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Cross-Regulation of JAK-STAT Signaling: Implications for Approaches to Combat Chronic Inflammatory Diseases and Cancers

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1. Introduction

The Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway is utilized by a range of cytokines (interferons, IL-2 and IL-6 amongst others) that control survival, proliferation and differentiation responses in diverse cell types. The realisation that unregulated activation of this pathway is a key driver of not only chronic inflammatory diseases such as rheumatoid arthritis, colitis and psoriasis, but also many cancers has identified its components as targets for therapeutic intervention by small molecule inhibitors and biologicals. In this article, we will discuss how an increased understanding of JAK-STAT pathway architecture, the basis for its dysfunction in pathological states, and its regulation by other intracellular signaling pathways are illuminating multiple strategies to manipulate this pathway in several disease arenas.

2. Basic architecture of the JAK-STAT pathway

2.1 Janus Kinases (JAKs)

JAKs encompass a family of four of cytoplasmic tyrosine kinases (JAK1–JAK3, TYK2) that function as essential signaling components immediately downstream of receptors for many haematopoietic cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (Epo), interferons (IFNs), interleukins (e.g. IL-2, IL-6) as well as growth hormone and leptin. While JAK1, JAK2 and TYK2 are widely expressed, JAK3 expression is limited to haematopoietic cells where it is used by the receptors for a selected group of cytokines that are critical in T cell, B cell and natural killer cell development (Ghoreschi et al., 2009). Importantly, functional deficiencies in JAK3 have been shown to account for autosomal recessive "severe combined immunodeficiency" (SCID) syndrome (O'Shea et al., 2004). However, despite these differences, JAKs are thought to function downstream of individual cytokine receptors in a similar manner.

Structurally JAKs comprise seven conserved domains (JAK homology (JH) domains 1-7) numbered from the carboxyl to the amino terminus (Fig. 1). The hallmark of the JAK family is the presence of JH1, which comprises a functional tyrosine kinase domain, and JH2, which was originally thought to be a catalytically inactive pseudokinase domain. However, it has recently been demonstrated that the JAK2 JH2 has dual-specificity protein kinase activity that phosphorylates Ser523 and Tyr570, which are critical negative regulatory sites, although the specific kinase activity of JH2 is approximately ten-fold less than that of JH1 (Ungureanu et al., 2011). It had long been established that JH2 has a negative regulatory function, as deletion of this domain has been shown to increase JAK2 and JAK3 phosphorylation and downstream activation of STATs. This is achieved *via* an intramolecular interaction between JH1 and JH2, which effectively suppresses basal kinase activity. Upon ligand binding, conformational changes relieve this interaction allowing activation of JH1 by phosphorylation of two activation loop Tyr residues (Tyr 1021/1022 in JAK1, Tyr 1007/1008 in JAK2, Tyr 980/981 in JAK3, Tyr 1054/1055 in TYK2) (Saharinen et al., 2000; Saharinen & Silvennoinen, 2002). Abrogation of JH2's dual specificity kinase activity was found to be sufficient to increase basal JAK2 activity and downstream signaling to STAT1, suggesting that this is a key element of the JH2 domain's suppressive effect on JH1. As described later in this chapter, this provides an essential "braking" function as mutations that disrupt the JH2 domain's suppression of JH1 activity are found in several haematological disorders.

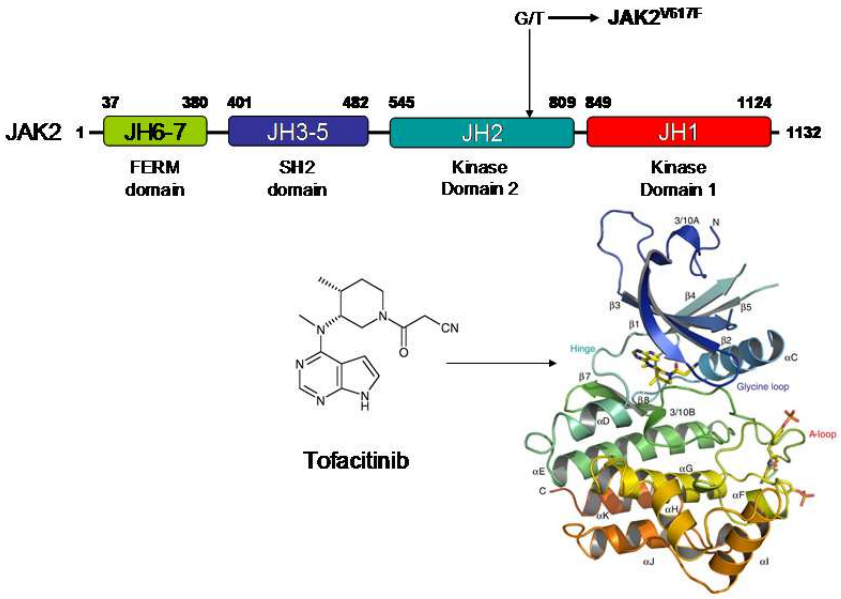

Fig. 1. Domain structure of human JAK2. The crystal structure of the JAK2 JH1 domain bound to JAK inhibitor tofacitinib is reproduced from Williams et al. (2009) with permission.

JH3-5 comprise an SH2 domain typically indicative of PTyr-dependent interactions with other signaling components but surprisingly partners for this domain have yet to be identified for any of the JAK family (Ingley & Klinken, 2006). Lastly, JH6-JH7 constitute a

Band 4.1, ezrin, radaxin, moesin (FERM) domain implicated in interactions with cytokine receptors required for cell surface expression (Huang et al., 2001) and also the JH1 domain to regulate its kinase activity (Zhou et al., 2001).

JAKs have typically been thought to be constitutively associated with dimeric cytokine receptor complexes at the plasma membrane, although a role for nuclear-localised JAKs in controlling the phosphorylation of histone H3 has emerged that may be particularly important in haematological malgnancies and embryonic stem cell renewal (Dawson et al., 2009; Griffiths et al., 2011). However, following cytokine binding, a conformational change within the dimeric receptor complex allows activation of receptor-bound JAKs due to transphosphorylation of JH1 activation loop Tyr residues. The structural basis of the JAK-receptor interaction and the mechanism by which the receptor re-orientates to receive the phosphorylation are currently unclear and the subject of intense research (Lupardus et al., 2011). Nevertheless, after receptors have been phosphorylated at specific Tyr residues, SH2 domain-containing proteins are recruited to activate downstream signaling. The major intracellular mediators commonly activated by multiple cytokine receptors are the signal transducer and activator of transcription (STAT) proteins.

2.2 Signal Transducers and Activators Of Transcription (STATs)

The STAT family of transcription factors were first described as interferon (IFN)-inducible transcription factors (Fu, 1992; reviewed by Darnell *et al.*, 1994;). The STAT family comprises seven mammalian members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6). However alternative splicing of STAT1, 3, 4, 5a and 5b primary transcripts yields isoforms with truncated C-terminal domains; for example the STAT3β isoform lacks the 55 C-terminal amino acids of STAT3α but acquires a unique 7 amino acid sequence (Dewilde et al., 2008). While the β isoforms have been reported to act as dominant negative regulators of transcription, it is now apparent that these isoforms can differ in their transcriptional activities, for example isoform-specific deletions of STAT3β and STAT3α have shown that STAT3β activates a distinct subset of STAT3 genes in response to IL-6 and may not act as a dominant negative *in vivo* (Maritano et al., 2004; Dewilde et al., 2008). Exactly how the discrete functional differences observed between STAT3α and STAT3β relate to their distinct subcellular trafficking kinetics remains to be determined (Huang et al., 2007).

STATs are activated by a wide range of cytokines and other growth factors. Depending on the cell type involved, a range of sometimes overlapping ligands can activate STAT1, STAT3, STAT5a and STAT5b, whereas only a few cytokines are capable of activating STAT2, STAT4 and STAT6 (Lim & Cao, 2006). For example, in addition to multiple IL-6 family members, STAT3 can also be activated by G-protein coupled receptors for angiotensin II and thrombin, as well as the VEGFR2 receptor tyrosine kinase (Madamanchi et al., 2001; Pelletier et al., 2003; Bartoli et al., 2000). Functionally, STAT3 has been implicated in cell proliferation, survival and anti-apoptotic responses in multiple cell types (Yu et al., 2009). Conversely, STAT6 is predominantly activated by IL-4 and is involved in the differentiation of $CD4^+$ T cells into T helper 2 (T_h2) cells (Hebenstreit et al., 2006).

Structurally, STATs comprise several distinct functional domains; these include an N-terminal domain, a coiled-coil domain, a DNA binding domain, a linker domain, an SH2 domain and a C-terminal transactivation domain. The N-terminal domain is involved in

STAT dimerisation and tetramerisation, and for some STATs the recruitment of regulatory proteins such as STAT E3 ubiquitin ligase SLIM (Tanaka et al., 2005). The coiled-coil domain is also implicated in protein-protein interactions; for example, the transcription factor c-Jun has been demonstrated to interact with this domain in STAT3, facilitating co-operation required for maximal IL-6-dependent acute-phase response gene activation driven by the 2-macroglobulin enhancer (Zhang et al., 1999b). This domain may also be involved in ensuring high affinity binding to cytokine receptors, as mutations within it impair recruitment of STAT3 to Tyr-phosphorylated gp130 (Zhang et al., 2000). The DNA-binding domain is highly conserved amongst the STAT family and as well as binding DNA, it also controls STAT translocation from the cytoplasm to the nucleus. It has been proposed to achieve this by maintaining the necessary conformation for binding of importins on the nuclear membrane (Ma & Cao, 2006). Once in the nucleus, STAT dimers can bind DNA motifs known as GAS (γ activated sequence) elements ($TTN^{5-6}AA$, N=any nucleotide) except in the case of the IFNα/β response, where complexes formed between STAT1, STAT2 and IRF9 (interferon regulatory factor 9) bind to the IFNα/β-response element (ISRE) $AGTTN_3TTTC$ (O'Shea et al., 2002). The linker domain has been implicated in transcriptional activation, since point mutations within this region of STAT1 have been found to abolish transcriptional responses to IFNγ (Yang *et al*., 1999). Additionally, this domain also participates in distinct protein-protein interactions, as demonstrated by the interaction of STAT3 with "genes associated with retinoid–IFN-induced mortality-19" (GRIM-19), which blocks STAT3-mediated transcriptional activity (Kalakonda et al., 2007).

The most conserved domain within the STAT family is the SH2 domain. This domain is essential for binding to activated receptors and is also responsible for cytokine-triggered dimerisation *via* specific phospho-Tyr residues (Shuai et al., 1994; Hemmann et al., 1996). Different receptor motifs determine which STATs are recruited; for example, STAT3 will bind to pYXXQ (Stahl et al., 1995) while STAT1 will only bind to pYXPQ (Gerhartz et al., 1996). This difference has been shown to be due to the SH2 domain through the creation of a chimaeric STAT3 molecule in which the SH2 domain of STAT3 was substituted with a STAT1 SH2 domain, resulting in a molecule that showed the receptor motif binding preference of STAT1 (Hemmann et al., 1996). On recruitment to an activated cytokine receptor, STATs are then phosphorylated by JAKs on a single conserved tyrosine residue at the carboxyl end of the SH2 domain (e.g. Tyr701 in STAT1 (Shuai et al., 1994) and Tyr705 in STAT3 (Kaptein et al., 1996)). This enables them to form dimers through an interaction of the P-Tyr on one STAT with the SH2 domain of another.

