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# Linking energy sensing to suppression of JAK-STAT signalling: a potential route for repurposing AMPK activators?

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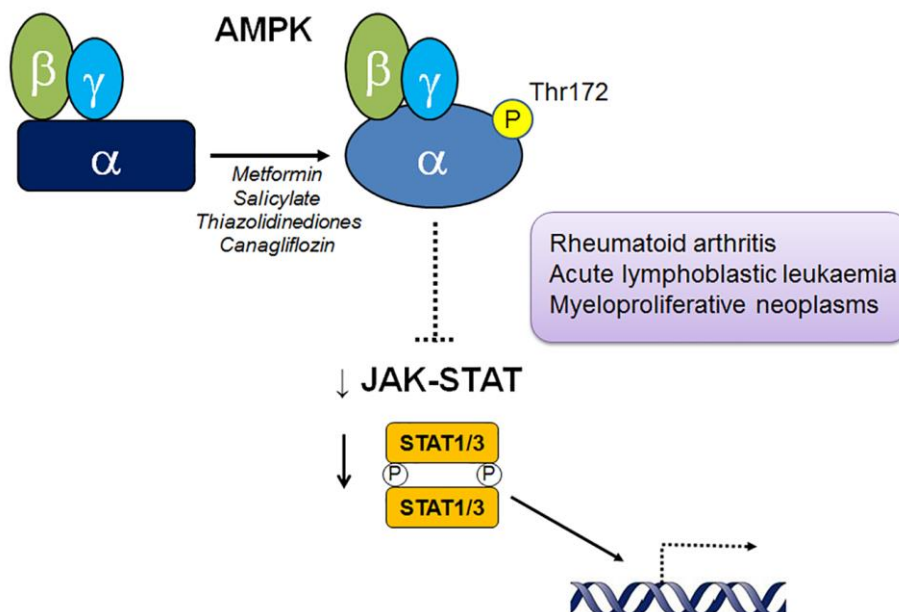
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## Graphical abstract



## ABSTRACT

Exaggerated Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling is key to the pathogenesis of pro-inflammatory disorders, such as rheumatoid arthritis and cardiovascular diseases. Mutational activation of JAKs is

also responsible for several haematological malignancies, including myeloproliferative neoplasms and acute lymphoblastic leukaemia. Accumulating evidence links adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK), an energy sensor and regulator of organismal and cellular metabolism, with the suppression of immune and inflammatory processes. Recent studies have shown that activation of AMPK can limit JAK-STAT-dependent signalling pathways via several mechanisms. These novel findings support AMPK activation as a strategy for management of an array of disorders characterised by hyper-activation of the JAK-STAT pathway. This review discusses the pivotal role of JAK-STAT signalling in a range of disorders and how both established clinically used and novel AMPK activators might be used to treat these conditions.

Keywords: AMP-activated protein kinase; Janus kinase; rheumatoid arthritis; myeloproliferative neoplasms; lymphoma

Chemical compounds: Metformin (PubChem CID: 4091); Tofacitinib (PubChem CID: 9926791); AICAR (PubChem CID: 17513); Salicylate (PubChem CID:338); A769662 (PubChem CID: 54708532); Aspirin (PubChem CID: 2244); Fludarabine (PubChem CID: 3367); Hydroxyurea (PubChem CID: 3657); Anagrelide (PubChem CID: 2182); Busulfan (PubChem CID: 2478); Ruxolitinib (PubChem CID: 25126798); Ciclosporin (PubChem CID: 5284373); Sulfasalazine (PubChem CID: 5359476); Methotrexate (PubChem CID: 126941);

## 1. INTRODUCTION

The JAK<sup>1</sup>-STAT pathway is activated by a range of cytokines, such as interferons, IL-2, and IL-6, which control survival, proliferation and differentiation in a range of

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<sup>1</sup>JAK, Janus kinase. STAT, signal transducer and activator of transcription. IL, interleukin. RA, rheumatoid arthritis. CVD, cardiovascular disease. AMPK, AMP-activated protein kinase. IL-6R, IL-6 receptor. sIL-6R $\alpha$ , soluble IL-6R $\alpha$ . Tyk2, Tyr kinase 2. MCP-1, monocyte chemoattractant protein 1. ERK1/2, extracellular signal-regulated kinase 1/2. PI3K, phosphatidylinositol 3-kinase. YAP, YES-associated protein. JH, JAK-homology. SH2, Src homology 2. FERM, N-terminal 4.1, ezrin, radixin, moesin. PK, pseudokinase. ALL, acute lymphoblastic leukaemia. MPN, myeloproliferative neoplasm. WT, wild type. LKB1, liver kinase B1. AICAR, 5-aminoimidazole-4-carboxamide riboside. MKP-1, mitogen-activated protein kinase phosphatase-1. SMCs, smooth muscle cells. mTOR, mammalian target of rapamycin. TSC2, tuberous sclerosis complex 2. NOTCH1, notch homolog protein 1. CDKN2A/B, cyclin-dependent kinase Inhibitor 2A/B. FBXW7, F-box and WD repeat domain containing 7. PHF6,

diverse cell types. Uncontrolled JAK-STAT signalling is not only a crucial driver of chronic inflammatory diseases such as rheumatoid arthritis (RA) and cardiovascular diseases (CVDs) but also several haematological disorders [1–3].

AMP-activated protein kinase (AMPK) is a Ser/Thr kinase that regulates cellular and organismal metabolism by sensing increases in the intracellular ratio of AMP to ATP following nutrient deficiency or hypoxia [4]. An increasing body of evidence has linked AMPK activation with the control of inflammation and immunity via a variety of mechanisms [4–6]. This novel finding provides a foundation for the evaluation and repurposing of well-tolerated, clinically available drugs, such as the anti-hyperglycaemic drug and AMPK activator metformin, for the treatment of a range of JAK-dependent disorders. Further insights into the novel inhibitory mechanism of AMPK might also provide a basis for the development of a new generation of selective JAK inhibitors.

Clinically available drugs can have positive effects beyond their intended targets e.g. statins, inhibitors of cholesterol production, also show anti-inflammatory effects which are beneficial for the treatment of CVDs [7]. The discovery of novel regulatory mechanisms important in disease and the positive secondary effects of existing drugs enables drug repurposing and thus expansion of available therapeutic options. Given the cost and time required to develop new drugs and test the efficacy and safety in clinical trials, re-purposing is advantageous as it increases the speed to clinic and availability of drugs while eliminating unknown variables in dosing and adverse drug reactions [8,9].

This review discusses the pivotal role of JAK-STAT signalling in inflammatory and myeloproliferative disorders and provides a molecular rationale for an additional way to manage such conditions via repurposing of clinically available AMPK activators.

