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The molecular regulation of Janus kinase (JAK) activation

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Abstract

The Janus Kinase (JAK) family members serve essential roles as the intracellular signalling effectors of cytokine receptors. This family, comprising JAK1, JAK2, JAK3 and TYK2, was first described more than 20 years ago, but the complexities underlying their activation, regulation and pleiotropic signalling functions are still being explored. Here, we review the current knowledge of their physiological functions and the causative role of activating and inactivating JAK mutations in human diseases, including haematopoietic malignancies, immunodeficiency and inflammatory diseases. At the molecular level, recent studies have greatly advanced our knowledge of the structures and organisation of the component FERM-SH2, pseudokinase and kinase domains within the JAKs, the mechanism of JAK activation and, in particular, the role of the pseudokinase domain as a suppressor of the adjacent tyrosine kinase domain's catalytic activity. We also review recent advances in our understanding of the mechanisms of negative regulation exerted by the SH2 domain containing proteins, SOCS (Suppressors of Cytokine Signalling) proteins and Lnk. These recent advances highlight the diversity of regulatory mechanisms utilised by the JAK family to maintain signalling fidelity, and suggest alternative therapeutic strategies to complement existing ATP-competitive kinase inhibitors.

Keywords

pseudokinase; kinase; cytokine receptor; Janus kinase

Introduction

The transmission of signals from extracellular stimuli across the plasma membrane via the cytoplasm to the nucleus in eukaryotes principally relies on the post-translational protein modification, phosphorylation. Protein kinases, enzymes that catalyse the transfer of the γ-phosphate from ATP to tyrosine, serine or threonine sidechains in substrate proteins, can thus be thought of “writers” of reversible marks, which function to modify their substrates, intracellular effectors, to regulate their signalling activities. Due to their essential roles in cellular signalling, protein kinases are subjected to many levels of positive and negative

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regulation to ensure fidelity of signals and restrict signal longevity to guard against aberrant signal activation. Defective kinase activity or regulation are known to underlie proliferative diseases, such as cancers, and consequently protein kinases have come to prominence as therapeutic targets.

Due to their essential roles as signal transducers downstream of cytokine receptor activation, the Janus Kinase (JAK) family of tyrosine kinases have garnered much attention since their discovery more than 20 years ago [1–6]. This family comprises four members: JAK1, JAK2, JAK3 and TYK2. In contrast to receptor tyrosine kinases, such as the c-Kit and Insulin receptors, cytokine receptors lack intrinsic protein kinase domains and consequently rely on the catalytic activities of constitutively-associated Janus kinase (JAK) family of tyrosine kinases to convey signals (Figure 1A). Crucially, cytokine receptors comprise two or more receptor subunits, each associated with a JAK monomer. Upon receptor ligation by a cognate ligand, receptor subunits are either reoriented or oligomerise leading to manoeuvring of receptor-associated JAKs into positions to facilitate their trans-phosphorylation and corresponding activation. Subsequently, activated JAKs phosphorylate tyrosines within the cytoplasmic regions of the receptor with which they are associated, generating docking sites for downstream adaptor and effector (“reader”) proteins that contain phosphotyrosine recognition domains, typified by its SH2 domain, including the signal transducers and activators of transcription (STAT) proteins. Depending on the receptor, and thus the docking sites generated by tyrosine phosphorylation within the cytoplasmic region, any one or more of six STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5 or STAT6) may be recruited via their SH2 domains. STATs exist as preformed dimers [7–9], and by being brought into proximity of the receptor-associated JAK can then be phosphorylated by JAK, leading to a reorientation of subunits within the STAT dimer and translocation into the nucleus where it functions as a transcription factor [10–12]. The resulting transcriptional program dictates whether the cell undergoes proliferation, differentiation, survival or death.

Whilst this pathway is notionally simple, many nuances of the pathway remain mysterious. For instance, how do cytokine receptors dictate that only a subset of JAKs (often only one of the family) associate with the receptor? What are the determinants that govern how a JAK typically signals via only a subset of the STAT family proteins? Additionally, it remains unclear why cytokine receptors would evolve a constitutive association with a protein kinase in preference to containing intrinsic protein kinase domains: does the combinatorial nature of JAK association with receptors facilitate signal pleiotropy or does the separation of receptor and kinase permit the kinase to perform additional functions, such as those proposed for JAK1 and JAK2 in the nucleus [13, 14] and reviewed in [15]? Furthermore, as summarized in Figure 1 and detailed below, regulation of the JAK/STAT signalling pathway is highly nuanced, with signalling flux tuned by layers of regulation by extrinsic regulators, by protein activators and inhibitors, including phosphatases which serve as “erasers” of both activating and inactivating phosphorylation events (Figure 1A,B).

JAKs and their cognate cytokine receptors

While cellular overexpression studies suggested JAKs could signal promiscuously downstream of many cytokine receptors, it is evident from genetic deletion studies that cytokine receptors have clear preferences for the JAK family members they utilize as signalling effectors (Table 1). In light of this, here we have focused our attention on the genetic deletion studies that have illuminated which JAKs couple with which cytokine receptors. The first insights into the specificity of JAKs within each signalling pathway arose from early cell-based genetic screens to identify components of the IFNα/β and IFNγ signalling pathways. Initially, TYK2 was found to complement a defect in IFNα signalling [6]; while subsequent studies implicated JAK2 in IFNγ signalling [16] and JAK1 in IFNα/β and IFNγ signalling [17]. A broader understanding of the relationships between JAKs and cytokine receptors has relied on the generation of knockout mice for each JAK family member and the detailed examination of signalling responses to cytokine stimuli by cells derived from these animals. Although *Tyk2*$^{-/-}$ and *Jak3*$^{-/-}$ mice are viable under stress-free conditions and thus readily able to be studied, examination of the signalling pathways mediated by JAK1 and JAK2 have been complicated by the fact that germline deletion of *Jak1* or *Jak2* causes lethality at postnatal day 1 [18] or E12.5 [19, 20] respectively. Lethality in *Jak1*$^{-/-}$ mice was attributed to neuronal defects arising from defects in gp130 receptor signalling [18]. The lethality of *Jak2* deletion was attributed to an absence of definitive haematopoiesis in knockout mice [19, 20] and the haematopoietic specificity of this phenotype was similarly observed upon conditional deletion of *Jak2* in postnatal and adult mice in recent studies [21–23], where defects in the haematopoietic organs, haematopoietic stem cells (HSC), erythropoiesis and thrombopoiesis were most profound. It is remarkable that like JAK1 and TYK2, JAK2 is ubiquitously expressed [18, 20] yet the defects in JAK2 signalling were largely confined to HSC, erythroid and thrombopoietic signalling. In contrast, JAK3 expression is confined to haematopoietic tissues, myeloid and lymphoid cells [4, 24], and deletion of *Jak3* principally caused lymphopoietic defects that manifested as severe combined immunodeficiency disease (SCID) in these mice [25, 26]. This phenotype is reminiscent of deletion or deleterious mutation of its cognate receptor, the IL-2, IL-4, IL-7, IL-13, IL-15, IL-21 common-γ (γc) receptor [27–29].