Lastly, the C-terminal transactivation domain mediates protein-protein interactions necessary for optimal transcriptional activation; these include interactions with the transcriptional co-activators "cAMP response element binding protein" (CREB)-binding protein (CBP) and p300 (Paulson et al., 1999). In the cases of STAT1 and STAT3, optimal interaction with p300/CBP requires phosphorylation of Ser727 by any of several Ser/Thr kinases, including extracellular signal-regulated kinases 1 and 2 (ERK1,2) (Schuringa et al., 2001; Heinrich et al., 2003).

To demonstrate how these proteins function in context, we will describe signaling pathway activation in response to the activation of two key cytokine receptor systems implicated in several haematological and non-haematological conditions: the IL-6 and IFN signaling complexes.

2.3 Activation of the JAK-STAT and ERK1,2 pathways by IL-6

The IL-6 receptor is composed of two different subunits, an 80 kDa IL-6-binding protein (IL-6Rα) and a 130 kDa signal-transducing subunit (gp130), which is shared by all IL-6-family cytokines, which include IL-11, IL-27, oncostatin M, leukaemia inhibitory factor (LIF), cardiotrophin-1 (CT-1) and ciliary neurotrophic factor (CNTF) amongst others (Heinrich et al., 2003). The gp130 subunit is ubiquitously expressed while IL-6Rα expression is restricted to hepatocytes, monocytes, neutrophils and some B and T cell subsets. However, IL-6 can also bind to a soluble form of the receptor (sIL- 6Rα) which is either shed from cell membranes by limited proteolytic cleavage of membrane-bound IL-6Rα by the metalloproteinases ADAM10 and ADAM17 or created by alternative splicing of IL-6Rα primary transcripts (Kallen, 2002). The resulting sIL-6Rα/IL-6 so-called "trans-signaling" complex can associate with gp130 on cells that do not express the membrane-bound IL-6R thereby widening the spectrum of IL-6-responsive cells. For example, vascular endothelial cells which express only gp130 are rendered responsive to IL-6 due to the shedding of sIL-6Rα from activated neutrophils (Marin et al., 2001).

Binding of the trans-signaling complex to gp130 or the interaction of IL-6 with IL-6Rα triggers dimerisation of the gp130 subunits and the formation of an active receptor signaling complex (Murakami et al., 1993; Fig. 3). JAK1, JAK2 and TYK2 have each been shown to be activated upon receptor stimulation (Stahl *et al.*, 1994; Narazaki et al., 1994) and can phosphorylate gp130 on Tyr's 683, 759, 767, 814, 905 and 915 (Stahl et al., 1994; Stahl et al., 1995; Gerhartz et al., 1996). (Fig. 2) Studies using cell lines lacking either JAK1, JAK2 or TYK2 have revealed that whereas JAK2 and TYK2 may be functionally interchangeable, effective downstream signaling absolutely depends on the presence of JAK1 (Guschin et al., 1995). Thus, phosphorylation of gp130 was demonstrated to be significantly reduced in the absence of JAK1 but was unimpaired in the absence of either JAK2 or TYK2. The membrane-proximal regions of gp130 are predominantly responsible for binding JAK1. These regions contain conserved box1 and box2 motifs which are both required for efficient JAK binding. Either deletions or mutations to box1 result in the impaired binding of JAKs to gp130 (Haan et al., 2000) while deletion of box2 only leads to JAK association when the kinase is over-expressed (Tanner et al., 1995), suggesting that box2 increases the affinity of JAK binding. In addition, an interbox1-2 region on gp130 is also involved in JAK binding and again, in studies where this region has been mutated, defective JAK signaling has been observed (Haan et al., 2000).

All IL-6 type cytokines are capable of activating STAT1 and STAT3 *via* gp130. However, STAT3 activation has been observed to a greater extent than STAT1 activation (Heinrich et al., 2003). STAT recruitment to activated IL-6 type receptors is mediated by the STAT SH2 domain and requires the phosphorylation of specific Tyr residues. In particular, STAT3 binds four phospho pYXXQ motifs of gp130 (Y^{767}RHQ, Y^{814}FKQ, Y^{905}LPQ and Y^{915}MPQ) (Stahl et al., 1995), whereas STAT1 is more restricted and binds two pYXPQ motifs in gp130 (Y^{905}LPQ and Y^{915}MPQ) (Gerhartz et al., 1996) (Fig. 2). Once recruited, STATs are phosphorylated by JAKs on a single Tyr residue (Tyr701 in STAT1 and Tyr705 in STAT3 (Kaptein *et al.*, 1996; Shuai *et al.*, 1993) (Fig. 2). In addition, as discussed earlier, both STAT1 and STAT3 can be phosphorylated by ERK1,2 on Ser727 to enhance p300/CBP recruitment for maximal transcriptional activity (Schuringa et al., 2001; Heinrich et al., 2003). Following phosphorylation, activated STATs form homo- and/or hetero-dimer complexes, consisting

of STAT1-STAT1, STAT1-STAT3 or STAT3-STAT3 dimers, which translocate to the nucleus to bind response elements of IL-6 inducible genes. STATs bind to essentially two types of response elements; interferon stimulated response elements (ISREs) and gamma-activated sites (GAS). The ISRE appears to be restricted to IFN signaling (Fu et al., 1990), whereas the GAS, including *sis*-inducible element (SIE), acute phase response element (APRE) and other GAS-like sequences are present in promoters of genes such as *c-fos* and the acute phase proteins that are well-defined STAT targets (Wegenka et al., 1993). Other target genes downstream of STAT3 include cell cycle regulators such as cyclin D1, and anti-apoptotic genes such as survivin and Bcl-X_L (Alvarez & Frank., 2004).

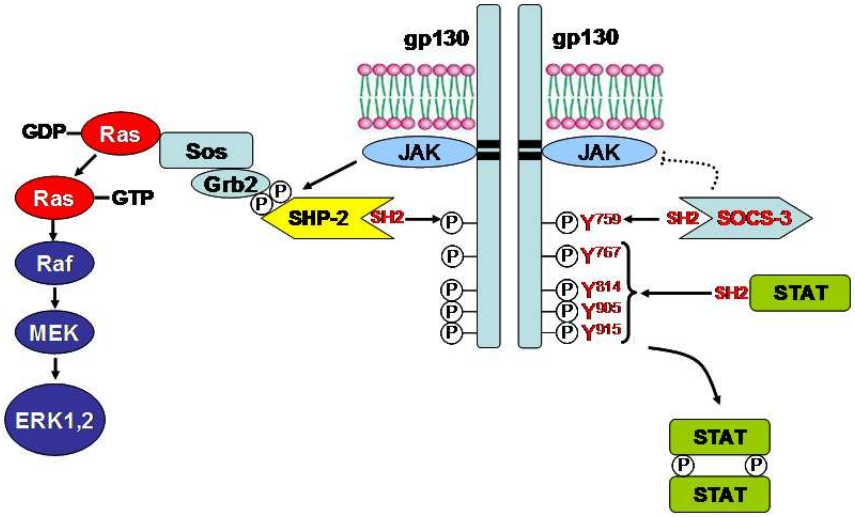

Fig. 2. JAK-mediated activation of ERK1,2 and STATs *via* gp130

Finally, it should be emphasized that SHP2 (SH2-domain-containing cytoplasmic protein tyrosine phosphatase), a ubiquitously expressed and highly conserved PTP, is also recruited to the activated IL-6 receptor. SHP2 comprises two N-terminal SH2 domains and a C-terminal PTP domain. It is recruited to pTyr759 on gp130 following receptor activation and is subsequently phosphorylated by JAKs. The phosphorylation of SHP2 provides SH2 domain docking sites for the adapter protein Grb2 (growth factor receptor binding protein 2), which is constitutively associated with the Ras guanine nucleotide exchange factor Son of sevenless (Sos) (Fig. 2). It has been proposed that the C-terminal domain residues Tyr542 and Tyr580 within SHP2 interact with the Grb2-Sos complex (Heinrich et al., 2003). Sos recruitment to the receptor complex allows for the activation of Ras at the plasma membrane, which in turn leads to the activation of the Ras-Raf-MEK-ERK1,2 cascade. ERK1,2 activation results in the preferential phosphorylation of substrates with the consensus sequence PXS/TP and more than 150 substrates have been identified to date (Yoon & Seger, 2006).

2.4 Activation of the JAK-STAT pathway by interferons (IFNs)

The IFNs comprise three families of secreted proteins that work in both an autocrine and paracrine manner to trigger intracellular signaling networks designed to combat viral

infection. Type I IFNs in humans are derived from seventeen genes clustered within chromosome 9. In humans, type I IFNs comprise thirteen IFNα's and a single version of each of IFNβ, ω, ε and κ (Borden et al., 2007). Interestingly, expression of IFN-κ and IFN-ε seems tissue specific, but all cells have the potential to produce other IFNs although the spectrum of IFNs actually induced appears to vary in a stimulus-specific manner (LaFleur et al., 2001; Hardy et al., 2004). IFNγ is designated as a distinct type II IFN because of its distant sequence homology with the type I IFNs, as well as its production by natural killer (NK) or activated T cells. The type III IFN family comprises three IFN-λ subtypes which are co-produced with IFN-β, and which activate the same main signaling pathways as type I IFNs but *via* different receptor complexes (Borden et al., 2007).

2.4.1 IFNγ signaling

The functional IFNγ receptor (IFNGR) comprises two 90 kDa IFNGR1 and two 62 kDa IFNGR2 chains. IFNGR1 is involved in ligand binding and signal transduction while IFNGR2 has a limited role in binding but is essential for downstream signaling (Stark et al., 1998). Originally, these subunits were not thought to interact in unstimulated cells but advances in imaging techniques in living cells have shown that the receptor is pre-assembled and ligand binding results in a conformational change to allow signaling to occur (Krause et al., 2002). IFNGR1 and IFNGR2 have binding motifs for JAK1 and JAK2 respectively. JAK1 binds to the membrane proximal sequence, L^{266}PKS on IFNGR1 (Kaplan et al., 1996) while JAK2 binds a Pro-rich noncontiguous motif of P^{263}PSIP followed by I^{270}EEYL on IFNGR2 (Bach et al., 1996).