## 2. IL-6-DEPENDENT JAK-STAT SIGNALLING

IL-6 does not signal directly but first forms a dimer with IL-6R $\alpha$  (CD126) prior to binding its cognate receptor gp130. While gp130 is ubiquitously and constitutively

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PHD finger protein 6. ECs, endothelial cells. HUVECs, human umbilical vein ECs. IFN, interferon. IFNAR, IFN $\alpha$ / $\beta$  receptor. ULK1, Unc-51-like autophagy-activating kinase. HSCT, haematopoietic stem cell transplant. mAbs, monoclonal antibodies. FLT3, FMS-like tyrosine kinase 3. PV, polycythaemia vera. ET, essential thrombocythemia. PMF, primary myelofibrosis. ACPA, anti-citrullinated protein antibodies. Th, T helper. Treg, regulatory T cell. DMARDs, disease modifying anti-rheumatic drugs. TNF, tumour necrosis factor. GLINT, glucose lowering in non-diabetic hyperglycaemia trial. CRLF, cytokine-receptor like factor 2.

expressed, membrane bound IL-6R $\alpha$  is restricted to hepatocytes, leukocytes, and lymphocytes. However, soluble IL-6R $\alpha$  (sIL-6R $\alpha$ ) is produced during inflammation thus increasing the repertoire of IL-6-responsive cells [10,11]. Signalling via membrane bound IL-6R $\alpha$  is called ‘classical signalling’ while that via sIL-6R $\alpha$  is referred to as ‘trans-signalling’ with the latter being predominant in disease-related pro-inflammatory responses. Gp130 has no intrinsic kinase activity but is constitutively associated with JAK family tyrosine (Tyr) kinases. JAKs comprise a family of four cytoplasmic tyrosine kinases (JAK1-3, and Tyk2). However gp130 only associates with JAK1-2 and Tyk2, while type I cytokine receptors for IL-2 and related cytokines use the common gamma chain ( $\gamma$ c) to associate with JAK3 and signal downstream [12]. Cytokine-receptor ligation precedes trans-phosphorylation and activation of JAKs, which Tyr phosphorylate gp130 on specific Tyr residues to generate docking sites for SH2-domain-containing STAT proteins. Receptor-bound STATs are then Tyr-phosphorylated by JAKs within their SH2 domains (Tyr<sup>701</sup> on STAT1, Tyr<sup>705</sup> on STAT3) prior to dissociation from the receptor and formation of homo- or heterodimers, or tetramers. Following nuclear translocation and binding to target gene promoters, STATs drive transcription of target genes, which include pro-angiogenic vascular endothelial growth factor, intracellular adhesion molecule 1, monocyte chemoattractant protein 1 (MCP-1), and matrix metalloproteases 2 and 9 [12]. IL-6 also activates extracellular signal-regulated kinase (ERK)1/2 and phosphatidylinositol 3-kinase (PI3K) pathways following recruitment of SH2-containing protein Tyr phosphatase 2 to JAK-phosphorylated gp130 and ERK1/2-mediated phosphorylation and activation of Gab1. Finally, gp130 has also been shown to couple directly with Src family Tyr kinases to trigger activation of transcriptional co-activator YAP (YES- associated protein) (reviewed in [12]).

### 3. JAK STRUCTURE AND ACTIVATION

JAKs share a common domain structure (Figure 1) consisting of seven JAK-homology (JH) domains (JH1, kinase domain; JH2, pseudokinase domain, JH3-5, Src homology 2 (SH2) domain, and JH5-7, N-terminal 4.1, ezrin, radixin, moesin (FERM) domain). While FERM and SH2 domains regulate binding to the box1/2 region of cytokine receptors, the so-called “pseudokinase” (PK) domain regulates kinase activity by binding the JH1 kinase domain. Specific events of JAK activation

remain unclear, however, cytokine-receptor ligation is thought to cause repositioning or reorientation of the JAK-receptor complexes, bringing JAKs into close proximity, leading to *trans*-phosphorylation of the activation loop within the JH1 kinase domain. Several distinct mechanisms have been proposed for JAK activation but in addition, unique receptor structures are also thought to enforce specific JAK orientations that might drive different modes of regulation [13]. This could be one mechanism that leads to preferential assembly of specific receptor-JAK complexes, thus fine tuning downstream signalling [14]. With such variations, specific targeting of distinct receptor-JAK complexes may represent an alternative approach to developing new therapeutics targeting this pathway in disease states [13].

Activating mutations are frequently found within the regulatory PK domain including JAK1<sup>V658F</sup> and JAK2<sup>V617F</sup>, which are associated with acute lymphoblastic leukaemia (ALL) and myeloproliferative neoplasms (MPNs) respectively. Receptor association is essential for sustained JAK activation suggesting the JAK-receptor interaction as a possible drug target [15]. Both phenylalanine mutations cause rearrangements within a highly conserved Phe-Phe-Val triad [16], leading to rearrangement and stabilisation of distinct structures within the PK domain, including the C helix and SH2-PK linker regions [17]. Conformational changes break the JH1-JH2 interaction, relieving auto-inhibition and causing an elevation in catalytic activity [13]. A similar process of rearrangement and activation is thought to occur in WT JAKs upon cytokine receptor stimulation [16]. The JAK2 PK domain has been shown to have weak catalytic activity, allowing inhibitory autophosphorylation of Ser<sup>523</sup> and Tyr<sup>570</sup> residues which stabilises the JH1-JH2 interaction [18]. The JAK2<sup>V617F</sup> mutation is thought to promote JAK2 activation in part by preventing PK catalytic activity [16]. JAK kinase activity is further regulated by phosphorylation of Tyr<sup>221</sup> which increases activity [19].

Interestingly, disrupting PK ATP binding reduces JAK2<sup>V617F</sup> activity while leaving WT JAK2 unaffected [20].

In summary, it is now clear that JAKs offer several sites that could be targeted by small molecules, including the JH1 and JH2 domains, ATP binding and regulatory phosphorylation sites, as well as FERM-SH2/receptor interaction sites. Furthermore, variations in receptor-JAK interactions and distinct regulatory modes of individual JAK isoforms may enable highly specific targeting of dysfunctional pathways and therefore reduce adverse off-target effects.

#### 4. CURRENT THERAPEUTIC STRATEGIES TARGETING THE JAK-STAT PATHWAY

Several agents have been developed that target the JAK-STAT pathway at multiple levels. In the case of IL-6 signalling, the monoclonal anti-IL-6 antibody siltuximab [21], and the humanised anti-IL-6R antibody tocilizumab [22] have been approved for clinical use. Anti-IL-6 therapies have been approved for Castleman disease [23], a benign lymphoproliferative disorder, and RA [24]. However, single cytokine-targeting therapies only partially limit a disease phenotype and become less effective over time due to immunogenicity [25]. In other disorders such as multiple myeloma, targeting IL-6 alone has only limited benefit, potentially due to reliance on a range of other growth factors including IL-6-family cytokines which also signal via the signal transducing gp130 receptor. Also, while inhibitors of STATs, which mediate many of IL-6's intracellular effects, have also been developed, they have only shown limited effects [26]. As such, current focus has been on the development of JAK inhibitors or "Jak inhibitors".

First generation Jak inhibitors such as tofacitinib (CP-690,550) are pan-JAK inhibitors which act as competitive inhibitors of the ATP-binding site in the JH1 domains conserved amongst all 4 JAK isoforms (JAK1-3, Tyk2: Figure 1). However, inhibition of all JAK isoforms will inhibit the action of multiple cytokines, which when coupled with possible off-target effects, can result in unwanted adverse reactions including disrupted haematopoiesis, innate and adaptive host defence responses. Future development of second generation Jak inhibitors, which target less conserved regions of JAKs, is aimed at producing more selective drugs with less side effects, although these may display reduced efficacies versus less selective inhibitors (reviewed by Banerjee et al. [25]). It will be beneficial to specifically target mutated JAKs since simultaneous inhibition of wild type JAKs can have negative effects. A recent finding that disrupting JAK PK domain ATP binding reduces JAK2<sup>V617F</sup> activity leaving WT JAK2 unaffected has led to the identification of PK domain small molecule binders which might lead to the development of a range of novel JAK inhibitors [27].