Findings in these mouse knockout cells reinforce the notion that there are obligate relationships between cytokine receptors and their JAK effectors, where a preference exists for which JAK family member serves as the signalling effector of each cytokine receptor. Although these studies have successfully identified obligate relationships between cytokine receptors and JAKs, one shortcoming is that they do not illuminate functional redundancy. This is exemplified by studies of G-CSF receptor signalling, where wild-type foetal liver cells and counterparts lacking *Jak1*, *Jak2* or *Tyk2* responded equivalently to G-CSF (Table 1) [18, 20, 30], indicating that in the absence of one JAK family kinase, another family member can fulfil the same signalling function. Relatedly, another confounding factor in interpreting the relationships between JAK family members and cytokine receptors using JAK knockout models is the idea that one JAK family member may function as the upstream activator of a neighbouring heterotypic JAK family member within a cytokine receptor complex. Consequently, where such hierarchies exist, deletion of a JAK family member

would be expected to compromise activation of a downstream JAK, which would serve the primary effector role. Broadly, this idea is supported by studies performed on cells derived from JAK-deficient mice. For example, while cells derived from *Jak1*$^{-/-}$ foetal livers exhibited defective, albeit measurable, signalling upon IL-3 stimulation [18], those from *Jak2*$^{-/-}$ foetal liver cells exhibited no detectable response to IL-3 [20]. These observations support the idea that the intracellular regions of the IL-3 receptor α and β subunits serve as scaffolds to recruit JAK1 and JAK2 to facilitate JAK1-mediated activation of JAK2 following IL-3 ligation. In such a model, JAK2 serves as the essential signalling effector, which relies on JAK1 for its optimal activation.

JAK mutations in human disease

Mutations in all four JAKs have been associated with human diseases. Inherited mutated JAK alleles lead to inactivated JAK3 and TYK2 in human immune deficiency syndromes (Table 1), while somatic mutations in JAK1, JAK2 and JAK3 result in constitutively active kinases in myeloproliferative diseases and leukaemia/lymphomas.

A single patient with hyper IgE syndrome, multiple infections and impaired responses to interferon and multiple other cytokines (including Th2 polarization) was shown to have a homozygous four nucleotide deletion leading to frameshift mutation and premature termination in the FERM domain resulting in no functional TYK2 (Table 1) [31]. However, a second patient, while also susceptible to viral and other infections, did not display hyper IgE syndrome [32]. There is an interesting discrepancy between the phenotypes of the *Tyk2*$^{-/-}$ mice and humans lacking TYK2, suggesting that interspecies differences likely exist (Table 1). Like cells derived from *Tyk2*$^{-/-}$ mice, peripheral blood cells from a *TYK2* deficient human exhibited defective IL-12, IL-23 and IFNα/β signalling, but additional severe defects in IL-6 and IL-10 signalling that were not observed in the mouse [31]. These studies may indicate that evolutionary divergence in either the receptor specificity underlying JAK utilization or the capacity for compensatory signalling in different organisms or genetic backgrounds.

Somatic activating mutations in JAK1 have been detected in up to 20% of adult T-cell acute lymphoblastic leukemia (T-cell ALL) and a lower fraction of B-cell ALL with the majority of activating mutations occurring in the pseudokinase domain [33, 34]. Low frequency mutations in JAK1 have also been detected in AML and non-haemopoietic tumours such as hepatocellular adenomas [35].

Somatic activating mutations in JAK2 (most commonly V617F) in the pseudokinase or adjacent domain are found at high frequency in myeloproliferative neoplasms [36–49]. The highest frequency (up to 95%) is in patients with polycythemia vera, with 50–60% in essential thrombocythemia, and idiopathic myelofibrosis [50]. In addition, lower frequencies of the mutation occur in Ph-negative chronic myeloid leukaemia, chronic myelomonocytic leukemia, megakaryocytic AML and juvenile myelomonocytic leukaemia (10–20%) [50]. Other mutations in the JAK2 pseudokinase domain (including point mutations involving R683) have been detected in about 20% of Down Syndrome-associated acute lymphoblastic leukaemia)[47] and other ALLs and AML. A number of JAK2 fusion proteins (usually

involving the N-terminal dimerization domains of the fusion partner and the C-terminal kinase domain of JAK2 with or without the adjacent pseudokinase domain) lead to activation of JAK kinase activity and have also been associated with myeloid and lymphoid leukaemias or atypical CMLs. They include the TEL-JAK2 [51, 52], PCM1-JAK2 [53, 54] and BCR-JAK2 fusion proteins [55].

Mutations in JAK3 have been described in 7–14% of patients with severe combined immunodeficiency (SCID) exhibiting a lack of T- and NK cells [56]. At least 34 mutations have been described covering all of the sub-domains of the protein and most result in a lack of functional protein expression. Most patients are compound heterozygotes having inherited a mutated allele from each parent. JAK3 associates with the common γ-chain of the IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors, mutations of which are associated with the most common form of SCID (X-linked) [56] (Table 1). Activating mutations in the FERM or pseudokinase domains of JAK3 have also been associated with acute megakaryocytic leukaemia [57].

