Tyr757 in mouse) in gp130 *via* its SH2 domain, the SOCS3 KIR associates with gp130-associated JAKs to non-competitively inhibit kinase activity (Figure 2B) [15].

In addition, the SOCS box motif enables formation of an elongin-cullin-SOCS3 **E3 ubiquitin ligase** complex that targets bound substrates for polyubiquitylation and proteasomal degradation. SOCS3 binds the cullin5 scaffold protein in part *via* an elongin B/C dimer. Cullin5 also binds the Really Interesting New Gene (RING) domain-containing protein Rbx2, which enables interaction with an E2 ubiquitin-conjugating enzyme (Figure 2A). Several protein families with roles in ubiquitylation have since been shown to include SOCS box domains which are critical for their function [16] [Box 1]. Targets for SOCS3-mediated ubiquitylation include JAKs and cytokine receptors [13] and mice in which the SOCS box domain has been deleted have specific immunological defects, suggesting ubiquitylation-dependent roles for SOCS3 in these processes [17].

Cavin-1 and CUEDC2: a new class of SOCS regulators

Interactions of SOCS1 and SOCS3 with target cytokine receptors, JAKs and proteins required to form a functional E3 ubiquitin ligase have been structurally defined [14,15,18]. In contrast, mechanisms for post-translational control of SOCS3 are poorly understood.

However, evidence has now emerged of additional SOCS3-interacting proteins required for optimal inhibition of cytokine signalling. These are cavin-1, an essential component of **caveolae** [19], and CUEDC2 (CUE [coupling of ubiquitin to ER degradation] domain-containing protein 2), known primarily through its role in cell cycle control [20,21]. Cavin-1 was identified in a search for new SOCS3 targets by comparing profiles of ubiquitylated proteins from wild-type ($SOCS3^{+/+}$) and SOCS3 homozygous knockout ($SOCS3^{-/-}$) mouse embryonic fibroblasts (MEFs). However, immunoblotting experiments in both $SOCS3^{+/+}$ and $SOCS3^{-/-}$ MEFs as well as wild type and SOCS3-null AS-M.5 human angiosarcoma-derived endothelial cells (ECs) demonstrated that loss of SOCS3 protein reduced cavin-1 protein expression in the absence of any decrease in mRNA. Thus, SOCS3 stabilised cavin-1 protein levels.

Cavin-1 is essential for maintaining the stability of caveolae such that cavin-1 deletion triggers a parallel loss of caveolin-1 and a reduction in cell surface caveolae. Thus, SOCS3 deletion triggered reductions in cavin-1 and caveolin-1 proteins which reduced the number of cell surface caveolae detectable in SOCS3-null AS-M.5 ECs [22].

Interestingly, interaction with cavin-1 required the SOCS3 SH2 domain but was independent of its ability to bind Tyr-phosphorylated targets. Instead, cavin-1 binding required a **PEST sequence** that links conserved motifs found in almost all SH2 domains. PEST sequences are unstructured regions involved in multiple cellular processes through controlling protein–protein interactions and protein turnover. Previous work had already implicated the SOCS3 PEST sequence in the control of SOCS3 protein turnover without altering its ability to inhibit JAK-STAT signalling. As well as SOCS3, a PEST sequence is also present at the same position within the CIS SH2 domain, which also interacted with cavin-1. Therefore, the PEST sequence within the SOCS3 SH2 domain is required for interaction with cavin-1 and may control cavin-1 interaction with CIS [22].

Subcellular fractionation experiments revealed that cavin-1 and SOCS3 are present in cytosolic and membrane fractions, and that loss of cavin-1 shifted SOCS3 to the cytosolic fraction, suggesting that cavin-1 may play a role in membrane targeting. This was confirmed by confocal imaging experiments which demonstrated that while SOCS3-green fluorescent protein expressed in cavin-$1^{-/-}$ MEFs has a punctate distribution in the cytoplasm, reconstitution with a cavin-1-mCherry fusion protein resulted in their co-localisation at the plasma membrane, where SOCS3 can target Tyr-phosphorylated cytokine receptors. Time courses showed that the magnitude and duration of IL-6-stimulated STAT3 phosphorylation are greater in cavin-$1^{-/-}$ *versus* cavin$^{+/+}$ cells. Importantly, SOCS3-dependent inhibition of STAT3 phosphorylation by IL-6 was abolished in cavin-$1^{-/-}$ MEFs, again suggesting a role for cavin-1 in localising SOCS3 to the plasma membrane for effective inhibition of signalling [22].

