



# Persistence of myelofibrosis treated with ruxolitinib: biology and clinical implications

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Haematologica 2021  
Volume 106(5):1244-1253

## ABSTRACT

Activation of JAK-STAT signaling is one of the hallmarks of myelofibrosis, a myeloproliferative neoplasm that leads to inflammation, progressive bone marrow failure, and a risk of leukemic transformation. Around 90% of patients with myelofibrosis have a mutation in *JAK2*, *MPL*, or *CALR*: so-called ‘driver’ mutations that lead to activation of JAK2. Ruxolitinib, and other JAK2 inhibitors in clinical use, provide clinical benefit but do not have a major impact on the abnormal hematopoietic clone. This phenomenon is termed ‘persistence’, in contrast to usual patterns of resistance. Multiple groups have shown that type 1 inhibitors of JAK2, which bind the active conformation of the enzyme, lead to JAK2 becoming resistant to degradation with consequent accumulation of phospho-JAK2. In turn, this can lead to exacerbation of inflammatory manifestations when the JAK inhibitor is discontinued, and it may also contribute to disease persistence. The ways in which *JAK2* V617F and *CALR* mutations lead to activation of JAK-STAT signaling are incompletely understood. We summarize what is known about pathological JAK-STAT activation in myelofibrosis and how this might lead to future novel therapies for myelofibrosis with greater disease-modifying potential.

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Received: September 15, 2020.

Accepted: December 11, 2020.

Pre-published: January 21, 2021.

<https://doi.org/10.3324/haematol.2020.262691>

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## Introduction

Primary myelofibrosis is a peculiar illness that has features of both a slowly progressive cancer and a chronic inflammatory disorder. It is a clonal neoplasm driven by a handful of somatic mutations that activate cell signaling, presumably residing in the long-term stem cell compartment,<sup>1-3</sup> but it also has a constellation of cytokine-mediated symptoms that are disproportionately severe. Myelofibrosis can also arise from antecedent polycythemia vera or essential thrombocythemia, leading to substantially overlapping clinical features. Progression of myelofibrosis leads to bone marrow failure with extensive marrow fibrosis or transformation to secondary acute myeloid leukemia. After the discovery of activating *JAK2* mutations in 50-60% of patients with primary myelofibrosis,<sup>4,5</sup> clinicians were hopeful that myelofibrosis would respond to tyrosine kinase inhibitor (TKI) therapy in a similar fashion to chronic myeloid leukemia, another myeloproliferative neoplasm (MPN). Chronic myeloid leukemia is driven by the *BCR-ABL1* fusion which responds to ABL1 TKI therapy with rapid log-fold reductions in the number of BCR-ABL1-mutated cells in the majority of patients.<sup>6-8</sup> Ruxolitinib was the first dual JAK1/JAK2 TKI approved for the treatment of myelofibrosis and was demonstrated to reduce splenomegaly and improve many symptoms related to myelofibrosis but, unlike TKI treatment in chronic myeloid leukemia, it does not result in elimination of the mutant clonal population of cells (a phenomenon termed “disease persistence” in contrast to the conventional understanding of TKI resistance) nor does it cause widespread regression of fibrosis. Understanding the molecular basis of clinical responses to ruxolitinib is of great relevance to cancer biology and has implications for prescribing (and stopping) therapy, as well as the future design of kinase inhibitors for other cancers. Here we review what is known to date about the

mechanism of ruxolitinib persistence, its relationship to various recurrent somatic mutations in myelofibrosis, and potential ways to circumvent persistence to improve outcomes.

### Physiological JAK-STAT signaling

JAK family kinases are non-receptor tyrosine kinases that are crucial for signal transduction of many cytokines and growth factors. The family comprises four members: JAK1, JAK2, JAK3, and TYK2.<sup>9</sup> JAK family kinases are pre-associated with the cytoplasmic portion of their cognate receptors via their FERM and SH2 domains. Cytokine-induced receptor dimerization facilitates JAK kinase *trans*-activation and phosphorylation of tyrosine residues in the activation loop, as well as local phosphorylation of receptor cytoplasmic-tail tyrosine residues and tyrosine residues on associated signaling molecules. JAK2 contains a carboxy-terminal JAK homology domain, JH1, which has tyrosine kinase activity and transfers ATP to a protein substrate (such as STAT3 or STAT5), together with a JH2 pseudokinase domain, which is believed to regulate the activity of JH1.