The above data point to a clear role for hyperactive JAKs in human hyper-inflammatory/autoimmune diseases and myeloproliferative diseases. Consequently there has been extensive interest by pharmaceutical companies in developing JAK inhibitors to target autoimmune disease/immunosuppression (anti-JAK1, JAK3) and myeloproliferative neoplasms and leukemia/lymphoma (anti-JAK2, JAK1). At least 10 small molecule JAK inhibitors are in current clinical trials for myeloproliferative neoplasms, rheumatoid arthritis, psoriasis and inflammatory bowel disease. All of these compounds target the ATP-binding site of JAKs and so none are absolutely specific for any JAK. Nevertheless Ruxolitinib™ (a JAK1, JAK2 inhibitor) has been approved for use in myelofibrosis and Tofacitinib™ (a JAK1, JAK3 inhibitor) has been approved for use in rheumatoid arthritis. Ruxolitinib™ treatment substantially reduced spleen size and improved quality of life in a significant proportion of patients with myelofibrosis but, surprisingly, treatment was also effective in patients without mutated JAK2 and there was not convincing evidence of reduction in mutated allele burden or progression to AML [58, 59]. Tofacitinib™ was more effective in improving symptoms in rheumatoid arthritis than placebo or existing anti-TNF receptor therapy [60]. While some of these results are promising, it is clear that a better understanding of the specificity of JAK inhibitors is required to draw firm conclusions about optimal therapies for these diseases.

Structure and activation of JAK kinases

All four members of the JAK family share a common domain architecture that is distinct from other tyrosine kinases (Figure 1B). The N-terminal half comprises a band 4.1, ezrin, radixin, moesin (FERM) domain and an SH2 domain that mediates JAK association with the cytoplasmic tail of a variety of cytokine receptors [61], while the second half contains a catalytically-defective pseudokinase domain (Jak Homology 2, “JH2”) that is critical for modulating the activity of a C-terminal tyrosine kinase domain (JH1) [62–64]. It is generally accepted that activation of JAKs is initiated by cytokine binding to their cognate receptor dimer, an event that triggers a change of conformation of the receptor dimer and repositioning of the associated JAKs. Such a rearrangement leads to the apposition of the

JAK tyrosine kinase domains, transphosphorylation of conserved activation loop tyrosines [65] and a concomitant elevation in catalytic activity [66] (Figure 2A). However the precise molecular mechanisms that drive JAK-receptor specificities and the sequential events that lead to JAK activation within the receptor-JAK complex still remain largely elusive.

With the overwhelming majority of the activating mutations located in the pseudokinase domain and the adjacent linker region connecting the pseudokinase domain to the SH2 domain (Figure 1B), considerable efforts have been deployed over the past decade to understand how the pseudokinase domain might exerts its inhibitory activity on the catalytic domain. While the recent crystal structures of the pseudokinase domains of JAK1, JAK2 and TYK2 in isolation did not unravel the process of activation of the JAKs, they provided us with some valuable insights [67, 68].

Despite missing key residues that are known to be crucial for catalytic activity, the pseudokinase domain has retained the capability to bind nucleotides in the presence of divalent cations [69]. However, only the JAK2 pseudokinase domain has been shown to possess weak catalytic activity and to autophosphorylate itself *in cis* on two auto-inhibitory phosphorylation sites, Ser523 and Tyr570 [64]. Those residues are not conserved amongst the other JAKs suggesting a JAK2-specific mechanism of activation. Intriguingly, the residues that compose the “activation loop” in the JAK2, JAK1 and TYK2 pseudokinase domains structures adopt a well-defined conformation reminiscent of inactive protein kinase structures and are predicted to occlude the putative substrate binding site, suggesting that a rearrangement of JAK2 pseudokinase activation loop would need to occur to allow phosphoryl-transfer activity [67]. Although evidently different from JAK1, it remains to be established whether the weak catalytic activity exhibited by the JAK2 pseudokinase domain plays an essential physiological role *in vivo*. One possibility is that nucleotide binding to these pseudokinase domains solely serves a role in modulating the overall conformation of the JAK. The significant stabilization conferred by ATP binding on purified recombinant JAK1 and JAK2 pseudokinase domains [69] may highlight the necessity to lock a particular conformation for intermolecular interactions to occur. An emerging idea is that nucleotide binding by pseudokinase domains may underlie or be indicative of a “molecular switch” mechanism [69, 70], although the physiological relevance of conformational switching amongst the JAK pseudokinase domains remains to be established.

Interestingly, the crystal structure of the JAK2 pseudokinase domain harbouring the activating mutant, V617F, points toward enhanced structural integrity within this domain underlying pathogenesis. The groups of Silvennoinen and Hubbard proposed that the activating effect of the V617F mutation arises from stabilisation of the αC helix via an aromatic stacking interaction between V617F and two phenylalanines (Phe595 and Phe594) from the αC helix, resulting in an increased length of the αC helix [68]. This is supported by biochemical analyses in which optimal activation of JAK2-V617F required an aromatic amino acid at residue F595 [71]. However, a subsequent structure of the JAK1 pseudokinase domain revealed that the length of the αC region can vary regardless of the presence of the corresponding activating mutation, V658F [67]. In the case of JAK1, Toms, Eck *et al*. proposed a model in which the V658F mutation in JAK1 promotes activation primarily via rearrangement of the pseudokinase domain and pseudokinase-SH2 linker, again through

aromatic stacking interactions. A highly conserved triad Phe-Phe-Val that include Phe575 (Phe537 in JAK2) located in the linker region, Phe636 (Phe595 in JAK2) located in the αC helix and V658 (V617F in JAK2) was shown to be critical for this rearrangement to occur in the JAK1 pseudokinase domain structure [67]. In the structure of the JAK1 pseudokinase domain harbouring the activating V658F mutant, the β4-β5 loop was found to fold toward the αC helix, and the phenyl ring of Phe658 to occupy the site normally occupied by Phe575 in the wild-type structure, pushing away the pseudokinase-SH2 linker region [67]. Nevertheless, it was also shown that this conformation can occur in the wild-type JAK1 pseudokinase domain, suggesting that this region is prone to movement but may be stabilized in an activated conformation by the oncogenic V617F (JAK2)/V658F (JAK1) mutation [67]. Importantly, mutations in the JAK2 pseudokinase-SH2 linker region, such as K539I, were recently identified in polycythemia vera (PV) patients and reported to induce constitutive JAK2 activation [72].