Several features of cavin-1 interaction with SOCS1 and SOCS3 are similar to those reported for CUEDC2 [23,24]. CUEDC2 has an established role in mitosis in which it controls release of the **anaphase-promoting complex/cyclosome (APC/C)** from checkpoint inhibition [20,21]. A search for transcription factors regulated by CUEDC2 showed that it could inhibit STAT3 activation in response to IFNα, IL-6 or forced co-

expression of JAK1. Importantly, CUEDC2 can also suppress cytokine-stimulated Tyr1022/1023 phosphorylation and activation of JAK1, which is immediately downstream of both IFNα/β receptor (IFNAR)1/IFNAR2 and gp130 signalling complexes responsible for IFNα and IL-6 signalling respectively. Crucially, the inhibitory effect of CUEDC2 on STAT3 activation requires SOCS3, and CUEDC2 can interact directly with the SOCS3 SH2 domain *in vitro* [23]. Interestingly, the SOCS3 SH2 region identified as being important for CUEDC2 interaction includes some of the PEST insert required for cavin-1 binding [22,23]. It also makes sense in that, like SOCS3, CUEDC2 would need to bind to the SOCS3 SH2 domain without blocking interaction with Tyr-phosphorylated targets such as gp130 (Figure 2A). However, CUEDC2 may potentiate SOCS3 inhibition by a mechanism distinct from cavin-1, as it stabilises SOCS3 by enhancing SOCS3-elongin C interaction to reduce SOCS3 ubiquitylation [23]. Moreover, unlike SOCS3, SOCS1 contains a PEST motif within its N-terminal region rather than the SH2 domain [25]. In addition, there is no clear effect of cavin-1 on SOCS3 stability [22]. Also, in contrast to cavin-1 and SOCS3, CUEDC2 is found in the nucleoplasm as well as the cytosol [26]. Thus CUEDC2 may stabilise specific intracellular pools of SOCS1 and SOCS3 so that there is more available for interaction with other targets, including plasma membrane-localised receptor complexes following cytokine stimulation.

Exploiting SOCS interactions as new therapeutic targets

Many studies have demonstrated that loss of SOCS1 and SOCS3 expression in myeloid cells and T cell subsets can enhance anti-tumour immunity [27]. Therefore, development of small molecules that can block SOCS1- and SOCS3-mediated inhibition of JAK-STAT signalling in myeloid and T cells represents one way to achieve this. Crucially, there is now detailed structural information available for both SOCS1 and SOCS3 interaction with JAKs and other components required for inhibition of cytokine signalling to inform these efforts. These each provide a detailed picture of SOCS KIR motif interaction with a GQM motif present in the JAK JH1 catalytic domain required for SOCS-mediated inhibition of JAK activity (Figure 2B) [14,15]. Cell-permeable peptide mimetics of SOCS1 function based on the KIR motif have already been developed that inhibit JAK-STAT signalling and have efficacy in murine models

of **relapse-remitting multiple sclerosis** [28] and **autoimmune uveitis** [29] as well as an ex vivo model of psoriasis [30]. Some of the observed efficacy may also be due to additional inhibition of NF-κB-mediated pro-inflammatory responses *via* SOCS1 interaction with and degradation of adaptor protein "MyD88-adaptor-like" (Mal) [31]. Whatever the mechanisms involved, functional assays suggest that current SOCS1 mimetic peptides have micromolar affinities and therefore require optimisation to increase target affinity. With detailed structural information available, this could now be achieved by structure-based design and molecular docking approaches that could also facilitate virtual screening to identify promising compounds from chemical databases for *in vitro* validation as SOCS mimetics [32,33].