Upon receptor activation, JAK2 is autophosphorylated, enabling it to trans-phosphorylate JAK1 (Briscoe et al., 1996). The activated JAKs then phosphorylate each IFNGR1 chain on Tyr440 within the sequence Y^{440}DKPH, which creates a pair of docking sites for STAT1 SH2 domains. STAT1 is then phosphorylated on Tyr701, dissociates from the receptor, forms homodimers and translocates to the nucleus. Activation of the phosphatidylinositol 3-kinase (PI3K) pathway also appears to play a role in IFNγ-induced STAT1-mediated transcriptional regulation, as inhibition of either PI3K or one of its downstream effectors, protein kinase C δ (PKCδ), blocks STAT1 phosphorylation on Ser727, thus reducing its transcriptional activity. Therefore, since IFNγ has been shown to activate PKCδ in a PI3K dependent manner, it has been suggested that PKCδ is an IFNγ-regulated Ser 727 kinase for STAT1 (Nguyen et al., 2001; Deb *et al.*, 2003), although a role for PKC-activated p38 MAP kinase has recently emerged (Borden et al., 2007).

2.4.2 IFNα/β signaling

Type I IFNs belong to the cytokine group that display similar secondary structures of a five α-helix bundle stabilized by two disulphide bonds (Borden et al., 2007). Binding of type I IFNs to their cognate receptor complex, which comprises one chain each of IFNAR1 and IFNAR2c, activates TYK2 and JAK1 constitutively associated on the respective chains. Interestingly, the intracellular domain of IFNAR2c plays the key role in the recruitment and docking of STATs while deletion of much of the cytoplasmic domain of IFNAR1 is without effect on signaling. The presence of a single Tyr residue within the IFNAR2c cytoplasmic

domain (either Tyr337 or Tyr512) is required for a full IFN response from this receptor (Borden et al., 2007; van Boxel-Dezaire & Stark, 2007).

Activated TYK2 then phosphorylates receptor-bound STAT2, an essential component of type I IFN signaling, on Tyr690 allowing it to interact with STAT1, which is then phosphorylated on Tyr701. STAT1 and STAT2 form a heterodimeric complex which dissociates from the receptor and translocates to the nucleus. It is now apparent that type I IFNs can activate all STAT members, but the transcription factor complex unique to type I IFNs is ISGF3 (IFN-stimulated gene factor 3), a trimeric complex of STAT1, STAT2 and a 48kDa DNA-binding protein IRF9 (IFN regulatory factor 9). Upon nuclear import, the STAT1 and IRF9 components of the complex specifically bind ISREs within target gene promoters. STAT2 does not contribute to the DNA binding activity of the ISGF3 complex, instead contributing a powerful C-terminal transactivation domain responsible for recruitment of p300/CBP co-activators (Borden et al., 2007).

3. Negative regulation of cytokine signaling

Cytokine signaling is typically transient, suggesting the involvement of negative regulatory steps aimed at terminating or resolving responses. Indeed, controlling these responses is crucial for avoiding detrimental inflammatory outcomes, including the development of diseases such as rheumatoid arthrisis (RA), Crohn's disease and Castleman's disease. There are many mechanisms with which to negatively control cytokine signaling but for the purposes of this chapter we will focus on soluble ligand traps, suppressors of cytokine signaling (SOCS) proteins and PTPs.

3.1 Soluble ligand traps

As previously mentioned, the IL-6 receptor is composed of two different subunits, an 80 kDa IL-6Rα and a 130 kDa signal-transducing gp130 subunit. A soluble form of the signal transducer protein gp130 (sgp130) was also detected in the circulation at relatively high concentrations (100–400 ng/ml in human plasma) (Narazaki et al., 1993; Chalaris et al., 2011). Sgp130 is produced by alternative splicing and has been shown to bind the sIL-6R/IL-6 complex in the circulation, thus acting as a selective inhibitor of IL-6 mediated trans-signaling, as classic signaling *via* membrane-localised IL-6R is not affected (Muller-Newen et al., 1998). Moreover, sgp130 appears to be specific for the IL-6/sIL-6R complex since signaling from gp130 in response to other IL-6-type cytokines such as LIF and OSM were only inhibited at 100–1000-fold higher concentrations (Chalaris et al., 2011).

As discussed later, the ability of either endogenous or genetically engineered soluble cytokine ligand traps to block binding to and activation of signaling from endogenous cytokine receptors has emerged as a very useful strategy to turn off excessive cytokine signaling associated with inflammatory and autoimmune diseases (Jones et al., 2011). In the case of IL-6, they have also increased our understanding of the contributions of classical *versus* trans-signaling in specific biological processes (Scheller et al., 2011).

3.2 Suppressor of Cytokine Signaling (SOCS) proteins

There are eight members of the suppressor of cytokine signaling (SOCS) family of proteins; CIS (cytokine-inducible SH2 domain-containing protein) and SOCS1 through to SOCS7 (Fig.

3). SOCS1 was the first member to be discovered in 1997 by three independent groups (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). Using the predicted amino acid sequence of SOCS1 as a probe, database searches identified 20 proteins with shared sequence homology within the C-terminal SOCS box region. Based on the presence of a central SH2 domain, the SOCS proteins were subdivided into a group of their own. The remaining proteins were divided into the following groups; WD-40-repeat proteins with a SOCS box (WSB proteins), ankyrin repeat proteins with a SOCS box (ASB proteins), sprouty (SPRY) domain-containing SOCS box proteins (SSB proteins) and GTPase domain-containing proteins (RAR and RAR-like proteins) (Krebs & Hilton, 2001). In addition to a central SH2 domain, all members of the SOCS family contain an amino-terminal region of variable length (50-380 amino acids) and a conserved 40 amino acid carboxyl terminal "SOCS box" (Alexander, 2002; Yoshimura et al., 2007). Analysis of the primary amino acid sequences of all SOCS members has revealed paired associations according to sequence similarity. Thus, CIS/SOCS2, SOCS1/SOCS3, SOCS4/SOCS5 and SOSC6/SOCS7 form related pairs. CIS and SOCS1-3 are the best characterised members of the family, while the remainder are poorly understood in comparison. Since both SOCS1 and SOCS3 are well studied, homology-paired and have been shown to potently inhibit cytokine signaling, focus will be placed on these SOCS members in particular.

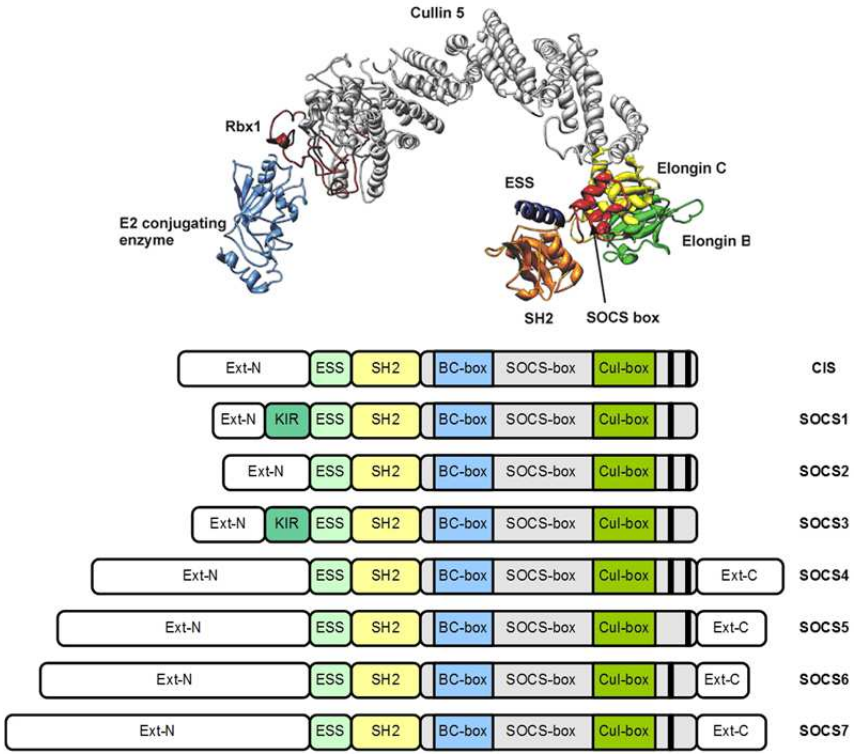

Fig. 3. Elongin-Cullin-SOCS-box (ECS) family of E3 ubiquitin ligases. Structure taken from Piessevaux et al. (2008) with permission.

3.2.1 SOCS proteins as inhibitors of cytokine signaling

SOCS proteins function as classical negative feedback inhibitors of cytokine signaling, since most SOCS proteins are themselves induced by cytokines. Cytokines shown to induce SOCS include the gp130 signaling cytokines, IL-2, IL-3, IL-4, IL-10, IFNs, G-CSF and leptin. (Alexander, 2002; Yoshimura et al., 2007). Other inducers include Toll-like receptor (TLR) agonists (e.g. LPS, CpG-DNA), growth hormone (GH), prolactin and cyclic AMP (cAMP) (Dalpke et al., 2001; Gasperini et al., 2002; Lang et al., 2003; Sands et al., 2006). The SOCS proteins can inhibit signaling by multiple mechanisms according to the SOCS member and signaling pathway involved. SOCS proteins can bind specific PTyr residues *via* their SH2 domain. Thus, SOCS3 binds to PTyr759 (PTyr757 in mouse) on gp130 (Nicholson et al., 2000) and physically occupies the same sites as other SH2 domain-containing signaling components such as SHP2, thereby competing with and blocking activation of other signaling pathways (De Souza et al., 2002; Heinrich et al., 2003) (Fig. 2). *In vitro* studies have shown that the phospho-peptide binding specificity of SOCS3 is very similar to that of SHP2, with optimal SOCS3 and SHP2 phospho-peptide ligands containing overlapping consensus sequences (De Souza et al., 2002). The same group also demonstrated that SOCS3 binds to the gp130 receptor with a much higher affinity than the leptin receptor ObRb. However, the finding that SOCS3 can bind two sites on the leptin receptor versus one site on gp130 may compensate for the low affinity each ObRb site exhibits (De Souza et al., 2002).

The kinase inhibitory region (KIR) of SOCS1 and SOCS3, located upstream of the SH2 domain, is capable of interacting with the substrate binding site of the JH1 domain of JAKs, acting as a pseudosubstrate and thus inhibiting catalytic activity and downstream signaling from the associated receptor (Sasaki et al., 1999; Yasukawa et al., 1999). Specifically, Tyr31 of SOCS3 and Tyr65 of SOCS1 have been identified as the critical residues responsible for the pseudosubstrate inhibition of JAK2 (Bergamin et al., 2006). Interestingly, structural data relating to this interaction has revealed that it is implausible for Tyr31 or Tyr65 to reach the active kinase domain of JAK2 if bound *via* the SH2 domain i.e. in *cis* (Bergamin et al., 2006). This does not rule out the possibility that the SOCS proteins could bind to one JAK molecule *via* their SH2 domain and inhibit another JAK *via* pseudosubstrate inhibition i.e. in *trans,* or the possibility that binding of the SOCS SH2 domain to the specific PTyr residues positions the KIR for binding to the kinase domain of associated JAK2. The latter possibility appears to be the more likely scenario, since the crystal structure of the SOCS3/gp130 complex and various structural data favour the physiological target of SOCS3 SH2 domain to be pTyr757/759 of mouse/human gp130 and not the activation loop of JAK2 (Bergamin et al., 2006).