Based on functional studies of activation, two distinct mutational hotspots within the JAK2 pseudokinase domain have been identified. Mutations that map to the N-terminal lobe of the domain, adjacent to V617F (e.g. R588M, N622I, S591L), and in the pseudokinase-SH2 linker region were found to confer factor-independent growth, despite not always exhibiting detectable elevations in catalytic activity [72]. In comparison, JAK2 pseudokinase domain mutations located close to the hinge region on the opposite face (L611S) and at either tip of an adjacent loop that is unique to JAK pseudokinase domains (I682F, R683S and F694L) induced elevated activation loop autophosphorylation within the kinase (JH1) domain and were shown to lead to activation in the absence of erythropoietin (EPO) stimulation [72]. Although two functionally-distinct regulatory interfaces appear to exist on opposite faces of the JAK2 pseudokinase domain, a unifying feature is the observation that cytokine receptor binding is crucial for their transforming potential [73–75].

While it is clear that the JAK pseudokinase domains act as protein interaction modules, whose primary function is to bind and inhibit the tyrosine kinase (JH1) domain activation, there are many conflicting reports on how this inhibition might occur. Two basic models of JAK activation have been proposed, in which the pseudokinase domain binds and inhibits the kinase domain (A) *in cis* or (B) *in trans* (Figure 2). Here, we discuss the evidence in support of each model, with particular emphasis on recent advances.

The recent crystal structure of the TYK2 pseudokinase-kinase tandem domains (PDB, 4OLI; [76] supports an inhibition mechanism occurring *in cis*, with an interacting interface between the pseudokinase domain and the kinase domain predominantly mediated by the N-lobes of each domain (Figure 3A). The pseudokinase domain interacts across the back of the kinase domain's ATP-binding site via an interface comprising residues located in the pseudokinase-SH2 linker region, between the αC helix and β4 strand, and extends out to residues near the unique pseudokinase domain loop in the C-terminal lobe. The interface on the kinase domain that mediates pseudokinase domain interaction is mainly located around the hinge region and the loop opposite to the glycine loop between the β2 and β3 strands. The pseudokinase-SH2 linker mainly interacts with the kinase domain hinge region, but it is yet to be demonstrated if this interaction remains in the full-length context. Importantly, the kinase domain adopts an active-like conformation with the activation loop expelled from the

active site, despite the use of a kinase-dead mutant (D1023N), suggesting that the release of the activation loop upon phosphorylation does not participate in the activation process. Instead, the N-terminal back-to-back interaction implies an *in cis*-autoinhibition mechanism that hinders the flexibility of the kinase domain's hinge region, hence restricting rotation between the N- and C-lobes of the kinase domain and the correct positioning of the αC helix and the catalytic elements that drive catalysis. Nevertheless, it is still unclear how this interplay between kinase and pseudokinase domains has a negative effect on the JAK activity in a receptor complex. The *in cis*-mechanism of autoinhibition described for TYK2 may represent a snapshot of a part of a process that drives autoinhibition/activation, but it is yet to be demonstrated if it is a conserved mechanism amongst the JAK family.

In contrast to the TYK2 pseudokinase-kinase crystal structure, our recent analysis of the JAK2 pseudokinase-kinase tandem domains using small angle X-ray scattering (SAXS) demonstrated that these domains exist in an elongated configuration in solution [77] (Figure 3B). While our data favour a model of inhibition *in trans*, whereby the pseudokinase domain of one JAK molecule inhibits the activation of the kinase domain of a second JAK molecule, it is important to note that the JAK2 pseudokinase-kinase protein was purified in an activated state with the kinase domain activation loop phosphorylated and presumably expelled from the active site. These data demonstrated that relative orientations of the pseudokinase and kinase domains of JAK2 are not fixed, a finding consistent with earlier cryo-electron microscopy studies of full length JAK1 [78]. If the *in cis* mechanism of inhibition is applicable to JAK2, this may suggest that only a subtle rearrangement of the catalytic elements around the hinge region is needed to drive pseudokinase domain binding, as seen in the TYK2 crystal structure. Should the *in cis* autoinhibition model arising from the TYK2 structure similarly operate in JAK2, it is likely that catalytically-dead mutants and/or inhibitors will be required to capture and crystallise the autoinhibited conformation.

Recently, Brooks and colleagues have proposed a model for the activation of JAK2 by the growth hormone receptor (GHR) that also supports an *in trans* autoinhibition mechanism [79] (Figure 2B). Using a combination of FRET analysis and computational modelling, Brooks et al. found that the GHR exists predominantly as a dimer *in vivo* held together through interactions of its the transmembrane helices. These helices are parallel in the inactive state and binding of the hormone induces the separation of the membrane proximal region and a rearrangement of the transmembrane helices, leading to the separation of the Box 1 motifs in the cytoplasmic receptor chains and their attached JAK2. Such separation triggered JAK2 activation by the repositioning the inhibitory pseudokinase domains away from their opposing kinase domains, bringing the two kinase domains into close proximity for *trans*-phosphorylation to occur. This model of activation predicts an interacting interface between the N-terminal lobe of the pseudokinase domain of one JAK2 molecule in the vicinity of the V617F mutation and the activation loop residues of the kinase domain of the other JAK2 molecule. This model is consistent with a scenario earlier proposed by Lee, where differences in proximity of the JAK2 kinase domains were hypothesised to underlie differential and graded signalling responses downstream of different cytokine receptors [80]. Further support for the *in trans* inhibition model has arisen from the demonstration that

activating mutations within the JAK2 pseudokinase domain led to deficits in the inhibition of the kinase domains of other JAK family members [81].