Research in Jak inhibitor development is progressing with a number of drugs entering clinical trials [26]. However, there is still a need for therapeutics that are efficacious but with minimal side effects. The discovery of unappreciated targets of clinically-available drugs and important regulatory cellular molecules has accelerated drug repurposing efforts. Repurposing offers the possibility of speedy transition through

clinical trials with the advantages of having known tolerable dosages and manageable side effects. Here, we discuss current understanding of AMPK and the possibilities of repurposing AMPK activators for the treatment of inflammatory and myeloproliferative disorders.

## 5. AMP-ACTIVATED PROTEIN KINASE (AMPK)

AMPK activation following cellular stress, such as nutrient deficiency and hypoxia, triggers a switch in metabolism from ATP consumption to ATP generation in order to maintain energy homeostasis. AMPK is a heterotrimeric complex consisting of a catalytic  $\alpha$  subunit and regulatory  $\beta$  and  $\gamma$  subunits. In mammals, seven genes encode two  $\alpha$  ( $\alpha$ 1-2), two  $\beta$  ( $\beta$ 1-2), and three  $\gamma$  ( $\gamma$ 1-3) subunit isoforms. The subunits of the  $\alpha$ 1  $\beta$ 1  $\gamma$ 1 complex are ubiquitously expressed and most intensively studied. Other subunit isoforms show tissue specific expression patterns, while variations in subcellular location have also been reported [28–31]. It is likely that tissue specific expression of distinct subunit isoforms will be important for therapeutically targeting specific disorders through AMPK activation [32–34].

AMPK is activated by allosteric binding of AMP/ADP to the  $\gamma$  regulatory subunit and Thr<sup>172</sup> phosphorylation of catalytic  $\alpha$ 1 and  $\alpha$ 2 subunits by the upstream Ser/Thr Kinase liver kinase B1 (LKB1). Binding of AMP also inhibits Thr<sup>172</sup> de-phosphorylation by protein phosphatase PP2A. However, AMPK can also be activated by Thr<sup>172</sup> phosphorylation alone independent of changes in cellular ATP and AMP levels via calcium/calmodulin-dependent protein kinase kinase  $\beta$  following elevation of intracellular Ca<sup>2+</sup> levels. Phosphorylation of the catalytic  $\alpha$  subunit and thus full AMPK activation is also dependent on N-terminal myristoylation of the  $\beta$  subunit, which interacts with both  $\alpha$  and  $\gamma$  subunits [35].

Several direct and indirect activators of AMPK (reviewed by Kim et al [36]) have been identified, including aminoimidazole-4-carboxamide riboside (AICAR), metformin, salicylate, and A769662. AICAR is metabolised to AICAR monophosphate (ZMP) which directly activates AMPK by mimicking AMP-dependent activation by binding  $\gamma$  subunits. A769662 is selective for  $\beta$ 1 subunit-containing complexes and similar to AMP, allosterically activates and inhibits Thr<sup>172</sup> de-phosphorylation, however, it employs a different mechanism in which Thr<sup>172</sup> phosphorylation is not required [37,38]. Aspirin, the pro-drug of salicylate, is structurally similar to A769662 and



activates  $\beta$ 1 subunit-containing complexes in a similar manner, although salicylate binds at an overlapping allosteric binding site and can thus antagonise activation by A769662 [39]. Metformin, a clinically available anti-hyperglycaemic drug used to manage type 2 diabetes mellitus (T2DM), indirectly activates AMPK by depleting ATP via inhibition of complex I of the mitochondrial respiratory chain [40], and mitochondrial glycerophosphate dehydrogenase [41] as well as an Axin-dependent lysosomal pathway [42]. However, many AMPK activators lack specificity and therefore it is necessary to differentiate between their AMPK-dependent and -independent effects [37,38,43–45].

## 6. AMPK LINKS METABOLISM AND INFLAMMATION

AMPK is a crucial regulator of energy metabolism, however its anti-inflammatory effects have been reported in several human cell types and multiple animal models of disease [46–49]. For example, prolonged STAT1 activation leads to accelerated neo-intimal formation and atherosclerosis while AMPK $\alpha$ 2 knockout mice have an enhanced STAT1-dependent inflammatory response. Pharmacological or genetic activation of AMPK, or treatment with the STAT1 inhibitor fludarabine reversed the response suggesting that AMPK is potentially protective against vascular inflammation and atherosclerosis [50]. The available literature suggests that AMPK inhibits multiple signalling pathways to mediate its anti-inflammatory effects [4]; these include inhibition of NF- $\kappa$ B signalling [51–53] and multiple mitogen-activated protein kinase pathways [54], and reducing reactive oxygen species production [54]. Other groups, including our own, have reported AMPK-dependent inhibition of JAK-STAT signalling [49,50,55–57]. Potential mechanisms for the observed AMPK-mediated suppression of IL-6 responses include the induction of orphan nuclear receptor small heterodimer partner (SHP) [57]. Long-term metformin treatment of primary rat hepatocytes was found to trigger an AMPK-dependent accumulation of SHP, which co-localised with STAT3 in the nucleus to reduce DNA binding and inhibit expression of pro-inflammatory genes including suppressor of cytokine signalling 3, serum amyloid A, IL-6, and tumour necrosis factor (TNF)  $\alpha$  [57]. In contrast, others have shown in HepG2 human hepatoma cells that chronic AICAR-mediated AMPK activation inhibits IL-6 signalling by suppressing JAK1/JAK2 tyrosine phosphorylation and activation, although the mechanism responsible was not

investigated [49,55]. In addition, AMPK-dependent induction of mitogen-activated protein kinase phosphatase-1 (MKP-1), a Thr/Tyr dual specificity protein phosphatase, has been proposed to inhibit STAT1 activation in human aortic smooth muscle cells (SMCs) [50]. MKP-1 has previously been reported to specifically target Ang-II-activated STAT1 whilst leaving STAT3 unaffected [58,59]. AMPK also reduced Ang-II induction of STAT1 target genes including MCP-1 in addition to STAT1-dependent vascular inflammation *in vivo*. Another mechanism by which AMPK could potentially inhibit JAK-STAT signalling is through its well-characterised suppression of the mammalian target of rapamycin (mTOR) pathway through direct phosphorylation and inhibition of Raptor and activation of the Rheb GTPase-activating protein tuberous sclerosis complex 2 (TSC2) [60,61]. However despite reports demonstrating that inhibition of mTOR signalling suppresses JAK-STAT activation [62,63], the molecular mechanisms responsible for this phenomenon remain unknown.