Uncontrolled signaling by JAK2 is prevented by at least three major negative regulatory mechanisms. Activation loop phosphorylation of Tyr1007/1008 can be removed by tyrosine phosphatases including PTP1B, TC-PTP, SHP1, SHP2, CD45, and PTP-RT. Secondly, SOCS proteins (SOCS1-7 and CIS) are transcriptionally upregulated following receptor activation and provide negative feedback loops that restrict the duration of active signaling by either directly inhibiting JAK or by promoting the degradation of the associated cytokine receptor.<sup>10-13</sup> Thirdly, phosphorylated JAK2 (p-JAK2) is ubiquitinated by CBL-family E3 ubiquitin ligases,<sup>14</sup> as well as SOCS1, leading to proteasomal degradation, normally within minutes of receptor activation. Ubiquitination is itself a reversible process mediated by de-ubiquitinases, such as USP9X.<sup>15</sup>

### Molecular aspects of JAK2 and ruxolitinib

The commonest somatic mutation in myelofibrosis is JAK2 V617F, which is present in 50-60% of patients with primary myelofibrosis and post-essential thrombocythemia myelofibrosis, and in 95% of patients with myelofibrosis following polycythemia vera.<sup>16</sup> The V617F point mutation has been shown to disrupt the normal auto-inhibitory function of the JH2 domain leading to dysregulated activation of JAK-STAT signaling which, both in animal models and in patients, contributes to many of the cardinal manifestations of the disease. Other mutations that occur in MPN may activate a cytokine receptor (e.g., thrombopoietin receptor) or downstream signaling proteins, including NRAS, KRAS, and PTPN11. The homodimeric type 1 receptor for thrombopoietin regulates platelet formation and is encoded by *MPL*.

The exact molecular mechanism of JAK2 V617F, in comparison with the action of wild-type JAK2 and other kinases such as JAK1 or TYK2, is still being elucidated. The molecular signature of this mutation is that it induces JAK autophosphorylation (activation) in the absence of cytokine. *In vitro* the presence of a homodimeric cytokine receptor is necessary for this to occur.<sup>17,18</sup> Interestingly, the

JH2 domain of mutated JAK2 in myelofibrosis lacks an Asp in the His/Arg/Asp motif of its catalytic loop and possesses no definitive kinase activity, even though it is required for cytokine receptor activation. The JH2 domain has, however, been shown to phosphorylate two negative regulatory residues of JAK2 (Ser523 and Tyr570), which may contribute to the increased kinase activity.<sup>19</sup> A biochemical study showed that the orthologous mutation in JAK1 (JAK1 V658F, which has been found in patients with acute lymphoblastic leukemia and confers cytokine-independence in transduced Ba/F3 cells<sup>20,21</sup>) does not enhance the catalytic activity of the isolated kinase or indeed any other measurable enzymatic parameter.<sup>10</sup> These findings are all consistent with the hypothesis that the V617F mutation does not render JAK2 more “active” when switched on but rather leads to its being switched on in inappropriate circumstances, such as in the absence of cytokine. Consistent with this, it was shown recently that JAK2 V617F promotes cytokine-independent receptor dimerization, thereby activating the kinase in the absence of an appropriate signal.<sup>22</sup> Understanding the exact molecular effects of V617F will be important in the design of V617F-specific therapies that have lower activity against wild-type JAK2. Emerging *in vitro* and clinical data suggest that various JAK-STAT pathway-activating mutations (*JAK2*, *MPL*, *SH2B3*, *NRAS*, *KRAS*, *PTPN11*), as well as mutations in the protein scaffold *CALR*, all activate JAK-STAT signaling by subtly distinct mechanisms. This may have implications for treatment outcomes and suggests the existence of mutation-specific differences in ruxolitinib sensitivity and mechanisms of persistence.