At this juncture, there is supporting evidence for both the *in cis* and *in trans* models of inhibition of JAK kinase domains by the adjacent pseudokinase domain. One possibility is that different mechanisms of inhibition and activation operate between different JAK family members (e.g. TYK2 *vs* JAK2) or that the mode of inhibition/activation is governed by the receptor to which the JAK molecules are tethered. Structural studies of full-length JAKs bound to receptor chains, in both active and inactive states, will be required to definitively address this conundrum and define how the pseudokinase-SH2 linker interface relays signals from an activated receptor to the kinase domain. A further step in this direction has been taken with the recent structure of TYK2 FERM-SH2 domain in complex with IFNAR1 receptor Box 2 motif [82] (Figure 3C). This structure highlights the critical scaffolding function of the SH2 domain in mediating receptor interaction and provides a glimpse of how receptor-JAK specificity can be achieved [82]. Of importance is the formation of an antiparallel β-strand near the SH2 domain–receptor interface between residues located in the linker between the F1 and F2 lobes of the FERM domain and the C-terminus of the SH2 domain. Despite poor sequence conservation within the linker between the F1 and F2 lobes of the FERM domain, a β-strand is predicted at this position for the other JAKs. The residues located within the C-terminal end of the SH2 domain are highly conserved within the JAKs and have been shown to be essential for the interaction of JAK2 with EPOR [72], raising the possibility that this region might play a key role in communicating the event of receptor ligation to the kinase domain, thus leading to activation.

Extrinsic regulation of JAKs

The fidelity of JAK signalling is essential to the health of an organism. Consequently, JAK activity is regulated at many levels, by intrinsic regulatory events, such as post-translational modifications (Figure 1B) and the inhibitory function of the pseudokinase domain (detailed above), in addition to the many extrinsic regulators described below and shown schematically in Figure 1A.

Phosphatases

Like many tyrosine kinases in the cell, JAK activity is specifically inhibited by the action of phosphatases. As well as catalysing dephosphorylation of JAK substrates (for example, STATs), certain phosphatases can act directly on JAK itself, inactivating it by dephosphorylating JAK phosphotyrosines that are critical for its activity, particularly phosphotyrosines in the activation loop. The phosphatases that directly target JAK will be discussed in detail below.

SHP1 and SHP2

SHP1 and SHP2 (PTPN6 and PTPN11 respectively) are ~600 amino acid phosphatases that both consist of a C-terminal phosphatase domain and two N-terminal SH2 domains. SHP1 is primarily expressed in haematopoietic cells and *Shp1* knockout and mutant mice display a range of haematopoietic abnormalities [83]. SHP1 directly associates with TYK2, JAK1 [84,

85] and JAK2 [86] as well as a number of different cytokine and haematopoietic growth factor receptors, such as those for IL-3 [87], erythropoietin [88] and IFNα [84] all in a cytokine-dependent fashion. Cells from *Shp1*$^{-/-}$ mice (also known as *motheaten*) display significantly enhanced JAK1 autophosphorylation in response to IFNα [84]. Detailed mechanistic insights into this phenotype were recently provided by the co-crystal structure of the SHP1 catalytic domain in complex with a phosphopeptide derived from the JAK1 activation loop [89]. *SHP1* is found to be hypermethylated in multiple myeloma, mantle cell lymphoma and follicular lymphoma leading to constitutive STAT3 phosphorylation [90, 91]

In contrast to SHP1, SHP2 is ubiquitously expressed. SHP2 binds JAK1 and JAK2 [92], and genetic knockout leads to increased JAK1 autophosphorylation and enhanced IFNα and IFNγ signalling [93]. These studies support the hypothesis that SHP2 directly dephosphorylates JAKs. In addition, activating mutations in SHP2 have been identified in juvenile myelomonocytic leukemia (JMML), B cell acute lymphoblastic leukemia, and acute myeloid leukemia (AML). SHP2 was the first identified phosphatase proto-oncogene [94]. In humans, mutations in SHP2 cause Noonan syndrome [95].

PTP1B and TCPTP

PTP1B (also known as PTPN1) is a 435 amino acid protein that consists of a phosphatase domain, a C-terminal domain with a proline rich motif that allows binding to SH3 domains, and a terminal targeting sequence that tethers it to the cytoplasmic face of the endoplasmic reticulum. PTP1B has a powerful role in regulating signalling via the insulin receptor, and *PTP1B* knockout mice are less susceptible to type 2 diabetes. However, in addition to this, PTP1B has also been shown to be an important regulator of JAK/STAT signalling, especially downstream of leptin receptor activation, making it an important metabolic regulator [96, 97]. Substrate trapping mutants of PTP1B have shown both TYK2 and JAK2 (but not JAK1) to be substrates for dephosphorylation [98, 99]. In addition to its effects on leptin signalling, over-expression of PTP1B inhibits IFNα and IFNγ signalling, presumably by dephosphorylation of the conserved tyrosines in the activation loop of TYK2 and JAK2, respectively. Finally, knockdown of PTP1B in a breast cancer cell line led to prolactin hypersensitivity due to enhanced JAK2 (and STAT5) phosphorylation [100].

TCPTP (T-cell protein tyrosine phosphatase, PTPN2) is a 415 or 387 amino acid phosphatase that, depending on processing, is similar in sequence to PTP1B. Like PTP1B, TCPTP is found tethered to the ER however the shorter form is also found in the nucleus. TCPTP has been shown to dephosphorylate JAK1 and JAK3 and TCPTP-deficient lymphocytes are hyper-responsive to IL-2 and IFNα and γ [101]. Inactivation of TCPTP occurs in a small percentage of T-ALL cases [102] and these mutations can cooperate with JAK1-activating mutations to give rise to disease [103]

CD45

Unlike the aforementioned phosphatases, CD45 (PTPRC) is a receptor tyrosine phosphatase. It consists of a large extracellular region that includes three fibronectin (FnIII) domains, a transmembrane domain and a cytoplasmic domain that contains two individual phosphatase domains. The major evidence for its role in regulation of JAK/STAT signalling was

discovered by Irie-Sasaki *et al*., who showed that CD45 dephosphorylates all four JAKs. In particular phosphotyrosines in the activation loops of the JAK catalytic domains were found to be CD45 substrates. Consistent with this, *CD45*$^{-/-}$ cells are hyper-responsive to IL-3 and EPO and show elevated levels of phospho-JAK2 [104]. Loss-of-function mutations of CD45 have been found in a small proportion of T-ALL patients and can be found in combination with activating mutations in JAK1 [105].

SH2 domain containing proteins

The trans-phosphorylation of JAKs, especially at the di-tyrosine motif found in their activation loop is a key step in their activation and is required to initiate downstream signalling pathways [66]. Consequently, physiological inhibitors of JAK signalling often contain an SH2 domain that allows them to target JAK-phosphotyrosines to induce its dephosphorylation, degradation or inhibition. Such inhibitors include the SOCS and LNK families of SH2 domain containing proteins, in addition to the phosphatases, SHP1 and SHP2 (described above). The SOCS and LNK family proteins will now be discussed in detail.