Using human umbilical vein ECs (HUVECs) as a model system, we showed that a panel of AMPK activators, including A769662, AICAR and a combination of metformin with salicylate, rapidly inhibited STAT3 activation by a sIL-6R $\alpha$ /IL-6 trans-signalling complex [64]. We have reported a similar rapid inhibition of sIL-6R $\alpha$ /IL-6-stimulated STAT3 phosphorylation by A769662 in 3T3-L1 adipocytes [65]. We also demonstrated that the inhibitory effect of AMPK by A769662 was observed on both STAT1 and STAT3 phosphorylation in response to oncostatin M and leukaemia inhibitory factor, both of which also utilise gp130 to signal downstream [64]. Importantly, the effect of AMPK was not restricted to cytokines utilising gp130, as STAT1 phosphorylation in response to interferon  $\alpha$  (IFN $\alpha$ ) was also inhibited, suggesting that AMPK was acting at a common post-receptor locus to mediate its effects [64]. JAK1 is immediately downstream of both gp130 and the IFN $\alpha$ / $\beta$  receptor (IFNAR)1/IFNAR2 complex responsible for IFN $\alpha$  signalling, and activation of AMPK resulted in a phosphorylation of JAK1 on Ser<sup>515</sup> and/or Ser<sup>518</sup> responsible for rapid inhibition of JAK-STAT signalling [64]. Importantly, AMPK was also able to inhibit constitutive signalling from oncogenic JAK1<sup>V658F</sup> prevalent in ALL [64]. In terms of the mechanism, AMPK-mediated JAK1 phosphorylation on Ser<sup>515</sup> and/or Ser<sup>518</sup> suppresses downstream signalling either directly or indirectly by promoting binding to 14-3-3 proteins, which regulate target protein function by disrupting protein-protein

interactions that induce changes in activity or subcellular localisation [66]. Several AMPK substrates, including B-Raf, Unc-51-like autophagy-activating kinase (ULK1) and Raptor, have been shown to bind 14-3-3 proteins following AMPK-dependent phosphorylation [60,67,68]. We have identified JAK1 as a similarly regulated AMPK substrate since pharmacological activation of AMPK promoted 14-3-3 binding to JAK1 via a mechanism requiring phosphorylation of Ser<sup>515</sup> and Ser<sup>518</sup> [64]. However the functional importance of 14-3-3/JAK1 interaction in intact cells remains to be tested.

In summary, the existence of several inhibitory mechanisms by which AMPK can suppress JAK-STAT signalling mirrors the multifaceted impact of AMPK on the mTOR signalling pathway, which it inhibits through direct phosphorylation of TSC2 on Ser<sup>1387</sup> and Raptor on Ser<sup>722</sup> and Ser<sup>792</sup> [60,61,69] (Figure 2A). These observations suggest that, like the mTOR pathway, AMPK-mediated regulation of JAK-STAT signalling has also evolved such that multiple mechanisms and targets are used to limit its activation.

## 7. REPURPOSING OF AMPK ACTIVATORS

AMPK is a critical global regulator of stress including nutrient deficiency, hypoxia, and inflammation. This provides a strong rationale for both the development of novel AMPK agonists and the repurposing of those already used in the clinic. Here we provide a molecular rationale to support the application of AMPK activation as a therapeutic strategy for management of conditions resulting from hyperactivation of the JAK-STAT pathway, such as ALL, MPNs and RA.

### 7.1 ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL)

ALL is an aggressive haematological malignancy affecting all age groups but primarily associated with young children, making it a particularly distressing disease. ALL is the most common paediatric cancer, accounting for 26% of all cancer cases in children [70]. While ALL can occur in adults, the patients age and ethnicity are important factors for prognosis; male, Hispanic children statistically have the most aggressive forms of ALL [70].

ALL is characterised by the accumulation of lymphoblasts arrested at specific stages of differentiation caused by the transformation of B/T cell progenitors. Initiating mutations in regulators of differentiation are subsequently perpetuated by activating

and inactivating mutations in pathways that drive proliferation/survival and tumour suppression respectively [71]. Classified into B-cell ALL (B-ALL), T-cell ALL (T-ALL), and Natural Killer ALL (NK-ALL), T-ALL is the more clinically aggressive. However, T-ALL is less common accounting for approximately 15% of paediatric ALL cases, NK-ALL is rare at 3% while the rest consists of B-ALL [70,72,73]. ALL classes are each split into primary genetic subtypes: for T-ALL, these are defined by mutations in signalling protein neurogenic locus notch homolog protein 1 (NOTCH1), cell cycle regulator cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B), E3 ubiquitin ligase component F-box and WD repeat domain containing 7 (FBXW7) and PHD finger protein 6 (PHF6), which has no known function [74]. Further secondary genetic mutations are also common such as those within the JAK-STAT pathway. Multiple activating mutations of JAKs, such as the constitutively active JAK1<sup>V658F</sup> and JAK2<sup>V617F</sup> which drive cell proliferation and survival [70], are associated with ALL while enhanced activation of JAK-STAT signalling is detected in 3-10% of all patients with all T-cell malignancies (reviewed by Waldmann and Chen [75]). Therefore at least some of these patients may benefit from therapeutic approaches that limit JAK-STAT signalling.

Chemotherapy is the first line treatment for ALL, involving delivery of an intensive combination of cytotoxic compounds. However, treatment often induces long-term adverse effects which either impair vital physiological functions or induce further complications, including secondary tumours and pulmonary and cardiovascular disorders [70,76]. 20-25% of children with T-ALL experience relapse and are often resistant to further chemotherapy; the outcome at this point being much worse [77]. Survival rates in paediatric ALL have improved dramatically in the last 50 years (currently 85–90%) following advances in genetic subtyping and personalised treatment regimens [78,79]. Allogeneic haematopoietic stem cell transplant (HSCT) therapy has been used to treat high risk patients following relapse. However, HSCT is associated with high risks of treatment failure and treatment-related mortality [70,80]. High risk patients are often treated with local or whole body radiotherapy which require antibiotics to prevent infection while other complications such as anaemia and risk of bleeding are treated with blood transfusions and platelet transfusions respectively [73].

Additional therapies are being developed with varying degrees of success [81,82]. These include targeting cell surface receptors overexpressed on T/B cells using

monoclonal antibodies (mAbs). Several cell surface antigens are common to precursor B-ALL (CD10, CD19, CD34) and T-ALL (CD2, CD3, CD7) cells [73]. Monoclonal antibodies are currently available which target CD20 specifically (rituximab) and both CD3 and CD19 simultaneously (the bispecific antibody blinatumomab) to induce complement activation, antibody-dependent cellular cytotoxicity and apoptosis [82]. Tyrosine kinases which drive survival and proliferation, such as BCR-ABL and FMS-like tyrosine kinase 3 (FLT3), are frequently overexpressed in leukaemic cells and have been targeted with tyrosine kinase inhibitors [70]. Multiple clinical trials are ongoing to investigate other novel agents including the use inhibitors towards the 26S proteasome, histone deacetylases [83], DNA methyltransferases [84], mTOR [85] and others [81,82] for use as either single or combination therapies.

It is apparent that new strategies are required which overcome the long-term side effects, relapse, and resistance associated with chemotherapies. One option is to specifically target aberrantly activated signalling pathways which drive proliferation and survival of cells. Aberrant JAK-STAT signalling is implicated in ALL pathogenesis [75]. Targeting the JAK-STAT pathway has shown positive effects on the disease phenotype [86] while activating mutations of JAK1 are frequently associated with cases of ALL [87–90]. The JAK1<sup>V658F</sup> mutation identified in T-ALL patients [90–92] drives the ligand-independent activation of STATs [93,94] and its expression can induce transformation of Ba/F3 pro-B cells [87,89,95]. Subtypes of leukaemia have also been reported with hyperactive JAK-STAT signalling in the absence of activating mutations [86]. As such, both WT and constitutively activate JAK mutants are an attractive therapeutic target for treating ALL.