The interaction of the SOCS1 and SOCS3 KIR motifs with the JAK JH1 domain substrate binding site and activation loop also support an approach for development of SOCS antagonists using JAK-derived peptides [14,15,28]. Development of SOCS1 antagonists using this strategy has focused on cell-permeable peptides based on the JAK2 JH1 activation loop (L^{1001}PQDKEpYYKVKEP, pY=phosphorylated Tyr). As SOCS1 is a potent negative feedback inhibitor of anti-viral IFN signalling [34–36], SOCS1 antagonists would be predicted to enhance anti-viral effects through potentiation of cellular IFN responses. In support of this approach, a SOCS1 antagonist has demonstrated anti-viral efficacy. *In vivo*, this is manifest as enhanced IFN and protective immune responses which reduce viral replication in infected cells [37].

While the above approaches exploit SOCS/JAK interactions, the identification of CUEDC2 and cavin-1 as regulators of SOCS function and stability represent another approach. As both cavin-1 and CUEDC2 enhance SOCS3 function (and SOCS1 function in the case of CUEDC2), small molecules that block SOCS/CUEDC2 or SOCS3/cavin-1 interactions should enhance JAK-STAT signalling from specific cytokine receptors by blocking SOCS-mediated negative feedback. Based on the successful exploitation of key protein-protein interactions (PPIs) for new cancer therapeutics currently in clinical trials, including nutlin-based inhibitors of p53/MDM2 interaction and bromodomain-containing protein 4 (BRD4) inhibitors that block interaction with acetylated Lys residues on target histones and transcription factors [38,39], the development of novel **orthosteric** small molecule SOCS PPIs for therapeutic applications is achievable. An important step forward would be crystal

structures of SOCS3 or its SH2 domain with either full length cavin-1, as multiple regions on cavin-1 appear to be required for interaction [22], or the CUEDC2 CUE domain that binds SOCS1 and SOCS3 [23,24]. This is because several key regions within the SOCS3 SH2 domain, including the PEST motif, are disordered and may therefore require interaction with binding partners to obtain high resolution structural information, similar to the increased ordering of the SOCS1 KIR observed when bound to JAK *versus* the lack of detectable electron density in its absence [14]. However, in the absence of structural information, **overlapping peptide array** technologies could be applied to identify and optimise high affinity SOCS-derived sequences that can function as SOCS/CUEDC2 or SOCS/cavin-1 PPI antagonists similar to approaches used for other proteins [40]. Identification of key regions within the CUE domain responsible for interaction with SOCS1 and SOCS3 would also allow for the development of optimised peptides that can mimic the effect of CUEDC2 in blocking SOCS ubiquitylation and thus act as SOCS stabilisers to enhance their expression and function [23,24]. In the case of SOCS3 stabilisation, this could be an alternative approach for chronic inflammatory conditions currently managed with JAKinhibs and anti-IL-6 receptor biologics, including RA, by enhancing SOCS3 expression and function to potentiate negative feedback inhibition of IL-6 signalling. In support of this approach, periarticular injection of a recombinant adenovirus encoding SOCS3 has been shown to reduce the severity of arthritis and joint swelling in two murine models of RA more effectively than a dominant-negative STAT3 adenovirus [41].

Finally, identification of novel mechanisms by which JAK-STAT signalling is regulated may also reveal opportunities to control signalling in new indications. One example is **pulmonary arterial hypertension (PAH)**, a rare but devastating condition with a poor prognosis [42]. While PAH-targeted therapies, including prostaglandin receptor agonists, phosphodiesterase-5 inhibitors, endothelin receptor antagonists and soluble guanylate cyclase stimulators, used alone or in combination can improve functional capacity and haemodynamics, they act predominantly as vasodilators to relieve symptoms, do not target key features of PAH pathogenesis and produce only modest reductions in mortality [42,43]. Studies in rodent models have shown that IL-6 is necessary and sufficient for the development of PAH, with significantly increased expression and function of membrane-bound IL-6R being observed in pulmonary arterial smooth muscle cells from pre-clinical PAH models and PAH patients [44,45].