SOCS family

The SOCS family members were initially discovered on the basis of their ability to bind JAK [106] and inhibit cytokine signalling [107, 108]. For the majority of the SOCS family, SOCS protein expression is induced by JAK/STAT signalling and they then act to inhibit the cytokine signalling cascade, forming a negative feedback loop [106–108]

The human genome encodes eight SOCS proteins [109]. Each member of the family consists of an N-terminal domain, a central SH2 domain and a C-terminal SOCS box domain. The latter is the defining characteristic of the SOCS family but is also found in a wider protein superfamily that, like SOCS proteins themselves, function as E3 ubiquitin ligases to promote the ubiquitination (and subsequent proteasomal degradation) of protein substrates. The majority of the SOCS family inhibit cytokine signalling by inducing the degradation of JAK-associated cytokine receptors once they are activated. This results in receptor (and possibly JAK) turnover. However, SOCS1 and SOCS3, alone of the SOCS proteins, can directly inhibit the catalytic activity of JAKs and these will now be discussed further.

SOCS1 and SOCS3

Extensive mutagenesis and characterisation of SOCS1 was used by the laboratory of Yoshimura to define a short region of the protein, upstream of its SH2 domain, that enabled SOCS1 to directly inhibit the catalytic activity of JAK [110]. This short motif was termed the Kinase Inhibitory Region (KIR). Further analysis by Yoshimura's group showed that SOCS3 was also capable of inhibiting JAK catalytic activity via the same KIR motif [111], a trait peculiar to SOCS1 and SOCS3.

Recent structural and mechanistic analyses have revealed the molecular basis of JAK inhibition by SOCS3 (and therefore by analogy, SOCS1). The KIR of SOCS3 is unstructured in the absence of JAK [112, 113], but upon binding to JAK, adopts an extended, β-strand-like conformation that sits in the substrate binding groove of the kinase

[114] (Figure 3D). This partially occludes the substrate-binding site and prevents JAK from interacting with substrates, thus inhibiting its ability to initiate downstream signalling. SOCS3 binds JAK2 with approximately micromolar affinity and this is reflected in a similar IC_{50} in *in vitro* kinase assays. SOCS1 appears to have the more potent KIR, as replacing the SOCS3 KIR with that of SOCS1 allows 10-fold more potent inhibition of JAK2 kinase activity [115].

SOCS3 can inhibit JAK1, JAK2 and TYK2 via its kinase inhibitory region, but not JAK3 [115]. This is due to the absence of an evolutionarily conserved “GQM” sequence in JAK3 that is present in all vertebrate forms of JAK1, JAK2 and TYK2, where it lines one edge of the substrate binding groove. Despite the ability to inhibit three of the four JAKs, knockout studies have shown that SOCS3 shows specificity, *in vivo*, for IL-6 family cytokines [116–119], G-CSF [120, 121] and leptin [122]. These cytokines all signal via receptors that contain binding sites for the SOCS3 SH2 domain [123] and this allows SOCS3 to bind to these receptors and their associated JAK1, JAK2 and TYK2 molecules simultaneously [114], indicating that it is specific JAK:receptor dimers that are the true high-affinity targets for SOCS3 action. To date there is no structural data regarding SOCS1, but high sequence conservation with SOCS3 in the KIR and the JAK binding surfaces suggest it may inhibit JAK via the same mechanism. Differences in the SH2 domain sequence, especially around the phosphotyrosine binding groove, indicate that the SH2 domain of SOCS1 will target different sites to SOCS3, potentially explaining why SOCS1 (but not SOCS3) is a potent inhibitor of interferon [124, 125] and IL-2 family [126] cytokine signalling.

LNK

LNK (also known as SH2B3) [127], together with the proteins APS and SH2B comprise a small family of signalling adapter proteins that contain both an SH2 and a PH (Pleckstrin homology) domain [128]. Whilst the SH2 domain of these molecules binds to specific JAK phosphotyrosines, the PH domains bind to phosphoinositides and direct these proteins to the cell membrane. Each member of the family also contains a phenylalanine zipper dimerisation motif and a proline rich domain.

Of the three members of the SH2B family, LNK has a well-characterised negative regulatory role in JAK2-based signalling. SH2B and APS, in contrast, appear to activate JAK signalling in certain contexts. These differential effects are intriguing given that the SH2 domains of LNK, APS and SH2B all target the same site, phosphotyrosine 813 of JAK2, which resides within the SH2-pseudokinase domain linker sequence [129]. It is also remarkable that an analogous motif is present in JAK3 (but not JAK1 or TYK2), yet neither SH2B nor APS impact JAK3 activity or tyrosyl phosphorylation [130]. Thus, it appears that the SH2B family proteins specifically modulate JAK2 activation, although the molecular basis for this specificity is currently poorly understood.

Consistent with a negative regulatory role in haematopoietic JAK/STAT signalling, $Lnk^{-/-}$ mice [131] [132] show increased numbers of HSCs [133] and haematopoietic cells showed increased responsiveness to a number of cytokines including IL-7 [132], EPO [134] and TPO [135, 136],. The effects of LNK action on TPO signalling have been especially well characterised; LNK expression is induced by TPO [137] and LNK and TPO play opposing

roles in HSC expansion as revealed by *Lnk* and *Tpo* double knockout mice [135]. Overexpression of LNK inhibits megakaryocyte development in mice [138], again consistent with a role in regulating TPO/JAK2 signalling.

Whilst the molecular details of LNK inhibition of TPO signalling have not been fully elucidated, LNK is known to interact directly with JAK2. In particular, the SH2 domain of LNK binds to phospho-Y813 of JAK2 [139, 140], which is located in the short linker sequence between the kinase and pseudokinase domains. The PH domain is also required for efficient regulation of TPO signalling as point mutations in this domain are found in human cases of V617F-negative MPNs [141]. This mutation led to TPO hyper-responsiveness, as did mutating the SH2 domain, as seen by aberrant growth as well as enhanced phospho-JAK2 and phospho-STAT in Ba/F3(MPL) presumably due to mislocalisation of LNK from the plasma membrane into the cytoplasm.