More than 90% of cases of myelofibrosis show mutational evidence of JAK-STAT activation,<sup>16</sup> suggesting that this pathway is a critical “necessary” driver of the pathology. However, the converse is not true: having a mutation in the JAK-STAT pathway does not inevitably lead to myelofibrosis, i.e., JAK2 V617F or mutant *MPL* is not sufficient for the disease phenotype. This is perhaps best exemplified by polycythemia vera, in which the JAK2 V617F variant allele frequency is commonly in the range 50-100%,<sup>23</sup> yet progression to myelofibrosis occurs in only 20-30% of individuals. This risk is time-dependent and was 15% with a median follow-up of 8 years in one study.<sup>24</sup> Emerging studies comprehensively detailing the genetic landscape highlight that myelofibrosis is a multi-mutation disease in the majority of patients.<sup>16</sup> Importantly, mutations in epigenetic genes such as *EZH2*, *ASXL1* or splicing factors as well as the presence of specific inflammatory cytokines may link to fibrotic aspects of the pathology.<sup>25</sup> In a large cohort of MPN patients, Grinfeld and colleagues performed an analysis to identify mutations that were associated with myelofibrosis *versus* chronic phase MPN. Five of the six genes with the highest odds ratio for myelofibrosis were epigenetic regulators (*ZRSR2*, *U2AF1*, *SRSF2*, *EZH2*, and *ASXL1*) and all had an odds ratio higher than that for JAK2 at an allele burden >50%.<sup>16</sup> Although some of these mutations are likely acquired during disease evolution, there is also evidence that epigenetic changes early in MPN development may affect the phenotype. In a few patients with MPN who had mutations in both *TET2* (which regulates DNA methylation) and *JAK2*, the progenitor cells that appeared to have acquired *TET2* mutations first were shown to be less sensitive to ruxolitinib than progenitors from other patients in whom *JAK2* was acquired first.<sup>26</sup> Determining the epigenetic mechanisms that contribute to ruxolitinib persistence/

resistance is an exciting area of ongoing research that is beyond the scope of this review.

### Molecular aspects of CALR and ruxolitinib

Somatic mutations in exon 9 of the gene coding for the endoplasmic chaperone protein calreticulin (*CALR*) are found in 70-80% of patients with *JAK2*-negative myelofibrosis and account for ~30% of myelofibrosis cases overall.<sup>2,27</sup> Virtually all *CALR* mutations in MPN are small insertions or deletions clustered in exon 9, resulting in a +1 frameshift and loss of the last four amino acids (KDEL) that form the endoplasmic reticulum retention signal, leading to altered distribution of *CALR*. The two commonest mutations are a 52 bp deletion (type 1, which is present in 45-53% of patients) and a 5 bp insertion (type 2, present in 32-41% of patients). Although more than 50 *CALR* mutations have been described, the majority can be classified as type 1-like or type 2-like based on bioinformatic predictions of protein structure.<sup>28</sup> This functional classification is clinically relevant as it influences prognosis in myelofibrosis.<sup>29,30</sup> The reason for differing prognosis is unclear, but there are biological differences that might be relevant: type 1 mutations eliminate all the negative charge of the C-terminal domain eliminating its calcium binding, thus potentially activating proteases and protein misfolding in the endoplasmic reticulum,<sup>31</sup> and potentially altering the chaperone function of *CALR*.

Data from independent laboratories have shown that mutant *CALR* protein requires *MPL* for signaling and factor-independent cell growth, and that the normal lectin domain of *CALR* is essential to bind glycosylated sites on *MPL*.<sup>32-34</sup> More recently, it was reported that both mutant and wild-type *CALR* proteins are present at higher levels in the plasma of myelofibrosis patients (compared to normal individuals),<sup>35,36</sup> and may function in a paracrine fashion by binding the extracellular domains of *MPL* to facilitate receptor dimerization.<sup>34,36</sup> However, the relative contribution of autocrine *versus* paracrine *versus* endosomal signaling of mutant *CALR* protein to aberrant activation of the *JAK-STAT* pathway in MPN has not been fully elucidated (Figure 1).