Consistent with a role for IL-6R in disease progression, treatment with either a neutralizing anti-IL6R antibody or a non-peptide IL-6R antagonist [46], reversed PAH in two rodent models of the disease [44]. However, while small molecule JAKinhibs and anti-IL-6R biologics could be re-purposed for PAH, development of additional therapeutics is essential as PAH patients can become resistant after initial responsiveness to new interventions [42]. Therefore, peptides targeting novel SOCS interactors such as cavin-1 and CUEDC2 that act as SOCS3 mimetics or stabilisers to enhance inhibition of IL-6-stimulated pro-inflammatory signalling could be used as starting points for the development of more drug-like molecules potentially via inhalation, which offers an attractive option for PAH by allowing drug delivery direct to the lungs [47].

SOCS3 mimetics might also be useful for a range of conditions in which STAT3 is hyperactivated due to epigenetic silencing of the SOCS3 gene. One such condition is **cholangiocarcinoma (CCA)**. Due to late clinical presentation and the lack of effective treatments, survival rates are only 10-40% over 5 years [48,49]. Therefore, there is a need for effective therapies for this devastating condition. STAT3 is hyperactivated in CCA epithelial cells, resulting in cellular resistance to apoptosis and induction of **epithelial-to-mesenchymal transition (EMT)** [50]. Importantly, ectopic expression of SOCS3 blocks IL-6 activation of STAT3 and EMT *in vitro*, while SOCS3 expression *in vivo* decreases metastasis, an effect associated with reduced STAT3 activation, N-cadherin and vimentin expression, and induction of E-cadherin [51]. In addition to the **repurposing** potential of anti-IL-6R biologics and JAKinhibs currently used for treatment of RA, development of SOCS3 mimetics also has the potential to reverse the detrimental effects of STAT3 activation in CCA to inhibit disease progression.

It should be noted that targeting PPIs to enhance SOCS function is not without potential pitfalls. For example, PPIs targeting the cavin-1 binding site on SOCS3 have the potential to uncouple SOCS3 from cavin-1, which could potentially destabilise cavin-1 in cell types in which cavin-1 is abundantly expressed, including adipocytes and skeletal muscle [19,22]. As loss of functional cavin-1 in mice and humans triggers lipodystrophy and insulin resistance, these would have to be monitored as potential adverse reactions to drugs targeting the SOCS3/cavin-1 interface.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Development of small molecule JAKinhibs and cytokine-targeted biologics has demonstrated that inhibition of the JAK-STAT pathway can be an efficacious way to treat inflammatory and autoimmune diseases. However, adverse effects and resistance to some of these regimes emphasises the need to develop more targeted therapeutics with novel mechanisms of action to overcome these limitations. Identification of molecular mechanisms by which JAK-STAT signalling is regulated may also reveal opportunities to control signalling in new indications.

Peptides targeting novel SOCS interactors such as cavin-1 and CUEDC2 that act as SOCS1 or SOCS3 mimetics or antagonists could be used as starting points for the development of more drug-like molecules for diseases in which these proteins play a role. One disease area in which SOCS3 mimetics have potential is RA, for which JAK inhibits and anti-IL-6R biologics are already options for those patients resistant to first line therapies.

Finally, the interaction of SOCS3 with cavin-1 represents a previously unforeseen link between JAK-STAT signalling and mechanoprotection. Several studies have shown that caveolae maintain plasma membrane integrity following mechanical stress (reviewed in [19]). Stretching of the plasma membrane causes an ATP- and actin-independent flattening of caveolae to buffer increased membrane tension, triggering a dissociation of cavins from the plasma membrane. Therefore loss of caveolin-1 expression in vascular endothelial cells results in a loss of caveolae and increased sensitivity to mechanical damage in vitro and in vivo [52,53]. Given that SOCS3 deletion destabilises cavin-1 and results in a loss of plasma membrane caveolae, this suggests a new role for SOCS3 and CIS in controlling mechanoprotective capacity via interaction with and stabilisation of cavin-1.

In summary, the identification of new mechanisms to control JAK-STAT signalling provides a starting point both for generation of new therapeutics for indications in which current options have significant limitations. However, exploiting these opportunities fully will require a greater molecular understanding of the mechanisms involved (see Outstanding Questions).

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BOX 1: The SOCS box superfamily

While initially identified in the SOCS family, SOCS box domains are found in over 30 genes within the human genome. All SOCS box-containing proteins have E3 ubiquitin ligase activity through formation of a complex with Cul5 and the RING domain protein Rbx2 similar to SOCS3 (Figure 2A). The SOCS box-superfamily comprises 6 subgroups which are defined by additional domains responsible for controlling localisation and function (Figure I). As the SOCS family is described elsewhere, this section will provide an overview of other key families.