The SOCS box present within all SOCS members can recruit elongins B and C, which together with cullin 5 and RING-box 2 (Rbx2) form an E3 ubiquitin-ligase complex (Fig. 3). This complex associates with enzymes E1, a ubiquitin-activating enzyme and E2, a ubiquitin-conjugating enzyme, to mediate Lys48 polyubiquitylation and subsequent proteasomal degradation of signaling components bound to the SOCS proteins *via* their SH2 domains (Kamura et al., 2004; Ungureanu et al., 2002; Zhang et al., 1999a) (Fig. 3). A possible ubiquitination site, Lys-6 is also present at the N-terminus of SOCS3, and an N-terminally truncated isoform of SOCS3 lacking this site is significantly more stable than wild-type SOCS3, suggesting that polyubiquitylation of Lys6 plays an important role in regulating turnover. Moreover, this demonstrates one level by which SOCS3 expression can be regulated post-translationally (Sasaki et al., 2003).

SOCS proteins can theorectically target the whole receptor-cytokine complex including the JAKs, plus the SOCS proteins themselves, for proteasomal degradation. Therefore, it raises the question of how SOCS proteins selectively block JAK signaling at specific receptors. This could possibly be explained by the realization that SOCS SH2 domains preferentially bind specific PTyr motifs on activated receptors rather than JAKs, thereby causing degradation of associated JAKs as well as the receptor-cytokine complex, and also achieving specificty at the receptor level. Indeed, it has been shown that mutation of the SOCS3 binding site Tyr757 to non-phosphorylatable Phe on murine gp130 is sufficient to cause enhanced IL-6-inducible gene expression (Anhuf et al., 2000). Furthermore, bone marrow-derived macrophages (BMDMs) isolated from mice with a Tyr757Phe mutation in gp130 switch the IL-6 mediated response to an "IL-10-like" *anti*-inflammatory response, in terms of inhibiting LPS-induced induction of pro-inflammatory cytokines (El Kasmi et al., 2006). Previous studies have linked the absence of SOCS3 with the establishment of the anti-inflammatory response following IL-6 treatment (Yasukawa et al., 2003), so the data suggests that mutation of only the specific PTyr that binds SOCS3 is sufficient to cause cytokine receptors to become refractory to SOCS inhibition, despite the presence of JAKs. This is in contrast to SOCS1, for which it has been shown that the phenotype of SOCS1-deficient mice can only be partially rescued in mice with SOCS1 lacking the SOCS box, but retaining the SH2 domain. This shows that both the SOCS box and SH2 domain are required for the inhibitory effects on IFN-signaling (Zhang *et al*., 2001). Mice in which endogenous SOCS3 has been replaced with a truncated SOCS3 mutant lacking the SOCS box have been shown to be viable but exhibit hyper-responsivenenss to G-CSF, suggesting that linkage of SOCS3 to the ubiquitin machinery is important in restraining G-CSF signaling in vivo (Boyle et al., 2007), possibly through controlling the ubiquitination-mediated routing of the G-CSF receptor to lysosomes (Irandoust et al., 2007).

In contrast to findings of SOCS interaction with elongins B and C leading to proteasomal degradation, some studies have found that interaction with the elongin BC complex can stabilise both SOCS3 (Haan et al., 2003) and SOCS1 (Kamura et al., 1998). Haan et al. (2003) showed that Tyr phosphorylation of SOCS3 disrupted interaction with elongins, which accelerated SOCS3 degradation. This may suggest that Tyr phosphorylation of SOCS3 is a prerequisite to its subsequent proteasomal degradation. Indeed, Haan *et al.* (2003) proposed that the elongin BC interaction with SOCS3 may function to associate SOCS3 with a latent ubiquitination complex that only becomes active when SOCS3 is phosphorylated. SOCS3 phosphorylation on Tyr204 and/or Tyr221 causes the dissociation of elongin C and the bringing together of the ubiquitination machinery into close proximity with SOCS3, subsequently triggering its degradation (Haan et al., 2003).

3.2.2 SOCS proteins as regulators of other Tyr phosphorylation-dependent pathways

In addition to the involvement of SOCS proteins in cytokine signaling, SOCS1 and SOCS3 have been shown to bind receptors for EGF and FGF receptors and affect downstream signaling events both positively and negatively (Ben-Zvi et al., 2006; Xia et al., 2002). With regards to EGF signaling, SOCS1 and 3 have been shown to facilitate EGFR proteasomal degradation in HEK293 cells (Xia et al., 2002), while SOCS1 has been shown to inhibit STAT1 phosphoryation and elevate ERK1,2 phosphorylation in response to FGF treatment of rat chondrosarcoma (RCS) cells (Ben-Zvi et al., 2006). Furthermore, SOCS1 and SOCS3

have both been shown to associate with insulin receptor substrates 1 (IRS1) and IRS2 following insulin stimulation, and interact with the elongin BC ubiquitin-ligase complex to promote their polyubiquitylnation and subsequent degradation (Kawazoe et al., 2001; Rui et al., 2002).

As mentioned earlier, SOCS3 offers another level of regulation by being able to become phosphorylated on Tyr204 and Tyr221 within the SOCS box in response to IL-2, Epo and other stimuli (Cacalano et al., 2001; Haan et al., 2003). Using a murine B cell line, it was found that WT SOCS3 could inhibit IL-2-mediated STAT5 phosphorylation, but maintain IL-2-mediated ERK1,2 phosphorylation, whereas a Tyr204/221Phe double mutant SOCS3 still inhibited STAT5 phosphorylation, but in contrast to the WT, induced the abolition of ERK1,2 phosphorylation, suggesting a phosphorylation-dependent maintenance of ERK signaling. (Cacalano et al., 2001). The inhibitory effect of the mutant was also observed following Epo and PDGF treatment. Thus, phosphorylation of Tyr204 and Tyr221 of SOCS3 following growth factor stimulation has been proposed to trigger PTyr221 interaction with the SH2 domain of RasGAP, thereby sustaining GTP accumulation on Ras and subsequent activation of ERK1,2. It is well established that the duration of ERK1,2 signaling is important for determining biological outcome; for example sustained activation of ERK has been shown to be required for the control of G1 progression by regulating cyclin D1 activation in some systems (Weber et al., 1997). SOCS3 therefore appears to have both pathway-specific and receptor-specific effects, and can positively regulate activation of specific signaling pathways, adding further complexities to its actions.

An additional level of complexity demonstrated by SOCS proteins is their ability to interact with other SOCS family members (Piessevaux *et al*., 2006; Tannahill *et al*., 2005). For example, although SOCS2 plays a major role in the negative regulation of GH signaling (Greenhalgh *et al*., 2002), it has also been shown to enhance GH signaling. This is believed to be caused by the binding of SOCS2 to other SOCS members and modulating their activity *via* the elongin BC complex, with subsequent proteasomal degradation (Piessevaux et al., 2006; Tannahill et al., 2005). This SOCS2-mediated inhibitory effect on other SOCS members has been observed on SOCS1- and SOCS3-dependent inhibition of GH signaling, thus potentiating it (Piessevaux et al., 2006). SOCS2 has also been shown to enhance IL-2 and IL-3 signaling (Tannahill et al., 2005) by accelerating proteasome-dependent degradation of SOCS3. Similar effects again have been shown on signaling *via* the IFN type 1 and leptin receptors (Piessevaux et al., 2006). These observations imply that SOCS2 is counteracting the effects of other SOCS proteins, rather like a secondary negative feedback mechanism, to limit the effects of excessive levels of SOCS proteins. This assumption is supported by the findings that SOCS2 induction is usually initiated after a significant delay following cytokine stimulation and is prolonged, whereas SOCS1 and SOCS3 expression is typically rapid and transient (Adams et al., 1998). Although poorly understood, SOCS6 and SOCS7 have also been shown to bind other SOCS members and similar effects to SOCS2 have been observed for SOCS6 (Piessevaux et al., 2006). Again, this data suggests that SOCS proteins can act as positive and negative regulators of signaling pathways and could explain some reported anomalies, such as the enhanced insulin signaling observed in transgenic mice overexpressing SOCS6 (Li et al., 2004) or the gigantism observed in transgenic mice overexpressing SOCS2 (Greenhalgh et al., 2002).

3.2.3 Functional roles of SOCS proteins

The functions of SOCS proteins *in vivo* have largely been elucidated by the generation of mice engineered to lack particular SOCS genes. These studies have greatly enhanced our understanding of their roles particularly with regards to the immune response, and have also identified key definitive roles of individual SOCS members, such as the non-redundant role SOCS1 appears to play in IFN signaling (Alexander et al., 1999). However, this is not always the case and knock-out models can encounter problems. Due to placental insufficiency, SOCS3-null mice die at mid-gestation (Roberts et al., 2001; Takahashi et al., 2003) and to overcome this, other ways of investigating SOCS3 deficiency have been explored. A genetic cross study conducted by Robb et al. (2005) showed that mice on a double LIF/SOCS3-null background were rescued from embryonic lethality due to placental failure, and the mice appeared normal at birth (Robb et al., 2005). It is believed that the deletion of SOCS3 leads to dysregulated LIF signaling, which alters trophoblast differentiation and causes placental defects (Boyle & Robb, 2008). In support of this is the finding that the number of trophoblast giant cells are reduced in LIFR-null mice, compared with an abnormally high number of trophoblast giant cells in SOCS3-null mice (Takahashi *et al*., 2003). Although embryonic lethality is rescued, a high neonatal mortality rate is observed in SOCS3$^{-/-}$LIF$^{-/-}$ null mice and adult animals develop a fatal inflammatory disease very similar to that seen in mice with a conditional deletion of SOCS3 in haematopoietic cells (Croker *et al*., 2003). On the other hand LIF$^{-/-}$ mice have a normal lifespan and do not exhibit any major haematopoietic abnormalities, suggesting that SOCS3 plays a vital role in the negative regulation of the inflammatory response.

Another way to overcome SOCS3 embryonic lethality is the generation of conditional knock-outs using the inducible Cre recombinase (Cre)-loxP system. In this way, the modified target gene can be ablated in adulthood, thus avoiding the placental insufficiency observed with constitutive global SOCS3 knockouts, and the ablation of the gene can be targeted to any tissue at a defined time. This is a powerful tool for the examination of genes that appear to be crucial during embryonic development but may also play important roles in particular adult tissues. Using these systems, the SOCS3 gene has been specifically deleted in the liver and in macrophages. The absence of SOCS3 results in sustained STAT3 and STAT1 activation following IL-6 treatment, but normal activation of STAT1 in response to IFNγ and normal activation of STAT3 in response to IL-10 (Croker et al., 2003; Lang et al., 2003). SOCS3 deficiency was also shown to trigger up-regulation of several IFN-responsive genes following IL-6 treatment, which is not normally observed upon IL-6 stimulation of cells with functional SOCS3 alleles. This suggested that in the absence of SOCS3, sustained STAT1 activation provokes a dominant IFN-like gene expression response. Furthermore, a mutation in gp130 (Tyr757Phe) in mice, which impedes SOCS3 and/or SHP2 recruitment, was shown to result in a phenotype displaying characteristics of RA, a condition already well established to be associated with deregulation of IL-6 signaling (Atsumi et al., 2002). Collectively, these studies demonstrate that SOCS3 is the main physiological regulator of IL-6 signaling and that SOCS3 can regulate the "identity" of the cytokine response as well as the duration of the signal (Croker et al., 2003; Lang et al., 2003).