As stated, activating mutations in the juxtamembrane domain of *MPL* are found in 5-10% of patients with myelofibrosis. Both *CALR* and *MPL* mutations presumably signal through *JAK2*, and there is evidence of therapeutic benefit from ruxolitinib in experimental models and in patients with these mutations. In these cases, the *JAK* inhibitor is binding wild-type *JAK2*, so there is limited selectivity of the TKI for MPN cells. Consistent with this, patients with *JAK2*-negative myelofibrosis may have slightly inferior clinical responses to ruxolitinib (discussed more fully below) but, because of the relatively small number of such patients in the COMFORT studies, additional studies are needed to confirm this.<sup>37</sup>

### Ruxolitinib is a “type I” ATP-competitive inhibitor

Historically, pan-*JAK* inhibitors (such as AG-490) were first developed as molecules that were substrate-competitive for tyrosine residues, and either mixed competitive or non-competitive for ATP. In recent years, drug devel-

opment has focused on ATP-competitive inhibitors, such as ruxolitinib. All clinically approved *JAK* inhibitors (ruxolitinib, fedratinib) bind and stabilize the kinase-active conformation of *JAK2*, and are known as “type I” inhibitors, in contrast to “type II” ATP-competitive inhibitors which bind and stabilize the protein in its inactive conformation. Exactly how these varying binding mechanisms play out in the clinic and their association with susceptibility to disease persistence are exciting areas of ongoing research.

*JAK2* V617F mutations are remarkable among recurrent oncogenic single nucleotide variants reported in the Catalogue of Somatic Mutations In Cancer (COSMIC) for having a consistent inflexible substitution, namely replacement of a small hydrophobic by a large hydrophobic residue outside the catalytic domain, generally implying a “change in function” rather than simply a loss or non-specific gain in function. Notably, the mutated JH2 pseudokinase domain does not bind ruxolitinib directly. This is in contrast to activating point mutations in *FLT3* (such as D835Y, D835H, F691L) or gate-keeper mutations

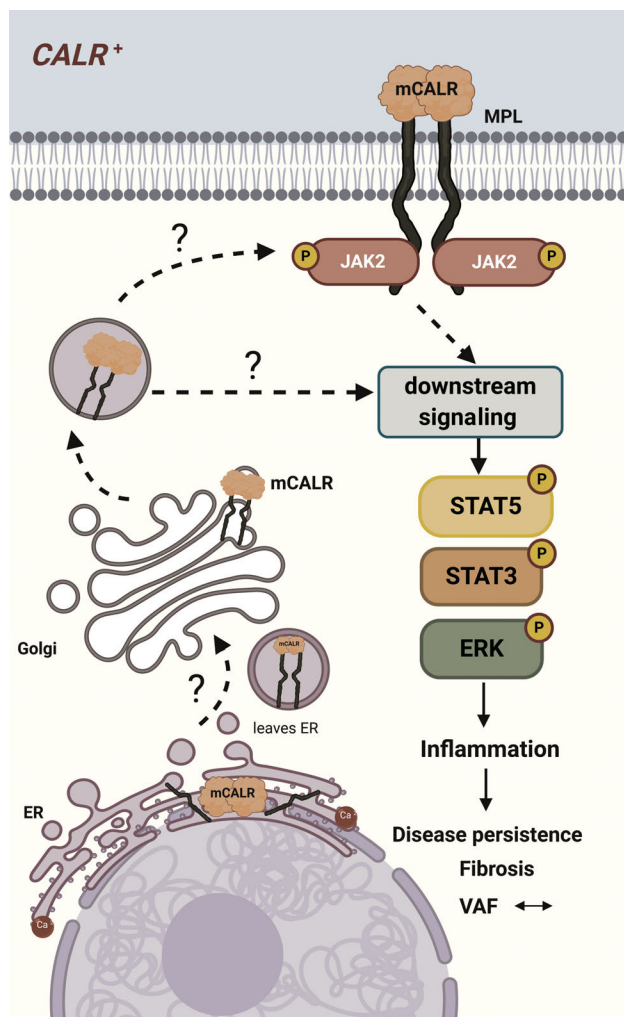


Figure 1. Activation of *JAK-STAT* signaling in *CALR*-mutant cells is inhibited by ruxolitinib. Schematic representation of mutant *CALR* (*mCALR*) trafficking in MPN cells showing inhibition by ruxolitinib. The exact mechanism(s) by which *mCALR* activates *JAK-STAT* signaling are still being elucidated. Mutant *CALR* leaves the endoplasmic reticulum and is associated with *MPL* to promote homodimerization and activation of *JAK2*.

found in *ABL1*, which involve a range of substitutions and directly alter binding of the drug with the enzyme.