Ankyrin repeat and SOCS box (ASB) family Comprising 18 members (ASB1-18), ASB proteins consist of between 1-12 N-terminal ankyrin repeats upstream of a C-terminal SOCS box. Ankyrin repeats mediate protein-protein interactions and thus determine the spectrum of substrates for individual ASB family members. ASB proteins can form oligomers with each other and additional E3 ubiquitin ligase complexes, although the functional significance of these interactions is unclear [54]. Key substrates of this family include signalling proteins such as insulin receptor substrate 4 (IRS4), an adaptor protein important for insulin and leptin receptor signalling in brain targeted by ASB4, and tumour necrosis factor receptor (TNFR) 2, which is targeted for degradation by ASB3 thereby inhibiting TNF-mediated apoptotic responses in Jurkat cells [55].

WD repeat and SOCS box-containing protein (WSB) family While there are 2 members of this family (WSB1-2), only WSB1 has been studied in any detail. WSB1 contains 8 WD40 repeats, which determine interaction with substrates, linked to a C-terminal SOCS box domain. The WD40 repeats form a four stranded anti-parallel beta sheets or “blades” which typically come together to form a propeller structure . WSB1 substrates include homeodomain-interacting protein kinase 2 (HIPK2), a regulator of gene transcription which prevents apoptosis, and the tumour suppressor von Hippel-Landau (VHL), which forms part of a separate E3 ubiquitin ligase complex that controls transcriptional responses to hypoxia [56].

SPRY domain-containing SOCS box protein (SPSB) family The 4 SPSB family members (SPSB1-4) each consist of an N-terminal SplA/ryanodine receptor (SPRY) domain responsible for target protein interaction linked to a C-terminal SOCS box domain. A well-characterised substrate of SPSB1, 2 and 4 is inducible nitric oxide synthase (iNOS), a key effector of the innate immune response. By promoting iNOS

protein degradation, SPSB proteins prevent the accumulation of cytotoxic levels of nitric oxide that could damage ultimately host tissue [57,58].

Figure Legends

Figure 1: JAK structure and activation of JAK-STAT signalling

A: Domain structure common to al JAK isoforms. The locations of Tyr phosphorylation sites within the JH1 domain activation loop required for kinase activity are shown. These correspond to Tyr1034,1035 (human JAK1), Tyr1007,1008 (human JAK2), Tyr980,981 (human JAK3) and Tyr1054,1055 (human Tyk2).

B: Interaction of a cytokine with its membrane-bound receptor complex triggers activation of receptor-bound JAKs, which then phosphorylate the receptor on key cytoplasmic Tyr residues. These act as docking sites for SH2 domain-mediated interactions with STAT proteins. JAKs phosphorylate recruited STATs on a single Tyr residue in the C-terminal domains, which allows the STATs to dissociate from the receptor and dimerise. Dimeric STAT complexes then translocate to the nucleus, bind target DNA and initiate gene transcription.

Figure 2: SOCS3 domain structure and functional interactions

A: SOCS3 domains include an extended N-terminal region, which includes a kinase inhibitory region (KIR) present in both SOCS1 and SOCS3, a central SH2 domain and a SOCS box, which contains sub-domains for binding elongins B and C and cullin 5 (Cul5) proteins. These link with Rbx2 to form a functional E3 ubiquitin ligase complex which interacts with an E2 ubiquitin-conjugating enzyme to transfer ubiquitin to bound targets. Targets for SOCS3-mediated ubiquitylation bind to the phospho-Tyr binding pocket of the SH2 domain. The SH2 domain is also the site for interaction with novel regulators cavin-1 and CUEDC2.

B: Ribbon diagram of the structure of a JAK1 (beige)-SOCS1-(red)-ADP (green) complex. The SOCS1 construct used did not contain a SOCS box. The broken circle highlights SOCS1 KIR and SH2 domain interactions with the C-terminal lobe of the JAK1 JH1 domain. The N-terminal JAK1 JH1 domain lobe are also indicated, along with an extended SH2 subdomain (ESS) motif present in SOCS1. The inset shows the SOCS1 KIR (red) overlaid with the SOCS3 KIR (green) within the JAK1 JH1 domain.