Interestingly, in the absence of SOCS3 in mouse macrophages, IL-6 has been shown to induce an "IL-10-like" anti-inflammatory response, as demonstrated by a reduction in LPS-induced production of TNFα and IL-12 (Yasukawa et al., 2003). This is an interesting

observation because until this point there was no obvious explanation as to why these two cytokines should have such diverse effects. Both cytokines use identical JAK-STAT members and yet have very distinct gene expression patterns (Murray, 2007; O'Shea & Murray, 2008). IL-10 has been shown to be anti-inflammatory in macrophages and dendritic cells, activating a different set of genes from IL-6, but both cytokines also activate a common pool of genes, including SOCS3 (Murray, 2007). Yasukawa *et al*. (2003) proposed that the difference in gene expression may be due to the intensity of the STAT3 signal. However, Murray (2007) has identified flaws in this concept; for example the strength of the signal does not account for the commonality of genes activated by the two cytokines. One obvious difference between the two cytokines is the involvement of SOCS3 as an inhibitory regulator of IL-6 but not IL-10 signaling. Studies have shown that if modified receptors are used, which are either naturally insensitive to SOCS3 (e.g. IL-22R) or engineered to be insensitive (e.g. IL-6, leptin receptors) but still activate STAT3, an anti-inflammatory response is triggered (El Kasmi et al., 2006). Thus, based on SOCS3 involvement, a hypothesis has been proposed describing the activation of a generic pool of STAT3 by the IL-10R, which is not subjected to any inhibition by SOCS3. The IL-6R, on the other hand may activate a different pool of STAT3 which can be specifically inhibited by SOCS3, possibly *via* post-translational modification by kinases, phosphatases, methylases or other regulators. These distinct STAT3 pools may therefore go on to activate different sets of genes. However, this is just one idea and ultimately ChIP-Seq experiments will be necessary to identify any differences in the genomic locations to which STAT3 can be recruited following stimulation with either IL-10 or IL-6.

With regards to leptin signaling, mice with a neural-specific deletion of SOCS3 have been generated using the Cre-loxP system. Similar to the observations for IL-6, SOCS3 deletion resulted in prolonged activation of STAT3 in response to leptin. Moreover, SOCS3-deficient mice exhibited a greater weight loss compared to their wild-type littermates. These knock-out mice were also resistant to high fat diet-induced weight gain and hyperleptinaemia, and retained insulin sensitivity. This study showed that SOCS3 is a key regulator of leptin signaling and hence plays an important role in diet-induced leptin and insulin resistance (Mori et al., 2004). A number of studies support this link between SOCS3 and leptin resistance, whereby leptin-mediated induction of SOCS3 has been associated with the attenuation of ObRb signaling (Bjorbaek et al., 1998). Chronic stimulation of ObRb has been shown to result in the desensitisation of ObRb signaling, whereby the receptor becomes refractory to re-stimulation. Mutation of the STAT3 binding site on ObRb (Tyr1138Phe), which mediates STAT3-induced SOCS3 induction, alleviates this feedback inhibition. Moreover, RNAi-mediated knock-down of SHP2 had no effect on the attenuation of ObRb signaling, suggesting a role for SOCS3 in the feedback inhibition of ObRb signaling and not SHP2 (Dunn *et al*., 2005). Consistent with a role for SOCS3 as a central regulator of leptin responsiveness, it has been shown recently that the ability of intracellular cAMP sensor Epac1 (exchange protein directly activated by cAMP-1) to trigger the induction of SOCS3 (Sands et al., 2006) blocks multiple signaling pathways downstream of the leptin receptor ObRb, thus suppressing leptin function in hypothalamic pro-opiomelanocortin neurons (Fukuda et al., 2011).

3.3 Protein Tyrosine Phosphatases (PTPs)

Protein phosphatases reverse the effects of protein kinases by catalysing the removal of phosphoryl groups to initiate, sustain or terminate signals (Andersen et al., 2001). Protein

tyrosine phosphatases (PTPs) comprise a large family of these proteins and are distinguished by a unique signature motif. Residues in this motif form the phosphate-binding loop and two residues (a Cys and Arg) are critical for the catalytic activity of PTPs (Andersen et al., 2001; Tiganis & Bennett, 2007). PTPs can be grouped into two general families; (1) the Tyr-specific PTPs, which can dephosphorylate substrate proteins on Tyr; these can be further sub-divided into transmembrane receptor-like PTPs and non-transmembrane PTPs, and (2) the dual-specificity phosphatases (DUSPs), which can dephosphorylate protein substrates on Tyr, Ser and Thr (Tiganis & Bennett, 2007). Our understanding of PTPs is greatly lagging behind that of PTKs, partly due to the discovery of PTKs a decade before PTPs. However like most kinases, PTPs exhibit a high degree of specificity towards their substrates. This is achieved by the PTP catalytic domain, which recognises phosphorylated Tyr residues and the flanking amino acids within the substrate, and the non-catalytic N- and C- terminal domains, which target the PTP to particular intracellular compartments for substrate recognition (Andersen et al., 2001). This is exemplified by the DUSPs, which dephosphorylate MAPKs on Tyr and Thr residues. Of the 10 members that make up this family, some can specifically target one class of MAPK (e.g. DUSP6/MKP-3 which dephosphorylates ERK1,2) while others can target more than one MAPK class (e.g. DUSP1/MKP-1 which can dephosphorylate ERK, JNK and p38 MAP kinases) (Owens & Keyse, 2007).

Given their importance in cytokine signaling, we will describe three PTPs in detail: PTP1B, TC-PTP and SHP2.

3.3.1 PTP1B

The prototypical PTP is PTP1B, which was first identified in 1988 (Charbonneau *et al*., 1988). It has been shown to have numerous substrates, but the most extensively studied of these include the insulin receptor (IR) and JAK2 (Tiganis & Bennett, 2007). Much of this information has come from analysis of PTP1B-null mice, which exhibit enhanced insulin sensitivity resulting from increased insulin-stimulated Tyr phosphorylation of the insulin receptor in muscle and liver. Furthermore, these mice are resistant to diet-induced obesity (Elchebly et al., 1999). Additional studies have since revealed the involvement of leptin signaling in the above phenotype and have demonstrated PTP1B inhibition of leptin signaling *via* dephosphorylation of JAK2 (Cheng et al., 2002).

3.3.2 T cell PTP (TC-PTP)

T-cell-specific protein tyrosine phosphatase (TC-PTP), which as the name suggests was originally cloned from a peripheral T cell cDNA library, is a ubiquitously expressed PTP. The primary transcript is processed into two splice variants that encode TC45 and TC48 isoforms. The resulting differences in primary sequence at the C-terminal domains of TC45 and TC48 are responsible for their distinct intracellular localization patterns (Lorenzen et al., 1995). Specifically, TC45 is localized to the nucleus due to a R^{378}KRK sequence, while TC48 is localized to the endoplasmic reticulum by its unique C-terminal 19 amino acids (Lorenzen et al., 1995). The nuclear TC45 isoform has several proposed substrates, including the IR and EGFR (Tiganis & Bennett, 2007), JAK1 and JAK3 (Simoncic et al., 2002), and also STAT1 and STAT3 (ten Hoeve et al., 2002; Yamamoto et al., 2002). Of interest, Yamamoto et al. (2002) have demonstrated TC45-mediated suppression of STAT3 activation in response to IL-6 in

293T cells, implicating the nuclear isoform of TC-PTP as an important negative regulator of IL-6 signaling. This is supported by studies of TC-PTP-deficient mice, which display a more complex phenotype as compared to mice deficient in the closely related PTP1B. Specifically, TC-PTP-null mice are viable but exhibit profound defects, resulting in splenomegaly, lymphadenopathy and thymic atrophy. As a result, the mice typically die at 3-5 weeks. Homozygous mice display defects in bone marrow, B cell lymphopoiesis, erythropoiesis, and impaired T and B cell functions. Taken together, the abnormalities displayed in TC-PTP-deficient mice strongly suggest a crucial role in haematopoiesis and immunity (You-Ten *et al.*, 1997). However, targeted deletion of TC-PTP in defined cell types will undoubtedly reveal previously unknown aspects of its function. For example, a recent study has shown mice lacking TC-PTP in neurons are hypersensitive to leptin and are resistant to diet-induced weight gain compared with WT animals (Loh et al., 2011). Animals in which both PTP-1B and TC-PTP were deleted showed additive effects compared with the single knockouts, suggesting that both PTPs regulate ObRb signaling. However, it was found that TC-PTP levels are elevated in hypothalamus extracts from obese mice, raising the possibility that this may contribute relatively more to the leptin resistance observed in obesity (Loh et al, 2011; Myers et al., 2008).

3.3.3 SH2 domain-containing PTP 2 (SHP2)

The SH2-domain containing PTPs (SHPs) are a subfamily of non-transmembrane PTPs comprising two vertebrate members, SHP1 and SHP2. While SHP1 expression is restricted to cells of the haematopoietic system, SHP2 is ubiquitously expressed. Both proteins contain two N-terminal SH2 domains (N-SH2 and C-SH2) and a C-terminal catalytic PTP domain. As such, both proteins have the unique ability to function as PTPs, dephosphorylating signaling components and thus *inhibiting* signal transduction, whilst also serving as adapter molecules *via* their SH2 domains to recruit further adapter molecules and *activate* downstream signaling (Heinrich et al., 2003; Neel et al., 2003). Both SHP1 and SHP2 appear to have non-redundant roles since homozygous deletion of either gene in mice results in death at 2-3 weeks due to severe inflammation. The SHP1-null phenotype is termed "motheaten" because of the patchy hair loss caused by sterile dermal abscesses (Neel et al., 2003) while SHP2 deletion produces embryonic lethality due to defective gastrulation or mesodermal differentiation (Neel et al., 2003). Thus, one SHP does not appear to compensate for the other in these phenotypes. The functional differences between SHP1 and SHP2 appear to be due to the differences in SH2 domain-mediated protein interactions, as well as differences in the PTP domains of both proteins (Salmond & Alexander, 2006).

The finding that ERK1,2 activation by IL-6 is inhibited in either knock-in mice expressing a Tyr759Phe-mutated gp130 (gp130$^{F759/F759}$) or cells transfected with a mutated Tyr759Phe gp130 construct, establishes a positive regulatory role of SHP2 on the ERK1,2 pathway (Kim & Baumann, 1999; Ohtani et al., 2000). Indeed, studies on growth factor receptor signaling, including EGF, insulin and platelet-derived growth factor (PDGF) signaling have each demonstrated a positive regulatory role of SHP2 on ERK1,2 signaling and gene expression (e.g. Yamauchi et al., 1995). In contrast to these positive effects, gp130$^{F759/F759}$ mice and cells transfected with a Tyr759Phe-mutated gp130 demonstrate impaired SHP2 activation, prolonged STAT3 and STAT1 activation and enhanced acute-phase protein gene induction, suggesting a negative role of SHP2 on the STAT pathway (Ohtani et al., 2000; Schaper et al.,

1998). In support of this negative regulatory role, the gp130$^{F759/F759}$ mouse displays splenomegaly, lymphadenopathy and an enhanced acute phase reaction (Ohtani et al., 2000). However, these results are complicated by the findings that SOCS3 binds to the same PTyr site on gp130 as SHP2 (De Souza et al., 2002) and could therefore potentially contribute towards these negative effects. To address this issue, studies have employed catalytically inactive dominant-negative SHP2 mutants in gp130 signaling. Expression of these mutants result in increased gp130, JAK and STAT3 phosphorylation as well as elevated gene induction (Lehmann et al., 2003; Symes et al., 1997), thus confirming the involvement of SHP2 in the negative regulation of the STAT pathway activated by gp130.