### What are the long-term outcomes for patients treated with ruxolitinib?

The COMFORT-1 study compared ruxolitinib with placebo for the treatment of myelofibrosis,<sup>38</sup> and the COMFORT-2 study compared ruxolitinib with best available therapy, including supportive care, hydroxyurea, and a range of other medical therapies.<sup>39</sup> The primary endpoint in both studies was the proportion of patients achieving a 35% or greater reduction in spleen volume. The proportion of patients who achieved this response after 24 weeks of ruxolitinib treatment was 42% in COMFORT-1 and 32% in COMFORT-2.<sup>38,39</sup> The response rates in the respective comparator arms of the two studies were 0.7% and 0%.

In a subsequent analysis of a subset of patients from the COMFORT-2 study the response rate after 48 weeks was 20% (confidence interval: 5.7-43.7%) in 20 patients with a *CALR* mutation versus 34% (confidence interval: 24.8-44.1%) in 100 *CALR*-negative patients, most of whom had a *JAK2* V617F mutation.<sup>37</sup> Overall, there was no statistically significant difference in splenic response rate or survival between patients with and without *JAK2* mutations, but the *JAK2*-positive patients had a numerically greater reduction in spleen size.<sup>40</sup> *JAK2* V617F is almost universally present in post-polycythemia vera myelofibrosis, which is characterized by loss of heterozygosity and high allelic burden,<sup>41</sup> and can perhaps be considered to epitomize *JAK2*-driven myelofibrosis. In this subgroup the hazard ratio for overall survival was 0.25, compared with 0.65 in primary myelofibrosis.<sup>40</sup> Whether this reflects the higher frequency of high-risk genomic lesions in primary myelofibrosis or 'on-target' efficacy through inhibition of *JAK2* V617F remains to be established. Overall, these clinical data emphasize that ruxolitinib is not a *JAK2* mutant-specific therapy, although patients with *CALR* mutations might have mildly inferior responses.

### Does ruxolitinib eradicate cells containing disease-causing driver mutations?

Although ruxolitinib is now established as the standard therapy for symptomatic myelofibrosis, evidence of a long-term effect on disease biology is limited. The average reduction in *JAK2* allelic burden was 7-22% after 48 weeks of treatment in evaluable patients;<sup>38,42</sup> regression of fibrosis in the marrow was seen in around 16% of patients at last follow-up (median 2.2 years),<sup>43</sup> and there was no change in the risk of leukemic transformation. Cases of complete hematologic or molecular remission have been reported,<sup>44</sup> but are very uncommon. Typically there is a gradual loss of response over time, with approximately 27% of patients remaining on first-line ruxolitinib treatment after 5 years in the COMFORT studies. In the subgroup of patients who achieved a 35% or greater reduction in spleen volume, the median duration of the response was 3.2 years.<sup>43,45</sup>

The immunophenotype of myelofibrosis-initiating cells has not been well studied, principally because of a lack of robust engraftment models, but in a few patients analyzed carefully, the stem cell population appeared to reside within the CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup> compartment, as determined

using a humanized bone marrow niche.<sup>3</sup> Flow cytometry studies suggest a high level of circulating CD34<sup>+</sup>CD38<sup>-</sup>hematopoietic stem-like cells in patients with mutant *CALR*, but all these cells were resistant to ruxolitinib in colony assays. New models of myelofibrosis will help to characterize the disease-propagating stem-like cells and identify critical cell surface markers for monitoring future disease-modifying therapies.

### What are the possible mechanisms of ruxolitinib persistence?

A number of models have been proposed to explain ruxolitinib persistence, i.e., the ongoing outgrowth of mutated cells despite *JAK*-*STAT* pathway sensitivity to ruxolitinib, with varying levels of evidence. These include activation of alternative kinases not inhibited by ruxolitinib,<sup>46</sup> epigenetic mutations leading to growth advantage,<sup>26,47</sup> phosphatase negative feedback inhibition (as noted with the failure of *RAF*K inhibitors in melanoma),<sup>48</sup> lack of specificity for *JAK2* V617F versus wild-type *JAK2*,<sup>10</sup> and accumulation of p-*JAK2* during exposure to ruxolitinib. The last of these phenomena is the most studied, with multiple lines of evidence across several independent studies suggesting that it is a contributory mechanism in clinically observed ruxolitinib persistence.<sup>49-51</sup> Various therapeutic strategies have been proposed in addition to targeting *JAK2* (reviewed by Bankar and Gupta<sup>52</sup>). Here we focus on the evidence regarding persistent activation of *JAK*-*STAT* signaling and its downstream proteins, and how these phenomena may be targeted by current or emerging therapies.