We have demonstrated that AMPK can block JAK-STAT signalling via inhibition of WT and constitutively active JAK1<sup>V658F</sup> via a mechanism that requires JAK1 phosphorylation on Ser<sup>515/518</sup> [64]. Thus inhibition of JAK-dependent ALL via AMPK activators might have positive effects on haemostasis. Furthermore, inhibiting JAK-STAT signalling might limit secondary side effects produced following current treatments regimens such as cardiovascular and pulmonary disorders. Therefore, this provides a basis for the evaluation and repurposing of clinically utilised AMP-activating drugs, such as metformin and thiazolidinediones, as potential treatment options for ALL associated with constitutive JAK1 signalling. In support of this, metformin has previously been investigated as a candidate to target T-ALL through

AMPK-mediated inhibition of the PI3K/mTOR pathway, which is hyper-activated in approximately 85% of T-ALL cases [96]. Grimaldi et al. demonstrated that metformin-activated AMPK inhibited mTOR and induced apoptotic cell death [96–99]. Thus AMPK activators might be effective in treating both JAK-STAT and PI3K/mTOR-dependent aspects of T-ALL cell dysfunction. To examine this, a clinical trial is currently on-going investigating the treatment of relapsed ALL with a combination of metformin and chemotherapy [96] (trial identifier:NCT01324180).

## 7.2 MYELOPROLIFERATIVE NEOPLASMS (MPNS)

Myeloproliferative neoplasms are a group of three haematological malignancies, termed polycythaemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), which are characterised by uncontrolled proliferation of CD34<sup>+</sup>CD38<sup>-</sup> haematopoietic stem/progenitor cells. An acquired JAK2<sup>V617F</sup> mutation analogous to T-ALL-associated JAK1<sup>V658F</sup> results in unregulated cytokine-independent JAK2 activation and is frequently detected in PV (>95% of cases), ET (~50%), and PMF (~50%) [100–102].

Thrombohaemorrhagic events and disease transformation into acute myeloid leukaemia (AML) are complications and main causes of morbidity and mortality in PV and ET [103]. It is not known how these complications arise and current treatments are limited to phlebotomy (PV patients only), low dose aspirin anti-platelet therapy (to reduce risk of thrombosis), and cytoreduction with hydroxyurea, anagrelide, IFN $\alpha$ , and alkylating agents such as busulfan [104]. A subpopulation of MPN patients have been found to express JAK2<sup>V617F</sup> in endothelial cells suggesting a further cellular target for MPN therapies [105]. Moreover, Etheridge et al. [106] found that mice engineered to express JAK2<sup>V617F</sup> in either ECs, haematopoietic cells or both cellular compartments developed an MPN phenotype with attenuated thrombosis following injury. It is thought that JAK2<sup>V617F</sup> contributes to the bleeding phenotype in part via the inhibition of function or secretion of the pro-thrombotic molecule von Willebrand factor, and reduced levels of agglutination. Together, these data suggest that targeting JAK-STAT signalling in both cellular compartments might be effective for the treatment of JAK2<sup>V617F</sup>-positive MPNs. Ruxolitinib, a dual JAK1/2 inhibitor, is approved for the treatment of intermediate/high-risk PMF and successful clinical trials have been carried out on JAK2<sup>V617F</sup>-positive individuals who were unresponsive to other treatments [104]. However, reductions in disease phenotype were frequently

accompanied by haematological side effects such as thrombocytopenia and anaemia as well as other non-haematological events including diarrhoea and pyrexia. Kawashima and Kirito [103] have reported that metformin treatment of JAK2<sup>V617F</sup>-positive MPN cell lines induced AMPK activation, leading to decreased levels of STAT5 phosphorylation and induction of apoptotic cell death. Furthermore, alignment of the primary sequences of JAK family members (Figure 2B) reveals conservation of the SH2 domain-localised AMPK phosphorylation sites we identified on JAK1 [107]. Together, these findings suggest that AMPK might also inhibit a range of constitutively active JAK mutants including JAK2<sup>V617F</sup>. As such, AMPK activators might also be beneficial in the treatment of JAK2<sup>V617F</sup>-associated MPNs via the management of both anti-proliferative effects and haemostatic defects.

### 7.3 RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis is a common autoimmune disease characterised by synovial inflammation and hyperplasia, autoantibody production and destruction of cartilage and bone. It is also accompanied by systemic features including cardiovascular, pulmonary, psychological, and skeletal disorders [108]. Although the aetiology of RA is not fully understood, recent evidence suggests that autoimmune responses to citrullinated epitopes predominantly present on matrix proteins plays a central role in pathology [109]. Anti-citrullinated protein antibodies (ACPAs) are a measure of B-cell activation [110,111] and elevated ACPA levels are a biomarker for aggressive RA [112]. Acute phase proteins such as IL-6 also play a crucial role in RA pathogenesis and elevated levels of IL-6 in serum and synovial fluid are markers of the disease [113]. IL-6 initiates and sustains inflammation and degeneration by inducing the acute-phase response, directly activating B and T lymphocytes, macrophages and osteoclasts, and promoting infiltration of inflammatory cells, and production of matrix metalloproteinases [113].

IL-6-activated CD4<sup>+</sup> T-cells, key drivers of RA, differentiate to specific T helper (Th) lymphocytes which drive distinct immune responses. Th1 cells secrete IFN $\gamma$ , IL-2, and TNF)  $\beta$ , which activate macrophages to enhance cell-mediated immunity and phagocytic responses, whereas Th2 cells generate IL-4, IL-5, IL-10, and IL-13 to stimulate B-cell-mediated antibody production and activate eosinophils. A recently identified CD4<sup>+</sup> T-cell lineage, Th17 cells, secrete cytokines such as IL-17 and IL-21

which activate JAK-STAT signalling in multiple cell types. This stimulates the secretion of chemokines, cytokines, and colony-stimulating factors which activate and recruit neutrophils and other myeloid cells leading to an exacerbated inflammatory response [114]. Th17 cells are less understood, in part because their pattern of differentiation in humans is distinct from that of mouse. However STAT3-dependent transcription is thought to be a common crucial factor in both systems [114]. IL-6 stimulates the differentiation of pathogenic Th17 cells from naive T-cells but is not required for Th17 maintenance following differentiation [115]. Furthermore, IL-6 inhibits the TGF- $\beta$ -induced development of immunosuppressive regulatory T cells (Treg) [116–118]. Therefore, upon sustained, elevated levels of IL-6, the fine balance between Th17 cells and Tregs is lost, thereby promoting an enhanced immune response [116–118]. IL-6 can also inhibit the differentiation of Th2 cells [119] while also promoting the differentiation of immature B-cells into antibody-secreting cells [120]. The latter is thought to be an indirect response of IL-6 stimulating the B cell helper capabilities of CD4<sup>+</sup> T cells through increased IL-21 production [120]. Thus, an increase in Th1 and Th17 cells and a decrease in Th2 and Treg T-cells promotes a pro-inflammatory, macrophage-dependent response. In an incompletely understood process, escalating levels of pro-inflammatory cytokines secreted by numerous cell types cause a vicious feedback cycle leading to local and systemic pathogenesis.

As a major driver of RA-dependent events, IL-6-dependent JAK-STAT signalling has been intensively studied and its components are targets for therapeutic intervention by small molecule inhibitors and biologicals [121,122].