Summary

Although the four members of the JAK family were first cloned more than 20 years ago, it is clear that our understanding of their physiology, molecular mechanisms of activation and negative regulation are far from complete. Guided by very recent molecular level studies, our fundamental understanding of JAK activation and negative regulation have vastly improved. Importantly, this work has opened up a number of novel possibilities for therapeutic development to counter diseases of JAK hyperactivation, including myeloproliferative neoplasms, leukaemias and inflammatory disease. For example, targeting the receptor:JAK interaction or emulating the negative regulation by the JAK pseudokinase domain or exogenous regulators, such as the SOCS proteins, with small molecules may offer a way forward to potent and highly specific JAK modulators to complement ongoing efforts to develop conventional ATP-mimetic kinase inhibitors.

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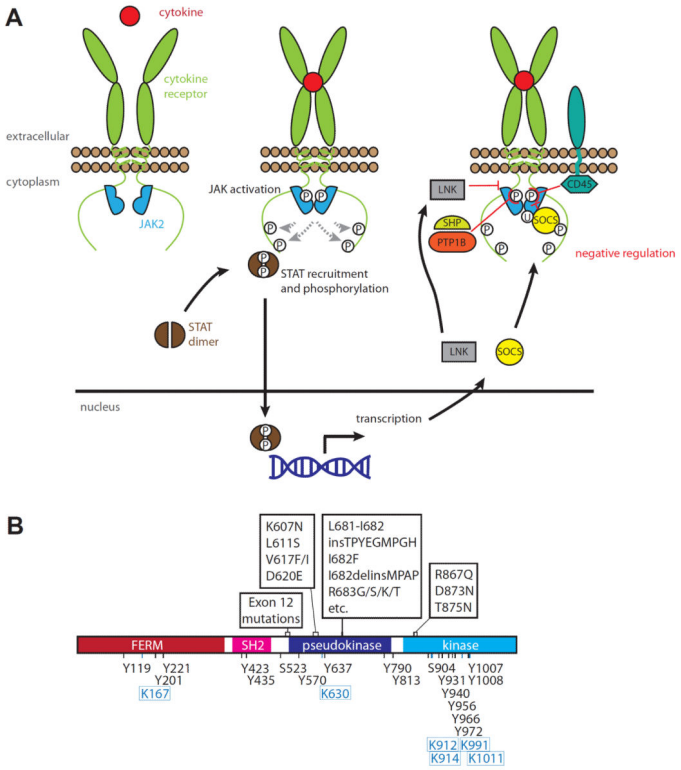

Figure 1.

Overview of JAK activation and regulation. (A) Summary of the JAK/STAT signalling pathway and its negative regulation. Ligation of a cytokine receptor leads to a transition of the associated JAK molecules from an inactive (left) to activated state (middle). Activated JAK is characterised by phosphorylation of activation loop residues within its kinase domain (encircled P; middle). Activated JAKs phosphorylate tyrosines within the receptor intracellular region to enable recruitment and phosphorylation of the principal downstream effectors, the STATs. Many layers of negative regulation have been identified (right), including phosphatases (CD45, PTP1B, SHP1 and SHP2) and SH2 domain containing regulators from the LNK and SOCS families. SOCS proteins inhibit JAK signalling by both inhibiting kinase activity and by mediating ubiquitylation (encircled U), leading to proteasomal degradation.

(B) Intrinsic regulatory events annotated on the human JAK2 domain structure. Mutational hotspots associated with myeloproliferative neoplasms are annotated above [36, 37, 39, 41–49, 142, 143]. Residues subject to phosphorylation [65, 144–152] and sumoylation [153] are annotated below in black text and blue boxed text, respectively. In addition to the crucial role of the activation loop tyrosines, 1007 and 1008 [65, 66], Y119, Y221, Y317, Y570, Y637, Y813, Y866, Y913, Y966 and Y972 have been reported to arise from JAK2 auto- or trans-phosphorylation and contribute variously to the modulation of JAK2 activation [146–148, 150, 154–156].

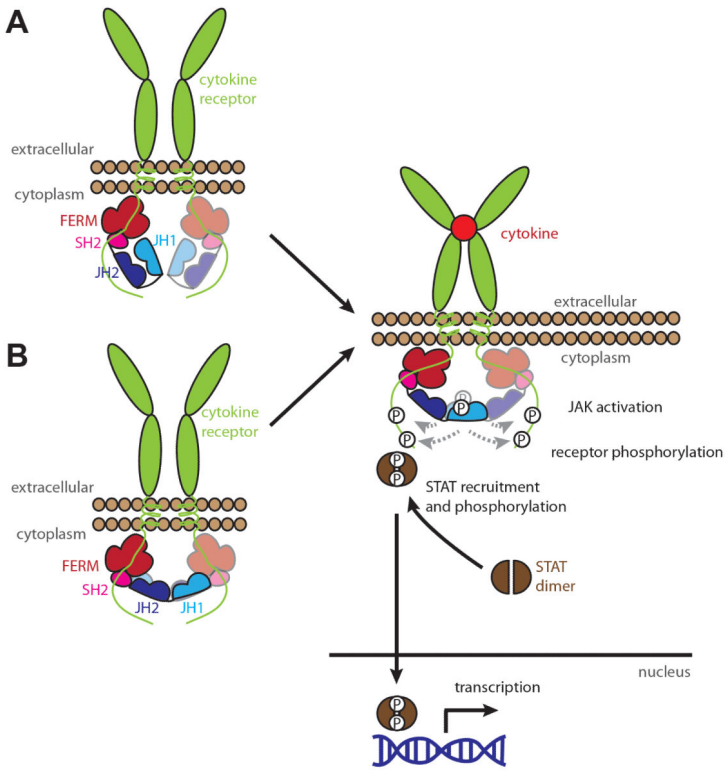

Figure 2.

The two prevailing models for regulation of JAK kinase domain catalytic activity by the pseudokinase domain: (A) *in cis*; (B) *in trans*. In the *in cis* inhibition model (A), the pseudokinase domain binds the kinase domain within the same JAK monomer, leading to a suppression in catalytic activity. The *in trans* model for inhibition (B) involves the binding of the pseudokinase domain from one JAK to the kinase domain of another within a receptor-assembled JAK dimer to suppress the kinase domain's catalytic activity. Activation of JAK in either model involves reorientation of the JAKs to facilitate mutual *trans*-phosphorylation and thus activation of the JAK kinase domains.