The GQM motif required for SOCS binding is indicated. Residue numbering refers to the human sequences. Figures adapted from [14] with permission.

Box 1, Figure I: SOCS box family protein: Domain structures of representative members from each of the six families of SOCS box family proteins. While members from each family can form function E3 ubiquitin ligases via the SOCS box, each family is defined by the other domains which define interactions with the substrates targeted by each complex. SH2, Src homology 2; ANK, ankyrin repeat; WD, WD repeat; SPRY, SPla/Ryanodine receptor domain; LRR, Leucine-rich repeat.

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Glossary

Activation loops: flexible loop structures within the catalytic domains of protein kinases. Most kinases have one or more residues in the activation loop that undergo phosphorylation as a prerequisite for activation of kinase activity.

Ankylosing spondylitis: condition in which chronic inflammation promotes the deposition of calcium where ligaments attach to vertebrae, and may eventually result in fusion of adjacent vertebrae.

APC/C: a large multi-protein complex of 11–13 subunits which ubiquitylates specific cell cycle proteins, thereby tagging them for degradation by the proteasome.

Autoimmune disorders: diseases in which the immune system mistakenly recognises body tissues as foreign material and attacks them. Examples include RA, multiple sclerosis and type 1 diabetes mellitus.

Autoimmune uveitis: chronic inflammation of the uvea, which is the middle layer of pigmented vascular structures of the eye, triggered by an autoimmune reaction.

Autosomal dominant hyper IgE syndrome: a rare immunodeficiency disorder caused by a single abnormal copy of the STAT3 gene, and characterised by elevated levels of IgE. Patients are susceptible to opportunistic bacterial infections of the skin and lungs.

Biologics: engineered protein- or nucleic acid-based drugs produced using biological processes involving recombinant DNA technology.

CAR T cell-induced cytokine release syndrome: adverse effect of treatment of B cell malignancies with CAR T cells, in which a rapid and pronounced release of multiple pro-inflammatory cytokines triggers fever, hypotension and respiratory insufficiency.

Caveolae: 50–100 nanometre flask-shaped plasma membrane invaginations found in many vertebrate cell types, and especially abundant in endothelial cells, adipocytes and muscle cells.

CCA: a malignant neoplasm originating from epithelial cells lining the biliary tracts.

Dominant-negative: a mutated gene product that antagonises the function of the wild-type/normal version.

E3 ubiquitin ligase: defined as a protein that recruits an E2 conjugation enzyme that has been loaded with ubiquitin, a small protein found in all eukaryotic organisms. After binding a protein substrate, the E3 ligase catalyses the transfer of ubiquitin from the E2 to the substrate.

EMT: epithelial-to-mesenchymal transition. This describes the process in which a polarised epithelial cell, immobilised to a basement membrane via its basal surface, undergoes biochemical changes that enable it to assume a mesenchymal cell phenotype, characterised by enhanced migration, invasiveness and resistance to apoptosis. Completion of EMT is marked by degradation of the underlying basement membrane and migration of the mesenchymal cells away from the epithelial layer.

FERM domain: typically involved in localising proteins to the plasma membrane via interaction with integral membrane proteins.

Giant cell arteritis: serious condition in which arteries, particularly those at the side of the head, become inflamed. This results in vessel narrowing, which interrupts blood flow. Symptoms include severe headache, dizziness and loss of vision.

Herpes zoster: also known as shingles, this is caused by the Varicella-zoster virus, which is also the causative agent of chicken pox. Once a person has had chicken pox, the virus remains in nerve cells near the spine. Herpes zoster develops when the virus is re-activated.

IFNs: a group of cytokines released by host cells in response to pathogens such as viruses, bacteria and parasites. The name derives from their capacity to “interfere” with viral replication and thus protect cells from viral infection.

JH: refers to seven regions (JH1-7) of structural similarity between the four JAK isoforms (JAK1-3, Tyk2).

KIR: a sequence within each of SOCS1 and SOCS3 that directly interacts with receptor-bound JAKs to inhibit kinase activity.