These negative effects of SHP2 on gp130 signaling may be receptor-specific as they do not appear to be observed for signaling from the leptin receptor ObRb, since mutation of SHP2 binding site Tyr985 of ObRb has no effect on STAT activation (Li & Friedman, 1999). Also, using dominant negative SHP2 strategies in COS-1 cells, SHP2 was shown not to have an effect on STAT3 phosphorylation or STAT3-mediated gene transcription from the SOCS3 promoter (Bjorbaek et al., 2001). However, these studies demonstrated no effect of SHP2 on STAT activation and STAT-dependent promoter activity following 15 minutes and 6 hours of leptin treatment respectively. Further research by this group has demonstrated that following 24 hours of leptin treatment, STAT-mediated transcription was enhanced in cells expressing mutated Tyr985Phe ObRb (Bjorbaek et al., 2001) and suggested that the induction of SOCS3 by leptin over prolonged leptin treatment could account for the enhanced STAT3 response, implicating SOCS3 involvement and not SHP2. In addition, over these extended periods of leptin treatment, SHP2 could possibly act as an indirect positive regulator of the STAT pathway, preventing SOCS3 binding to the ObRb at Tyr985 (Bjorbaek et al., 2001).

Thus, in general, a positive role of SHP2 in cytokine-induced ERK1,2 activation and a negative or positive role of SHP2 in cytokine-induced STAT activation have been proposed. How SHP2 mediates this positive effect on ERK activation and the contribution of SHP2 to the negative regulation of STAT signaling in relation to SOCS3 are still areas under investigation. To address the first point, SHP2 appears to exert a positive effect on ERK signaling by acting as an adapter protein, wherein SHP2 becomes recruited to PTyr residues on activated cytokine receptors and following activation, associates with the adapter protein Grb2, which is bound to the Ras GDP-GTP exchange factor Sos. The membrane-localised Grb2/Sos complex can then go on to activate the Ras-Raf-MAK-ERK pathway (Li et al., 1994). With regards to gp130 signaling, it has been proposed that the SHP2-Grb2/Sos mode of ERK activation is adopted (Heinrich et al., 2003). However, in addition to this model, Grb2-associated binder-1 (Gab1) has also been shown to become tyrosine phosphorylated to associate with SHP2 and phosphatidylinositol 3-kinase (PI3K) to activate ERK in response to IL-6 (Takahashi-Tezuka *et al*., 1998). Alternatively, SHP2 may act as a PTP, thus dephosphorylating and inactivating specific substrates that normally suppress ERK1,2 activation. For example, SHP2 has been shown to dephosphorylate tyrosine residues on EGFR required for RasGAP recruitment to the receptor, thereby inhibiting phosphorylation-dependent translocation of RasGAP to the plasma membrane and thereby maintain Ras-GTP levels to sustain ERK1,2 activation (Agazie & Hayman, 2003).

Finally a model has been proposed whereby the binding of SHP2 to PTyr residues has been shown to activate its PTP activity *via* either of two mechanisms (Barford & Neel, 1998); (1) the SH2 domains can bind to PTyr motifs on activated receptors such as pTyr759 on gp130,

which leads to unfolding of the protein and subsequent activation of phosphatase activity, or (2) the SH2 domains can bind to PTyr542 and 580 on the C-terminal tail of SHP2 itself and cause conformational changes leading to activation. If PTyr binding does not occur, SHP2 remains in an inactive state whereby the N terminal SH2 (N-SH2) domain sterically hinders access of phosphorylated substrates to the PTP domain, as demonstrated by its crystal structure (Hof et al., 1998). Therefore, the N-SH2 domain of SHP2 can either bind and inhibit the phosphatase, or bind to PTyr residues on substrates to activate the enzyme. Interestingly, in the human autosomal dominant disorder Noonan Syndrome, approximately 50% of all cases are caused by mutations in the SHP2 gene *PTPN11*, and specifically in portions of the amino N-SH2 domain. These mutations lock SHP2 in its active conformation and subsequently cause excessive SHP2 activity (Tartaglia et al., 2001). Noonan Syndrome is characterised by short stature, cardiac defects, facial dysmorphia and an increased risk of developing leukaemia (Salmond & Alexander, 2006). Interestingly this syndrome displays aberrant hyperactivation of the ERK1,2 pathway (Bentires-Alj et al., 2006). Thus, Noonan Syndrome demonstrates an involvement of SHP2 in regulating ERK activation in human disease although further research is required to better understand the exact mechanisms involved.

Likewise, the mechanisms by which SHP2 negatively or positively regulates STAT activation are not well understood and the relative contribution of both SHP2 and SOCS on gp130 or ObRb signaling is still unclear. There are reports showing that SHP2 can act directly as a STAT phosphatase (Chen et al., 2003) and STAT3/SHP2 complexes have been detected in cells (Gunaje & Bhat, 2001). A large body of evidence supporting a negative role of SHP2 in gp130-mediated STAT activation exists, whereas conversely, SHP2 has been shown to have no effects on ObRb-mediated STAT3 phosphorylation (Bjorbaek et al., 2001). Therefore the possibility exists that SHP2 acts as an indirect positive regulator of STAT3 by impeding SOCS3 recruitment at PTyr985 (Bjorbaek et al., 2001).

4. Cross-regulation of signaling pathways via SOCS proteins

Cells are typically exposed to a changing milieu of cytokines and other extracellular regulators. Therefore to produce an appropriate response, effective crosstalk between distinct signaling pathways is essential. In terms of cytokine receptor signaling, the levels at which such crosstalk can occur are now beginning to be revealed, and here we will discuss two recently characterized examples of this type of regulation.

4.1 Regulation of SOCS3 expression by cAMP/Epac1

Many studies have shown that prototypical second messenger cAMP exerts profound anti-inflammatory and immunosuppressive effects on many target cell types, including vascular endothelial cells, neutrophils, monocytes, CD4 T cells and regulatory T cells (Mosenden & Tasken, 2011). Many of these effects are mediated *via* defined gene transcription programmes, which include the up-regulation of protective molecules, such as the immunosuppressive cytokine IL-10, while down-regulating pro-inflammatory molecules such as TNFα and IL-1 (e.g. Wall et al., 2009). A large body of research has identified members of the cAMP-response element binding (CREB) protein family as the principal mediators of changes in gene expression in response to cAMP following their phosphorylation by PKA. However, observations of cAMP-mediated induction of specific

genes occurring *via* PKA-independent mechanisms have demonstrated that other mechanisms must also exist. For example, we have demonstrated that cAMP elevation is capable of inducing the SOCS3 gene *via* a PKA-independent mechanism that requires Epac1 (Sands et al., 2006; Woolson et al., 2009). Epac1 and Epac2 were originally identified as guanine nucleotide exchange factors (GEFs) for the Rap 1 and Rap2 small GTPases. Each has several important structural features, including a cyclic nucleotide binding domain (Epac2 has two such domains), a DEP (Dishevelled, Egl, Pleckstrin) domain, a REM (Ras exchanger motif) domain and a Cdc25 homology domain that exhibits GEF activity (Borland et al., 2009b). Identification of SOCS3 as an Epac-regulated target gene revealed a new mechanism by which cAMP could inhibit signaling from specific SOCS3-targeted cytokine receptors, such as the leptin receptor ObRb and gp130 (Sands et al., 2006; Fukuda et al., 2011). The mechanism of SOCS3 induction is mimicked by expression of a constitutively active Val12 mutant of Rap1a and is blocked by either siRNA-mediated depletion of Rap1 or overexpression of RapGAPII, suggesting that the ability of Epac1 to stimulate the accumulation of GTP-bound active Rap1 is essential (Yarwood et al., 2008). Rap1 then activates phospholiase C ε to generate intracellular *sn* 1,2-diacylglycerol (DAG) and mobilize intracellular calcium, resulting in the activation of specific protein kinase C isoforms (Borland et al., 2009a), although exactly how PKC regulates SOCS3 transcription is unclear.

We have also identified the transcription factor(s) targeted by Epac1, demonstrating that Epac1 activation increases the binding of the CCAAT/enhancer binding protein β (C/EBPβ) to a region within the human SOCS-3 promoter enriched in C/EBP-responsive elements in vascular endothelial cells *in situ*. In addition, overexpression of specific C/EBP isoforms in endothelial cells can potentiate Epac-mediated induction of SOCS-3, while selective knockdown of either C/EBPβ or C/EBPδ abolishes induction. Similar experiments performed on C/EBP knockout murine embryonic fibroblasts have confirmed these findings. Interestingly, SOCS3 induction in response to cAMP requires activation of the ERK1,2 pathway, which triggers phosphorylation of C/EBPβ on Thr225. The importance of this event is demonstrated by the ability of Thr225Ala-mutated C/EBPβ to function as a dominant-negative inhibitor of SOCS3 induction (Borland et al., 2009a). However, siRNA-mediated depletion of Epac1 fails to diminish cAMP-stimulated ERK1,2 phosphorylation, which occurs *via* a PKA-independent mechanism, suggesting that an additional PKA-independent pathway is involved in C/EBP-mediated SOCS3 induction (Woolson et al., 2009).

While a role for specific transcription factors has yet to be addressed, characterisation of the induction of the pro-glucagon and AQP2 genes in response to cAMP has revealed some similarities to the induction of SOCS3. In each case, induction by cAMP-elevating agents is resistant to inhibition of PKA and can be mimicked by selective activation of Epac by the Epac-selective activator 8CPT-2′-O-Me-cAMP. There also appears to be a requirement for activation of the ERK1,2 pathway by cAMP (Lotfi et al., 2006; Umenishi et al., 2006). These similarities would suggest that the PKA-independent mechanisms by which the pro-glucagon, AQP2 and SOCS3 genes are induced in response to cAMP may be related and presumably apply to other yet to be identified Epac-inducible genes.

4.2 Regulation of multiple steps within the NF-κB pathway by SOCS proteins

Given that SOCS family members each contain an SH2 domain, it is not surprising that their influence on cellular processes have been found to extend beyond classical JAK-STAT

signaling processes. These include the inhibition of insulin signaling due to SOCS3-mediated ubiquitylation and degradation of IRS1 and/or IRS2 (Kawazoe et al., 2001; Rui et al., 2002), and the regulation of FAK1 (Liu et al., 2003; Stevenson et al., 2010). Importantly, many studies have also revealed that SOCS proteins are able to regulate the NF-κB pathway at several levels, providing a key mechanism by which JAK-STAT-mobilising cytokines can control pro-inflammatory responses.