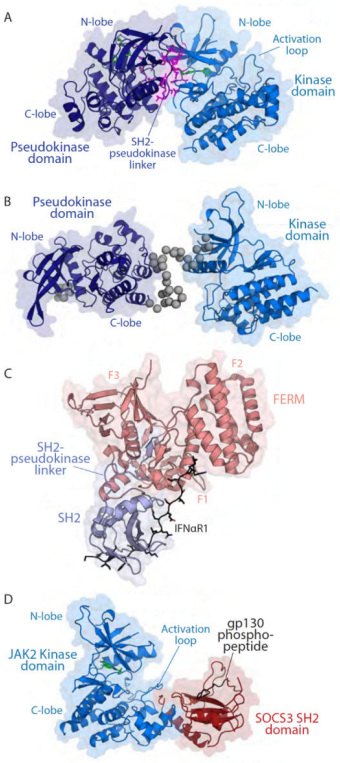

Figure 3.

Structures of component domains within JAK family members.

A. Crystal structure of TYK2 pseudokinase-kinase tandem domains (PDB, 4OLI; [76]). The pseudokinase domain is coloured in deep blue and the kinase domain is coloured in marine. Residues involved in the pseudokinase-kinase domain interface are shown as magenta sticks.

B. The solution structure of JAK2 pseudokinase-kinase tandem domains determined by SAXS [77]. Domains are coloured as for TYK2 in panel A, but with grey beads to model the N-terminus and interdomain linker. The component crystal structures of the JAK2 pseudokinase (PDB, 4FVP; [68]) and kinase (PDB, 2B7A; [157]) domains were used to prepare this rigid body model.

C. Crystal structure of TYK2-FERM SH2 domain in complex with IFNAR1 (PDB, 4PO6; [82]). The FERM domain is coloured in salmon with F1, F2 and F3 subdomains labelled. The SH2 domain is coloured pale blue. The IFNAR1 Box 2 peptide is coloured black.

D. Crystal structure of the JAK2 kinase domain (blue) in complex with the SOCS3 SH2 domain (red) and the gp130 phospho-Y757 peptide (black) (PDB, 4GL9; [114]). Small molecule inhibitors are shown as green sticks in panels A and D.

Table 1

The phenotypes caused by *JAK* deficiency

Gene		Knockout model	Viability	Knockout phenotype	Defective cytokine signalling	Normal cytokine signalling	Reference
Jak1	Mouse	Germline	Postnatal day 1 lethality	Death attributed to neurological defects from defective gp130 signalling. Small thymi and defective thymocyte production.	IL-3; IL-2, IL-4, IL-7; IL-6, LIF; IFNα; IFNγ; IL-10	GM-CSF, IL-5; M-CSF; G-CSF	[18]
Jak2	Mouse	Germline	Embryonic lethality at E12.5	Failure of definitive erythropoiesis.	IL-3, IL-5, GM-CSF; IFNγ; EPO; TPO; GH; PRL	IL-6, LIF; IFNα/β; G-CSF;	[19, 20, 158, 159]
Jak2	Mouse	Conditional: systemic at in postnatal day 4 (PN4) and 2 month old animals.	Lethal	PN4 lethality due to defective erythropoiesis. Adult mice succumbed to severe anaemia, thrombocytopenia, HSC loss and bone marrow failure.			[21–23, 158]
		Conditional: mammary epithelia	Viable	Impaired mammary gland development and maintenance of functionally differentiated alveolar cells	PRL		[159]
Jak3	Mouse	Germline	Viable	Develop SCID. Block in B-cell maturation in the bone marrow; smaller thymi and spleen size; defective T cell function	IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-21		[25, 26, 160]
JAK3	Human	Nonsense mutation	Viable	Diminished T and NK cell numbers; normal B cell numbers.	IL-4		[161, 162]
Tyk2	Mouse	Germline knockout. B10.Q/J strain devoid of TYK2 protein.	Viable	Macrophages unresponsive to LPS stimulation. Reduced IL-12 mediated T-cell response following viral challenge.	IL-12; IFNα/β and IFNγ (at low doses); IL-23;	IL-3; IL-6, LIF; IL-10; G-CSF, TPO	[30, 163–165]
Tyk2	Mouse	Conditional: myeloid, dendritic, T-cell, ubiquitous	Viable	Myeloid deletion reduced MCMV defence. Ubiquitous, but not lineage specific deletions, impaired tumour immunosurveillance			[166]
TYK2	Human	Nonsense mutation	Viable	Susceptibility to viral, fungal and mycobacteria infection. Atopic dermatitis	IL-12; IFNα/β; IFNγ; IL-6; IL-10; IL-23		[31]

Gene	Knockout model	Viability	Knockout phenotype	Defective cytokine signalling	Normal cytokine signalling	Reference
			SCID: severe combined immune deficiency; LPS: lipopolysaccharide.			

Class I cytokines. TPO, EPO, G-CSF, GH, PRL signal via homodimeric receptors (c-MPL, EPOR, G-CSFR, GHR, PRLR, respectively). GM-CSF and IL-5 signal via receptors composed of common-β (βc) and cytokine specific α-subunits; IL-3 via βc or an IL-3 specific β-subunit ($\beta_{IL\text{-}3}$) and an IL-3 specific α-subunit. IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-21 signal via receptors composed of a common γ-subunit (γc) and cytokine-specific α-subunits. IL-6, IL-11, LIF, CNTF, cardiotrophin-1 and oncostatin-M signal via receptors composed of the shared subunit, gp130, and cytokine-specific α subunits. IL-12 signals via a receptor composed of IL-12β1 and β2 subunits. IL-23 signals via a receptor composed of IL-12β1 and an IL-23-specific α-subunit.

Class II cytokines. IFN-α and IFN-β signal via a receptor composed of Interferon α receptors 1 and 2 (IFNAR1 and IFNAR2). IFNγ signals via a receptor composed of IFNGR1 and IFNGR2. IL-10 signals via a receptor composed of IL-10α and IL-10β subunits. IL-22 signals via a receptor composed of IL-10Rβ and a cytokine-specific α-subunit.