### 7.3.1 TARGETING RA BY INHIBITING IL-6-JAK-STAT SIGNALLING

RA is commonly treated using conventional disease modifying anti-rheumatic drugs (DMARDs) (cyclosporin, sulfasalazine, and methotrexate), slow-acting drugs that reduce disease progression. There is an on-going development of novel biologics that specifically target the cause of inflammation including anti-TNF $\alpha$  (infliximab, adalimumab, certolizumab and golimumab) [123], co-stimulatory receptors CD80/CD86:CD28 required for full T-cell activation (abatacept), anti-CD20 which targets B cells (rituximab), and anti-IL-6R (tocilizumab)[124].

Tocilizumab, a humanised anti-IL-6R antibody which binds and inhibits both IL-6R and sIL-6R $\alpha$ , has been used successfully for treatment of RA [116–118]. While the



previously mentioned biologics are often combined with methotrexate to increase efficacy, no additive effect is seen with tocilizumab and methotrexate suggesting targeting of the same pathway [124]. This finding is supported by Thomas et al. [125] who demonstrated that, in addition to several possible mechanisms of action [126], methotrexate also targets JAKs. It is therefore possible that, while improving RA prognosis, IL-6/JAK-directed mono-therapy might also have fewer side-effects. Most recently, tofacitinib, a ATP-competitive JAK1/3 inhibitor, was approved by the US FDA for RA treatment of patients who are intolerant/resistant to first line therapies such as methotrexate (reviewed by Nakayamada et al. [127]). A small number of studies have demonstrated that AMPK activators, metformin and A769662, suppressed inflammatory arthritis in murine models of RA [128,129]. It has also been reported that AMPK $\alpha$ 1-deficient mice exhibit a mildly enhanced inflammatory arthritis versus wild type controls [130]. Moreover, Kang et al. reported that metformin inhibits Th17-cell differentiation both *in vivo* and *ex vivo*, and AMPK activation inhibits STAT3 phosphorylation in CD4+ T cells *in vitro* [129]. Together with our own data, this suggests that metformin might suppress Th17 cell differentiation by inhibiting STAT3 phosphorylation in part *via* AMPK-mediated phosphorylation of JAK1.

## 8. SUMMARY AND PERSPECTIVES

This review has evaluated data which provides a mechanistic rationale for repurposing clinically available drugs for the targeted activation of AMPK to treat an array of JAK-dependent disorders. An increasing body of evidence has shown that changes in cellular nutritional status impact on immune and inflammatory status via AMPK [131]. Upon stress, AMPK activation acts to limit JAK-STAT-dependent pro-inflammatory signalling via several mechanisms to initiate a protective response. JAK-STAT signalling is crucial in the development of several pro-inflammatory disorders such as rheumatoid arthritis, cardiovascular disease, as well as myeloproliferative neoplasms such as acute lymphoblastic leukaemia. Novel findings suggest that AMPK can limit JAK-STAT signalling driven by constitutively active JAK1<sup>V658F</sup> and, based on sequence similarity between JAK isoforms (Figure 2B), the same mechanism may inhibit other constitutively active JAKs associated with disease [107]. Such mutations are common in haematological malignancies for which current treatments are either limited or harmful. Therefore, repositioning current medications or developing novel small molecule AMPK activators or biologics, used

alone or in a combined regimen, might provide a more efficacious but less harmful therapy.

Repositioning of drugs is the reuse of existing pharmaceutical agents to alleviate symptoms unconnected with the primary disorder. Successful repositioning of existing drugs has been documented over the decades [9], although it is not without its own drawbacks [8]. Given the cost/time of research and development and clinical trials with new drugs, re-purposing is advantageous as it increases the speed to clinic and availability of drugs while eliminating unknown variables such as tolerable dosages and side-effects. There is potential for current therapeutics for arthritis and proliferative diseases i.e. tocilizumab, tofacitinib and ruxolitinib, which target IL-6-dependent JAK-STAT signalling, in the treatment of vascular disease [132]. While approved for RA, tofacitinib is also being investigated for use against other autoimmune diseases [133]. Moreover, statins, which inhibit cholesterol production and are widely prescribed for management of cardiovascular disease, have shown to target IL-6-dependent disorders by limiting STAT3 activation through blocking IL-6 production [134,135]. Activation of AMPK by clinically available drugs including the anti-hyperglycaemic drug metformin and salicylate, the active metabolite of aspirin [136,137], have been shown to have protective effects in the vasculature [138]. We have shown that metformin, salicylate, and A769662 can each limit IL-6-dependent STAT3 phosphorylation in HUVECs. AMPK was found to directly phosphorylate Ser<sup>515</sup> and Ser<sup>518</sup> within the SH2 domain of JAK1 [64]. AMPK-mediated inhibition of JAK-STAT signalling was attenuated upon either mutation of Ser<sup>515</sup> and Ser<sup>518</sup> residues or knockdown of AMPK $\alpha$ 1 catalytic subunits. Together these data support AMPK-dependent regulation of JAK signalling as a mechanism that could be further investigated with a view to the repurposing AMPK activators for a range of immune and inflammatory diseases.

The AMPK activator metformin is a widely used, relatively safe and inexpensive anti-diabetic drug and therefore might be beneficial for long-term treatment regimens. Common adverse effects of metformin are relatively mild and include hypoglycaemia and gastrointestinal intolerance, which can occur in up to 30% of patients. A more serious but rare adverse effect is lactic acidosis due to drug overdose or administration in contraindicated conditions [139]. It is possible that AMPK-

dependent inhibition of JAK-STAT signalling might limit secondary side effects of current treatments i.e. chemotherapy, such as cardiovascular and pulmonary disorders. Several observational studies have reported that treatment with metformin limits cardiovascular morbidity and mortality independent from its glucose-lowering action in patients with T2DM [140–143]. However, clinical studies showed little or no effect on several surrogate markers of cardiovascular disease in non-diabetic patients with high cardiovascular risk, taking statins [144], or during cardiac surgery [145]. As such, clinical trials of metformin monotherapy are necessary to evaluate the cardiovascular protective effects of metformin treatment in non-diabetic patients with CVD. One such study is currently ongoing is the Glucose Lowering In Non-diabetic hyperglycaemia Trial (GLINT; ISRCTN34875079). Over 12 000 patients with high cardiovascular risk and abnormal blood glucose levels but without diabetes, will be assigned to metformin or placebo for 5 years with data cut-off in December 2024. Aspirin is an acetylated prodrug which is rapidly broken down to the allosteric AMPK activator salicylate within the bloodstream [146] and is a well-established anti-platelet drug for secondary prevention in patients at high risk of cardiovascular events [147]. However, aspirin may adversely impact on haemostasis and cause complications in patients prone to bleeding. While low dose aspirin is used to reduce risk of thrombosis, myocardial infarction and stroke, it can also cause gastrointestinal ulcerations and bleeding [148]. Additionally, further work is necessary to dissect AMPK-dependent and -independent effects of current AMPK activators, which lack specificity [37,38,43–45].

While a pipeline of novel agents is being developed to complement and replace existing chemotherapy regimens, the accumulation of genetic changes rapidly results in the generation of unresponsive sub-clones of primary tumours. This requires the development of a variety of personalised combination therapeutic regimens and as such, there is an on-going need to expand the arsenal of anti-cancer agents. For example, genetic background will be an important factor which determines the use of JAK inhibitions and/or treatment regime either at diagnosis or relapse. A recent finding suggested that loss of USP9X, a deubiquitinase and activator of JAK-STAT signalling, in cytokine-receptor like factor 2-positive (CRLF<sup>POS</sup>) ALL might contribute to cell survival by limiting JAK signalling [149]. Importantly the same group found that low-dose ruxolitinib also had the same effect in USP9X knockout O18z cells expressing CRLF2/JAK2<sup>R683G</sup>. Therefore, it is critical to assess disease status and

evolution prior to stratification of patients to the most appropriate therapeutic regimen.