MPNs: a group of four rare bone marrow disorders (chronic myeloid leukaemia, polycythaemia vera, essential thrombocythaemia, myelofibrosis) that cause an

increase in the numbers of specific blood cell types. MPNs are distinguished from each other based on symptoms and the type of blood cells affected.

Multicentric Castleman's disease: condition in which there is abnormal growth of cells within multiple lymph nodes.

Neutropenia: deficiency of neutrophils, which play an important role in fighting infection by destroying invading bacteria and fungi.

Orthosteric: refers to a primary binding site, as opposed to an "allosteric" binding site which is spatially distinct from the primary/orthosteric site.

Overlapping peptide array: Describes an organised pattern of peptides spotted onto a glass or cellulose support. In an overlapping peptide array, a protein's full sequence is subdivided into partially overlapping 10-20 residue peptides. These arrays can then be used for several downstream applications, including identification of protein-protein interaction sites.

PEST sequence: Pro-Glu-Ser-Thr-enriched sequences found in many proteins and which play important roles in controlling protein half-life.

Psoriasis: chronic autoimmune condition characterised by the presence of dry, itchy and inflamed patches of skin. The most common form is plaque psoriasis which presents as red patches topped with white scaly skin.

PAH: defined as high blood pressure in the small blood vessels that supply the lungs (pulmonary arterioles). Pulmonary arteriole walls become thick, stiff and resistant to expansion to allow blood flow. The reduced blood flow makes it harder for the right-hand side of the heart to pump blood through the arteries, ultimately causing heart failure.

Relapse-remitting multiple sclerosis: autoimmune condition in which the sheath of myelin that protects nerve cells is damaged. This disrupts communication in the nervous system, resulting in a range of physical symptoms including muscle weakness, blindness and poor coordination. In the relapse-remitting form, symptoms occur in isolated attacks.

Repurposing: application of approved drugs to treat a different disease. Also termed "repositioning". One of the best known examples is sildenafil, which was originally

developed as an anti-hypertensive medicine but has been repurposed for treatment of erectile dysfunction and PAH.

RA: a long-term autoimmune condition that typically causes pain, swelling and stiffness in joints of the hands, feet and wrists. Other organ systems can be also affected, including the blood and cardiovascular systems.

Severe combined immunodeficiency: a group of rare genetic disorders that cause major abnormalities of the immune system, leading to greatly increased risk of infection and other life-threatening complications.

SH2 domain: a structurally conserved protein module that allows interaction with phosphorylated tyrosine residues on other proteins.

Thrombocytopenia: deficiency of platelets required for effective clotting following tissue damage. Therefore, thrombocytopenia can lead to easy or excessive bruising (purpura), prolonged bleeding times and the appearance of red or purple blood spots in the skin (petechiae).

Outstanding Questions

Are cavin-1 and CUEDC2 members of a wider family of SOCS1 and SOCS3-interacting regulatory proteins that control SOCS function and/or subcellular localisation?

To what extent is interaction with cavin-1 and CUEDC2 shared within the SOCS family?

Does SOCS3 maintain caveolae directly through its interaction with cavin-1? And does SOCS3 play a role in determining cellular resistance to mechanical damage in vivo by regulation of caveolae?

EDITOR IN CHIEF

We have restructured the manuscript in the suggested running order, in the process taking out a lot of mechanistic detail from the introductory sections and reducing the total word count in line with the recommendation. The length of the main text of the article is now 3460 words and includes 58 references.

We have also simplified the figures as suggested (particularly Figs 1A and B), taking out extraneous detail and better integrating them into the main text so they illuminate the key points being communicated.

In terms of the specific comments in the original manuscript:-

1. The introductory paragraph now has more appropriate references.
2. The article is much less focused on IL-6 so much of this detail has been removed.
3. In Fig 1A, a generic JAK domain structure has replaced the original figure. The 2 phosphorylation sites in the figure responsible for activation of all 4 JAK isoform are now given in the legend. For clarity, we have also removed the term “pseudoSH2” throughout the article.

REVIEWER 1

We thank the reviewer for describing our article as “very well written and timely”. With respect to specific points:-

1. Cbl E3 ligases – while it is an excellent suggestion to include some information about this class of E3 ligases which also target JAKs, the restricted word length of the Opinion article means we cannot include this.
2. The text on JH1-JH2 interactions has now been removed.
3. CIS1 has been corrected to CIS.