Members of the TLR family of receptors and the receptor for IL-1 trigger an inflammatory response via a conserved mechanism. The cytosolic region of these receptors contains a Toll/IL-1 receptor (TIR) domain that interacts with a panel of adaptor molecules to activate defined intracellular signaling cascades, including p38 and c-Jun N-terminal kinase MAP kinase pathways and the NF-κB pathway (O'Neill, 2009). While several TIR domain-interacting proteins have been identified, the best characterized are MyD88 (myeloid differentiation primary response gene 88), Mal (MyD88 adaptor-like), TRIF (TIR domain-containing domain that induced IFNβ) and TRAM (TRIF-related adaptor molecule). All TLRs activate the NF-κB pathway but different receptors utilize specific subsets of adaptors to achieve this; for example, both TRAM and TRIF have been shown to be required for activation of IRF3/7 and NF-κB by TLR4, while the absence of Mal abolishes TLR2- and TLR4-stimulated pro-inflammatory cytokine production (O'Neill, 2009; Brikos & O'Neill, 2008). The requirement for Mal is thought to be due to an interaction with TRAF6 that promotes phosphorylation of the RelA subunit of NF-κB on Ser536, which is required for binding of transcriptional co-activators, rather than any effect on transcription factor translocation to the nucleus from the cytosol (Verstak et al., 2009). The ability of SOCS1 to regulate TLR-mediated responses has been reported by multiple investigators, but no one unifying mechanism has emerged. One study has demonstrated that activation of either TLR2 or TLR4 with selective agonists can trigger the proteasomal degradation of Mal but not MyD88 (Mansell et al., 2006). Further characterization of this phenomenon revealed that this process required the Tyr phosphorylation of Mal by Bruton's tyrosine kinase (BTK), which is also activated by TLRs. Upon SOCS1 induction in response to TLR activation, the SOCS1 SH2 domain is thought to bind phosphorylated Mal. Once bound, SOCS1 functions as an E3 ubiquitin ligase to catalyse the Lys48 polyubiquitylation of Mal, thereby targeting it for degradation by the proteasome, blocking RelA phosphorylation and turning off NF-κB-mediated gene transcription (Mansell et al., 2006) (Fig. 4). However it should be noted that others have found that SOCS3 plays an important role in the suppression of IL-1 signaling by binding and inhibiting TAK1, a kinase which is required for IL-1 receptor-mediated initiation of MAP kinase and NF-κB pathways (Frobøse et al., 2006). The significance of this interaction for SOCS-mediated suppression of TLR signaling has yet to be investigated.

Several recent studies have also shown that SOCS1-mediated negative regulation of NF-κB is not restricted to activation by TIR domain-containing receptors due to its incorporation into a multimeric complex by COMMD1 (copper metabolism gene MURR1 domain-containing protein 1) (Maine & Burstein, 2007). The defining ~70 residue domain conserved within all 10 members of the COMMD family is located at their C-termini, and mutation of conserved residues within this domain has been shown to ablate inhibition of NF-κB. Interestingly, COMMD1 appears to associate with the RelA component of NF-κB complexes on target gene promoters, i.e. after translocation of NF-κB from the cytoplasm to the nucleus (Maine et al., 2007; Geng et al., 2009). Current research suggests that RelA-bound COMMD1 then recruits a SOCS1-elongin B/C-Cul2-Rbx1 E3 ubiquitin ligase complex which promotes

the polyubiquitylation and proteasomal degradation of RelA, thus turning off NF-κB-mediated target gene transcription (Fig. 4). Accordingly, COMMD1 deficiency results in a sustained nuclear accumulation of RelA without affecting nuclear entry of NF κB (Maine et al., 2007; Geng et al., 2009).

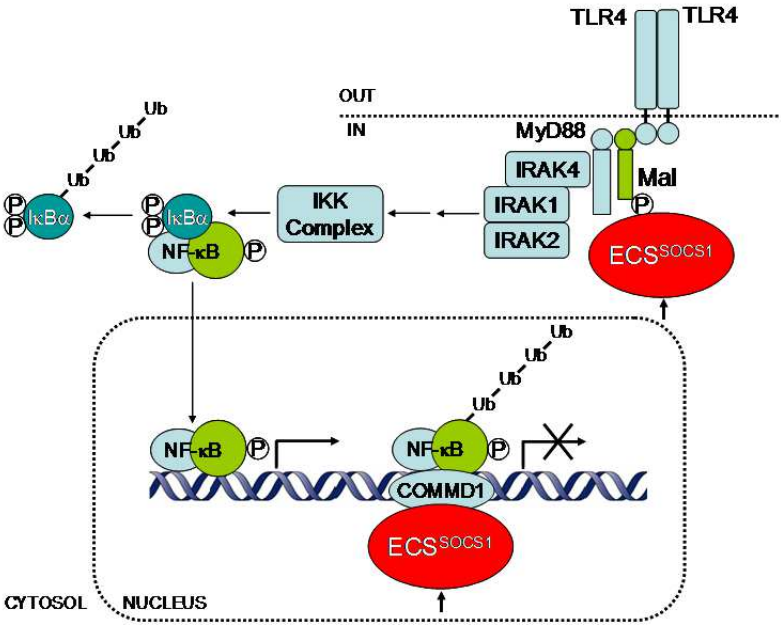

Fig. 4. Control of the NF-κB pathway at multiple levels by a elongin-cullin-SOCS1 (ECS^{SOCS1}) ubiquitin E3 ligase complex.

5. Implications for therapeutic strategies to treat chronic inflammatory diseases and cancers

Over the last decade, the importance of chronic inflammation to the pathophysiology of non-infectious diseases, including cancers, cardiovascular disease and diabetes mellitus, has been established (O'Shea & Murray, 2008). In parallel with these developments, genome wide association studies have identified polymorphisms in cytokine receptors and their associated signaling molecules associated with autoimmune diseases such as type 1 diabetes and inflammatory bowel syndrome (e.g. Rossin et al., 2011), while new therapies have been approved deriving from our understanding of how pro-inflammatory signaling drives pathology (e.g. anti-TNF and IL-6 antibodies for RA and other indications) and several more are in development (e.g. JAK inhibitors) (Taylor & Feldmann, 2009; Ding et al., 2009; Quintás-Cardama et al., 2011).

5.1 Polycythaemia Vera (PV) and other myoproliferative neoplasms

Myoproliferative neoplasms arise from either haematopoietic stem or progenitor cells, and are characterized by the proliferation of terminally differentiated myeloid cells (Quintás-

Cardama et al., 2011). Along with essential thrombocythaemia (ET) and primary myelofibrosis (PMF), polycythaemia vera (PV) represents one of the most studied classes of myoproliferative neoplasms, with patients displaying hyperactivation and proliferation of haematopoietic stem cells in bone marrow, resulting in increased production of platelets, white and red blood cells (Quintás-Cardama et al., 2011). The importance of JAK2 in haematopoiesis had already been established from studies demonstrating that homozygous JAK2 deletion produces embryonic lethality due to insufficient erythropoeisis. This arises due to the pivotal role of JAK2 activation downstream of receptors for cytokines that drive this process, including Epo, thrombopoietin (Tpo), G-CSF, and GM-CSF (Rane & Reddy, 2002). It was subsequently found that approximately 95% of patients presenting with PV have a G-to-T substitution at position 1849 of the JAK2 gene that results in a Val617Phe dominant gain-of-function mutation within the JH2 domain ($JAK2^{V617F}$) (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005) (Fig. 1). As mentioned previously, a recent study demonstrating that the JH2 domain functions as a dual specificity kinase module has shown that the ability of the JH2 domain to suppress JAK2 Tyr kinase activity is disrupted by the Val617Phe mutation due to a reduced JH2-mediated regulatory phosphorylation of Ser523 and Tyr570 (Ungureanu et al., 2011). Additional studies examining those PV patients who are $JAK2^{V617F}$-negative have found that the phenotype is associated with additional mutations within exon 12 of the JAK2 gene. Thus, essentially all cases of PV appear to be driven by somatic activating mutations within the JAK2 gene.

Not surprisingly, expression of Val617PheJAK2 activates signaling pathways that drive proliferation and resistance to apoptosis in a cytokine-independent manner. STAT3 and STAT5, as well as the ERK1,2 and PI3K pathways, are the main effectors of JAK2 activation responsible for the PV phenotype. However it has also recently been proposed that Val617PheJAK2 is resistant to the suppressive effects of SOCS induction. In fact, SOCS3 appears to specifically enhance downstream activation of STAT5 in Epo receptor- and Val617PheJAK2-expressing cells (Hookham et al., 2007). The mutated JAK2 was also found to enhance the Tyr phosphorylation of SOCS3, and this has been proposed as the mechanism by which SOCS3 is rendered incapable of blocking signaling from the Epo receptor and activated Val617PheJAK2. Conversely, others have found that both SOCS1 and SOCS3 are still capable of binding and regulating Val617PheJAK2 expression levels by targeting it for degradation: indeed the mutated constitutively active JAK2 was found to have a reduced stability compared with WT JAK2 (Haan et al., 2009). Furthermore, the authors found that Val617PheJAK2 expression levels have to exceed a minimum threshold before cytokine-independent constitutive activation of downstream signaling is observed, and they propose that such a scenario may explain progression to homozygocity in myoproliferative neoplasms such as PV (Haan et al., 2009). Perhaps related to this observation, the expression ratio of mutated to wild-type JAK2 seems to modulate the phenotypic manifestations of myoproliferative neoplasms, with high ratios favouring the development of a PV-like phenotype and low ratios inducing an ET-like phenotype (Kralovics et al., 2005). In this context, the hypermethylation-mediated silencing of SOCS gene induction seen in idiopathic myelofibrosis (Fourouclas et al., 2008) would be predicted to play an important role in determining pathological severity arising from somatic activating JAK2 mutations.

Given their role in disease and their ability to recapitulate disease phenotypes in mouse models, mutated JAK2 proteins represent attractive targets for drugs to treat myoplastic neoplasms. Incyte's ruxolitinib, an orally available non-selective JAK1/JAK2 inhibitor and one of the first of these agents to be developed, has been shown to improve symptoms such as itching and reduce spleen size in PMF patients but without producing observable remission (Verstovsek et al. 2010; Tefferi et al., 2011). Remarkably, these effects occur regardless of whether the $JAK2^{V617F}$ allele is present. Therefore, determining exactly how this class of drugs can exert its effects is necessary if clinical efficacy is to be improved. It has been suggested that the lack of selectivity of ruxolitinib may be responsible for its limited efficacy in PMF, but comparable studies with more selective inhibitors such as LY3009104 (Lilly, currently in phase II trials for RA), which is approximately 40-fold more selective for JAK2 *versus* JAK1, will be required to address this issue. Tofacitinib (Pfizer, currently in phase III trials for multiple immune/inflammatory disorders including psoriasis and RA) was initially developed as part of a strategy to develop JAK3-selective inhibitors but upon detailed characterization was also shown to have significant activity against JAK1 and JAK2. Encouragingly, JAK inhibitors such as tofacitinib have shown efficacy in several models of $JAK2^{V617F}$–mediated proliferation (Manshouri et al., 2008) and therefore may prove useful if ultimately approved for their primary indications.