AMPK functions as a heterotrimeric complex of consisting of single  $\alpha$ ,  $\beta$ , and  $\gamma$ , subunits with the  $\alpha1\beta1\gamma1$  complex being ubiquitously expressed and most studied. Other subunits show tissue specific expression patterns while variations in subcellular location have also been reported [28–31]. Therefore expression profiles and functions of specific AMPK isoform complexes might be exploited to design AMPK activators which target defined tissues such as the vasculature. This is supported by the identification of several isoform specific direct activators of AMPK [45] including salicylate and A769662, which selectively target  $\beta1$ -containing AMPK complexes. Furthermore, lymphocytes have been reported to express AMPK $\alpha1$  catalytic subunits exclusively, thus fewer heterotrimeric complexes are possible which might allow specific targeting of diseases such as ALL [150]. Despite numerous reports suggesting that AMPK activators may be beneficial for the treatment of cardiovascular disease and a number of human pathologies [47,50,130,138] no direct AMPK activators have yet reached clinical use. However, two newly developed direct allosteric AMPK activators, PF-739 [151] and MK-8722 [152], have shown efficacy in improving glycaemic control in primates and may therefore prove useful in patients with specific indications.

The novel finding the AMPK targets JAK1 [107] suggests the possible development of AMPK-targeting molecules with enhanced specificity to target JAK1/2-dependent tissue specific disorders. JAK inhibitors, such as ruxolitinib and tofacitinib, competitively inhibit the JH1 domain ATP-binding site. Structural similarities between JAK isoforms in this region means that development of specific JAK inhibitors targeting this site is challenging, thus requiring a more novel approach [153] such as the not yet exploited approach of targeting the cytokine receptor-JAK interaction. Recent advances in JAK structural studies have found that the SH2 domain interacts with the cytokine receptor box 2 motif [18,154]. We have shown that AMPK phosphorylates JAK1 at Ser<sup>518</sup> and Ser<sup>515</sup> which are positioned directly beside this interaction suggesting this modification disrupts the cytokine receptor-JAK1 SH2 interaction and downstream signalling. Thus targeting this interaction might allow for the design of small-molecule inhibitors with specificity towards distinct JAK isoforms and JAK-receptor combinations.

Together, current data suggests that targeting AMPK with clinic-ready drugs and exploiting its novel regulatory mechanisms might usher in a new generation of selective therapeutics for chronic inflammatory disorders and a range of haematological malignancies.