REVIEWER 2

We thank the reviewer for describing our article as “well written and thoughtful”. With respect to specific points:-

1. Other approved biologics – we have reduced the focus on IL-6 throughout the revised manuscript. In reference to the reviewer’s specific point, we have now included references to approved IL-17 and IL-5 biologics (page 3).
2. JAK inhibitors – we have now included anaemia as a common side effect as requested (page 3). We have also included filgotinib as a JAK1 inhibitor in the context of reducing side effects with more selective drugs (page 3). The word

restriction on an Opinion article means we cannot go into depth on this topic. Finally we mention how increased risk of infection is a side effect of biologics such as tociluzimab and JAK inhibitors (page 3).

3. Cavin-1 – the reviewer makes the valid point that cavin-1 expression in immune cells is limited. However our manuscript proposes targeting the distinct regions on SOCS3 that interact with cavin-1 and CUEDC2 as approaches to enhance endogenous SOCS3 function. While these approaches are independent of whether cavin-1 or CUEDC2 are co-expressed with SOCS3, given the importance of SOCS3 in determining cavin-1 stability, small molecules that bind SOCS3 and block interaction with co-expressed cavin-1 may impact function of those cells in which cavin-1 is abundantly expressed. This is now elaborated on p11.

REVIEWER 3

We appreciate the reviewer's criticisms and have taken them on board as follows:-

1. Figures – in response to feedback from the Editor in Chief to simplify the background section, we have changed Figure 1A to show a simplified scheme for cytokine receptor activation of the JAK-STAT pathway. In Figure 3, the construct used for SOCS1-JAK1 JH1 domain structural studies did not contain a SOCS box. This information is now included in the legend for clarity. In terms of similarity to a previously published figure (singular), the reviewer is referring to a schematic of the domain structure of JAK1 and JAK2. We have simplified Figure 1A in line with the Editor in Chief's feedback and, as a result, it is now distinct.
2. We have incorporated some clarification to describe how modulation of SOCS function would alter cell function to alleviate disease. For example, when discussing anti-viral effects of SOCS1 antagonists, this is discussed as a consequence of enhancing anti-viral interferon responses by blocking SOCS1-mediated negative feedback of interferon signaling (page 8). Likewise, we discuss how SOCS3 agonists or stabilisers could be developed for RA (page 9) and PAH (page 10) through potentiation of SOCS3-mediated inhibition of pro-inflammatory IL-6 signalling.

REVIEWER 4

We thank the reviewer for describing our article as “well organized and written”. With respect to specific points:-

1. SOCS as targets is counterintuitive – we take the point that receptor-targeted small molecules generated by targeting cytokine receptors would be a more conventional approach to generate selective therapeutics. However, we do not believe this is enough of an argument to discount SOCS-targeted therapeutics. IN fact, a criticism of targeting “more broadly acting downstream regulators” could also be levelled at currently approved JAKinibs, which non-selectively act downstream of multiple JAK-STAT-mobilising receptors yet demonstrate efficacy in a range of disorders (pages 3-4 and associated references). Also, our manuscript is now being considered as an Opinion piece and is therefore a more appropriate platform for proposing our perspective.
2. As literature in this area is limited, we have been advised by the Editor in Chief to reconfigure our manuscript as a shorter Opinion piece, which we have now done. Also, while many of the experiments in the supporting literature used overexpression systems to identify and characterize key observations, in the case of SOCS3/cavin-1, these were validated in cells deficient in endogenous SOCS3 and cavin-1. To be frank, the work would not have been published if experiments in overexpression systems were not validated in cells expressing the endogenous proteins.
3. Given that the focus of the article is SOCS proteins, we respectfully disagree with the reviewer that the Box material is not a good fit. In support of this, none of the other referees commented on this.
4. Inclusion of the SHP2 binding site in Figure 1A – in response to the Editor in Chief’s feedback, we have reduced the focus on IL-6 and have instead produced a new Figure 1A which shows a simplified scheme for cytokine receptor activation of the JAK-STAT pathway.