However, as with almost all kinase inhibitors currently used for treatment of hyperproliferative disorders such as chronic myelogenous leukaemia (Diamond & Melo, 2011), there is the strong possibility of drug resistance developing following chronic drug administration. Thus, new drug-resistant activating JAK2 point mutations will likely emerge and alternative signaling pathways able to drive proliferation and resistance to apoptosis may be up-regulated to bypass drug-mediated inhibition of JAK2 (Deshpande et al., 2011). As a result, there will undoubtedly be a need to develop additional therapeutics for myeloproliferative disorders to account for these possibilities once first generation JAK inhibitor drugs have been approved.

5.2 RA and other autoimmune disorders

RA is a chronic, systemic autoimmune/inflammatory disease that predominantly results in the deterioration of synovial joints, although other tissues may be affected. The disease process often typically leads to the destruction of articular cartilage and ankylosis of the joints, resulting in discomfort and pain for affected individuals. Key cytokines involved in driving pathogenesis include TNFα, IL-1 and IL-6, which exert their effects on immune cells and synovial fibroblasts (Feldman et al., 1996). Up until approximately ten years ago, the treatment options for RA were limited to disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate, sulphasalazine and leflunomide. While DMARDs are effective for the majority of patients, a significant proportion display adverse reactions to chronic administration, including renal and hepatotoxic effects. This has led to the development of several biological drugs designed to block cytokine receptor activation by binding and sequestering free cytokine to prevent engagement with the cognate receptor, and each is typically administered either as part of a combination therapeutic regimen with one or more DMARDs or as monotherapy in patients not able to tolerate DMARD therapy or for whom it has proven ineffective. They include TNFα blockers (e.g. etanercept, infliximab and adalimumab, the first fully human antibody drug to be FDA approved) and IL-1 blockers (e.g. anakinra) (Taylor & Feldmann, 2009; Gabay et al., 2010).

One of the more recently approved biological therapies is the humanized antibody tocilizumab, which blocks IL-6 signaling by binding to the IL-6R to prevent receptor activation (Ding et al., 2009). The development of IL-6 blockers came from a wealth of research implicating this pleiotropic cytokine in the progression of RA and other autoimmune conditions at several levels. Seminal studies demonstrated that patients with RA, but not osteoarthritis, had elevated levels of synovial IL-6, thereby linking the cytokine to an autoimmune syndrome (Hirano et al., 1988). Studies of knock-in mice expressing a Tyr759Phe-mutated gp130 resistant to SOCS3-mediated inhibition and incapable of activating ERK1,2 demonstrated that these animals spontaneously developed an RA-like autoimmune disease, implicating IL-6 activation of STAT3 as a key mediator of the pathology (Atsumi et al., 2002). Additionally, IL-6 *via* activation of STAT3 can induce the expansion of Th17 cells, which are intimately involved in the development of several autoimmune diseases including RA. In addition, IL-17A can trigger the induction of IL-6 in synovial fibroblasts, thereby creating a positive feedback loop that sustains the chronic pro-inflammatory state (Ogura et al., 2008). IL-6 has also been found to suppress generation of Treg cells, which restrain effector T cell responses (Hirano, 2010). The balance between these two subsets is critical for maintaining homeostasis, and so the ability of IL-6 to drive the balance in favour of Th17 cell dominance is likely to be a be a major cause of pathology in RA and other disorders. Interestingly, it was also found that Tyr759Phe-mutated gp130 must be present in non-haematopoietic cells, and that in response to IL-6 stimulation non-haematopoietic cells expressing Tyr759Phe-mutated gp130 show enhanced production of IL-7, leading to the activation and proliferation of $CD4^+$ T cells (Sawa et al., 2006). These observations demonstrate that IL-6-regulated immune and non-immune cell interactions are critical in the development of RA, and suggest that such interactions may play a similar etiological role in other autoimmune diseases in which IL-6 plays an integral role such as psoriasis, uveitis and inflammatory bowel disease.

5.3 STAT3 activation and tumour development

Mutational activation of proto-oncogenes, coupled with the mutational inactivation of tumor suppressors, are critical events in pathogenesis of cancers (Hanahan & Weinberg, 2011). However several genes that play pivotal roles in the development of malignancies are not activated by mutation. These include the STATs (particularly STAT3) and NF-κB transcription factors, which are activated by extracellular stimuli generated within the tumor microenvironment (Grivennikov & Karin, 2010). As described earlier in this chapter, STATs are typically activated transiently due to the existence of multiple negative feedback mechanisms that act at several levels to terminate signaling, and the same is true for activation of both the canonicical and non-canonical NF-κB pathways (Liu & Chen, 2011). Thus, activated nuclear STAT3 is found in many cancers, including breast, colon and prostate among others (Grivennikov et al., 2009; Marotta et al. 2011; Hedvat et al., 2009). Hyperactivation of STAT3 in infiltrating immune cells and stromal cells is due to the presence of multiple STAT3-activating stimuli, including IL-6 and other cytokine capable of utilizing gp130, IL-10 family members and also receptor tyrosine kinases for VEGF and EGF. Importantly, several of the STAT3-inducible gene products generated by these stimuli, including IL-6 itself, IL-10, IL-23, sphingosine-1-phosphate receptor $S1P_1$, and angiogenic growth factor VEGF, perpetuate a positive feedback loop that promotes sustained pathogenic STAT3 activation within the tumour (Yu et al., 2009; Lee et al., 2010). In

conditions such as hepatocellular carcinoma, malignant melanoma, prostate cancer and others, this STAT3 activation loop can be amplified further due to methylation of the CpG island within the SOCS3 gene promoter, thereby blocking its induction (Niwa et al., 2005; Fourouclas et al., 2008; Pierconti et al., 2011). Indeed, SOCS3 hypermethylation has been shown to be associated with prostate cancer tumours with a high Gleason score, suggesting a causative role in pathogenesis (Pierconti et al., 2011).

Importantly, while STAT3 and NF-κB trigger distinct gene expression programmes in various cell types, many gene products are induced by both, including IL-6, VEGF, chemokines CCL2 and CXCL2, anti-apoptotic proteins Bcl-X_L and Bcl-2, and SOCS3 (Yu et al., 2009). The different mechanisms by which STAT3 and NF-κB (predominantly the RelA/p50 heterodimer) interact to enhance target gene transcription have been shown to be context-dependent but typically result in enhanced target gene induction. For example, Tyr705-phosphorylated STAT3 has been shown to bind promoter-bound RelA to facilitate the recruitment of the transcriptional co-activator p300, thereby triggering RelA acetylation. As a result, interaction with IκBα is inhibited, resulting in reduced export of RelA/p50/IκBα complexes and thereby prolonging its nuclear localization and potentially its transcriptional capacity even if upstream IKK-mediated signaling is only weakly activated (Lee et al., 2009). Other groups have identified a different mechanism whereby non-phosphorylated STAT3 can displace IκBα from inactive RelA/p50 complexes in the cytoplasm, thereby facilitating nuclear entry in the absence of IKK activation and the subsequent induction of a subset of κB-responsive genes such as the chemokine RANTES (Yang et al., 2007). Interestingly, evidence suggests that NF-κB/STAT3 complexes are capable of binding to target sequences distinct from those utilized by either transcription factor in isolation (Yang et al., 2007; Yang & Stark, 2008). While this phenomenon has only been studied in detail for the serum amyloid A gene mobilized as part of the acute phase response, it raises the possibility that NF-κB/STAT3 complexes may be able to initiate a unique transcriptional programme, although any role in tumour progression is still unclear.

Despite strong evidence that targeting STAT3 might be a useful therapeutic strategy to suppress tumour formation, one major limitation stems from its importance in stromal as well as haematopoietic cell function. As a result, while pharmacological inhibition of STAT3 in the former may exert anti-tumourigenic effects, inhibition in the latter may actually enhance tumourigenesis, with the net result being limited efficacy. For example, homozygous deletion of the murine STAT3 gene in enterocytes has been shown to block the development of colitis-associated colon cancer (Grivennikov et al., 2009), whereas its deletion in macrophages and neutrophils results in the development of enterocolitis due to the inability of IL-10 to suppress myeloid cell function *via* STAT3 (Takeda et al., 1999). Moreover STAT3 has important pro-survival functions in healthy tissues and is central to immune cell homeostasis, making it difficult to predict the consequences of chronic global inhibition of function. Thus, alternative strategies might include specifically targeting tumourigenic cytokines that utilize STAT3, such as IL-6 or IL-23, which would spare the protective anti-inflammatory/immunosuppressive functions of STAT3-mobilising cytokines such as IL-10. Related to this point, it is been demonstrated in a mouse model of colitis-associated cancer that TGF-β-dependent suppression of T cell stimulation could enhance IL-6 expression. A concomitant reduction in membrane-bound IL-6R on epithelial cells coupled with an increase in ADAM17 in stromal tissue resulted in the generation of sIL-6R and

subsequent trans-signaling (Becker et al., 2004). Tumour growth could be suppressed by the administration of sgp130Fc, which specifically blocks trans-signaling by sIL-6Rα/IL-6 but not classical IL-6 signaling through membrane-bound IL-6R. Given that a similar loss of IL-6R and upregulation of ADAM17 has been described in human colon cancer (Becker et al., 2007), selective inhibition of IL-6 trans-signaling using sgp130Fc or related therapeutics might prove a useful approach to arrest the development of colon cancer and other tumours in which a pathophysiological role for IL-6 trans-signaling could be identified.

6. Conclusions

Progress in our understanding of the molecular basis of cytokine signaling and the subsequent identification of the importance of JAK mutations and hyperactivation of STAT3 in disease states has improved our understanding of the pathogenesis of RA, PV and several cancers. A direct result of this progress has been the development and introduction of JAK inhibitors and anti-IL-6 therapies for several inflammatory and autoimmune conditions. However our understanding of JAK-STAT signaling, particularly its integration with the NF-κB and cyclic AMP signaling pathways to regulate immune and inflammatory responses, remain incomplete. Moreover, new insights continue to emerge into roles both for nuclear-localised JAKs in chromatin remodeling and cell cycle regulation (Zouein et al., 2011) and links with Rho kinase that control actomyosin contractility, cell migration and even STAT3 activation (Sanz-Moreno et al., 2011). Consequently it is likely that the first wave of therapies targeting cytokine-activated JAK-STAT pathways is the beginning of several new approaches with which to treat the wide range of diseases in which they play a critical role.

7. References

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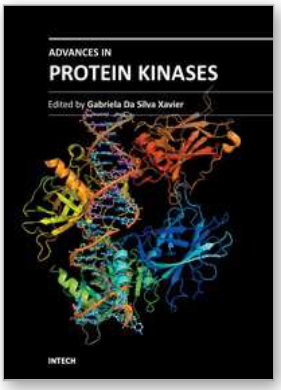

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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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