Conflicts of interest: none

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## FIGURES

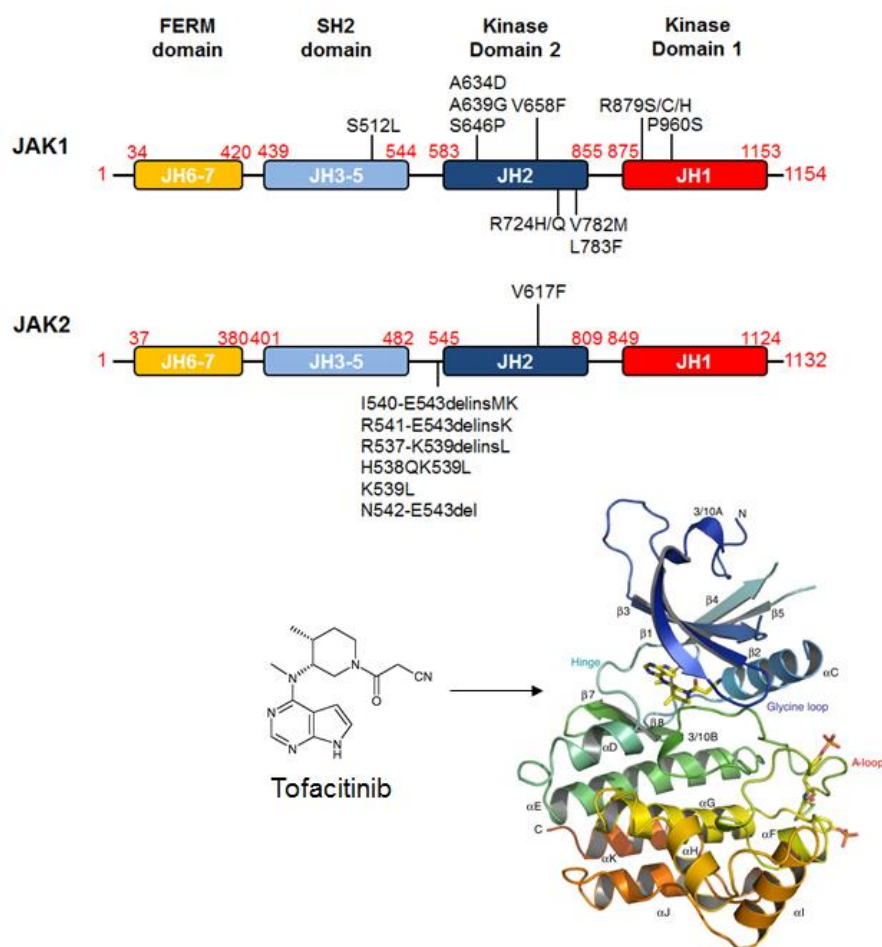
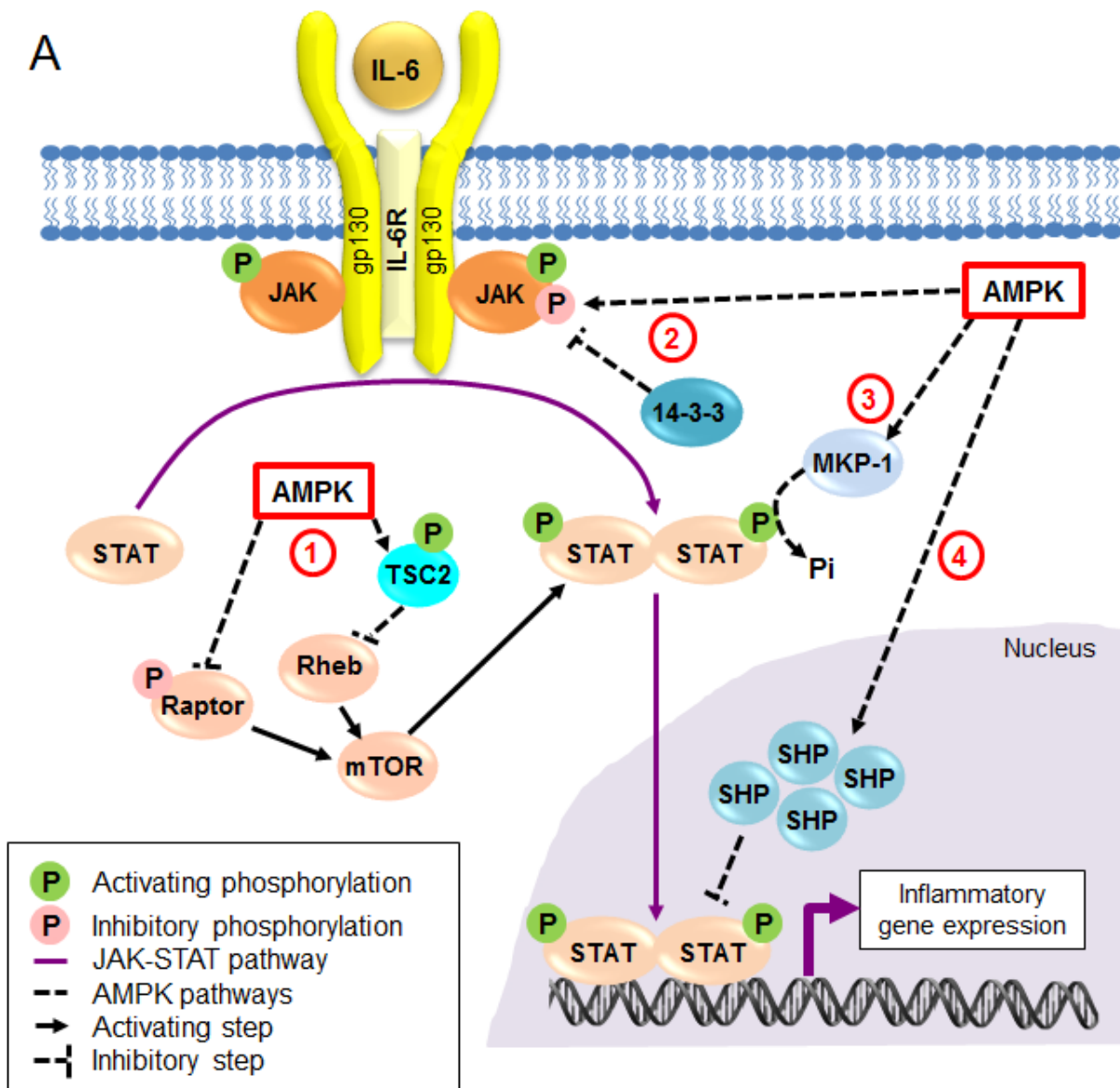
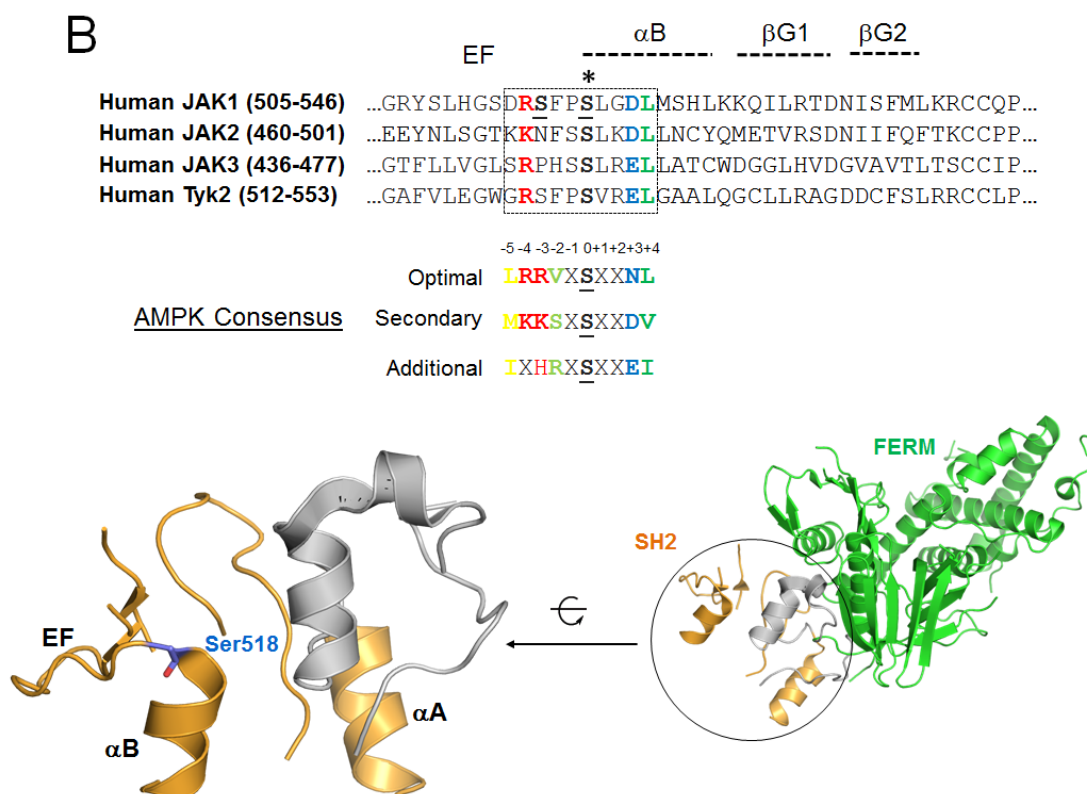


Figure 1: Human JAK1 and JAK2 domain structure and disease-associated mutations resulting in constitutive kinase activation.

Upper: Schematic diagram of human JAK1 and JAK2 showing the common structure conserved in all four JAK isoforms consisting of seven JAK-homology (JH) domains (JH1, Tyr kinase domain; JH2, pseudokinase domain; JH3-5, Src homology 2 (SH2) domain; JH5-7, N-terminal 4.1, Ezrin, Radixin, Moesin (FERM) domain). While the FERM and SH2 domains regulate receptor binding, the pseudokinase domain, which has weak catalytic activity [18], regulates JH1 domain Tyr kinase activity. The locations of activating mutations in JAK1 detectable in patients with ALL and activating mutations in JAK2 observed in MPN patients are indicated. JAK1<sup>V658F</sup> and JAK2<sup>V617F</sup> mutations within the regulatory JH2 domains are the most frequently detected JAK mutations in ALL and MPN respectively [75].

Lower: Crystal structure of the JAK2 JH1 domain bound to JAK inhibitor tofacitinib, reproduced with permission [155].





**Figure 2: Control of the JAK-STAT pathway at multiple levels by AMPK**

Panel A: Schematic representation of the molecular mechanisms by which AMPK has been proposed to inhibit JAK-STAT signalling. 1- inhibition of mTOR via Raptor inhibition and TCS2 activation. 2- Ser phosphorylation of JAK and downstream binding of 14-3-3. 3- dephosphorylation of STAT by MKP-1. 4- increased SHP accumulation and prevention of STAT DNA binding and transcription.

Panel B: Upper - Alignment of SH2 domain regions from the four human JAK isoforms. The lettering above the alignment indicates secondary structural elements. The residues corresponding to Ser<sup>515</sup> and Ser<sup>518</sup> in JAK1 are underlined and in bold, and the conserved Ser residues in JAK2, JAK3, and Tyk2 equivalent to Ser<sup>518</sup> are in bold and marked with an asterisk. Box highlights the sequence surrounding the conserved Ser residue in each JAK isoform and is colour-coded to show conformity to the AMPK phosphorylation consensus sequences indicated below [60]. Lower - Structure of the human JAK1 FERM-SH2 fusion (Protein Data Bank: 5IXD) [154]. Zoom: Location of Ser<sup>518</sup> within the  $\alpha$ B helix immediately downstream of the EF loop in the SH2 domain. The  $\alpha$ A and  $\alpha$ B loops present in all SH2 domains are also indicated. Figure adapted from Rutherford et al. [64].