### Why does phosphorylation of *JAK2* increase during ruxolitinib treatment?

Initial laboratory studies studying ruxolitinib activity *in vitro* noted that, paradoxically, phosphorylation of *JAK2* on Tyr1007/1008 located in the activation loop was observed at increased levels in V617F-positive cells following prolonged exposure to ruxolitinib.<sup>49</sup> Both the total level of phosphorylation and the total amount of *JAK2* protein were increased.<sup>49</sup> Similar observations were also made for the type I inhibitor "JAK inhibitor 1" and Go6976, a protein kinase C inhibitor with potent activity against *JAK2*.<sup>53,54</sup> Accumulation of p-*JAK2* was noted to occur in the presence of total blockade of kinase function and inhibition of *STAT* and *ERK* phosphorylation downstream (Figure 2). It was noted that ruxolitinib-induced phosphorylation of *JAK2*: (i) was staurosporine-sensitive and ATP-dependent; (ii) required cytokine receptor interaction and intact JH1, FERM and JH2 domains; and (iii) could occur in V617F<sup>+</sup> SET2 cells in the absence of *JAK1* and *TYK2*.<sup>50</sup> In contrast, *JAK2* wild-type cells (such as TF-1 cells) showed little or no type I inhibitor-induced loop phosphorylation after growth factor starvation. A carboxyterminal-directed antibody could not immunoprecipitate *JAK2* after exposure to type I inhibitor. All these findings are consistent with a conformational change in the kinase domain generated by ruxolitinib. Because of presumed structural flexibility, high resolution crystallography data are not available for the activation loop during type I inhibitor binding but, critically, activation of downstream *STAT* phosphorylation could be repro-

ducibly detected following removal of the drug. This implies that the accumulated p-JAK could act as a pathological signaling node as the drug level falls in patients if drug is abruptly stopped or a dose missed.

More recently, Tvorogov and co-authors extended these findings to show that accumulation of p-JAK2 is due to resistance of p-JAK2 to ubiquitination and degradation while bound to ruxolitinib.<sup>51</sup> Specifically, immunoprecipitation with an anti-p-JAK2 antibody produced a band for p-JAK2 in untreated cells, but not in ruxolitinib-treated cells, consistent with an altered conformation induced by the drug. Accordingly, it was noted that cells exhibiting delayed phosphorylation kinetics (i.e., those with wild-type JAK2) were significantly more sensitive to ruxolitinib cytotoxicity and growth inhibition than cells with rapid phosphorylation kinetics (cells with JAK2 V617F). This was an important observation that raised the possibility that ruxolitinib allows drug-bound JAK protein to escape the negative feedback loops of dephosphorylation and degradation (Figure 2B).

To test this, recombinant JAK2 kinase was treated with a phosphatase in the presence of ruxolitinib. Ruxolitinib

blocked the dephosphorylation by PTP1B for up to 20 h whereas reduction of Tyr1007/1008 phosphorylation normally occurred within 2 h.<sup>51</sup> In keeping with this, ruxolitinib prevented any detectable ubiquitination of JAK2 after cytokine stimulation. These results suggested that binding of ruxolitinib induces a conformational change that conceals Tyr1007/1008 from phosphatase access, and ruxolitinib-bound p-JAK2 is no longer susceptible to dephosphorylation or degradation.

Interestingly, primary *CALR*-mutant cells did not exhibit either JAK2 phosphorylation in the presence of ruxolitinib or striking withdrawal signaling to the same degree as the JAK2 V617F samples. *CALR*-mutant myelofibrosis cells showed undetectable levels of activated JAK2 Tyr1007/1008 phosphorylation in the presence of ruxolitinib and in some *CALR*<sup>+</sup> samples and in a *CALR*-mutated cell line (MARIMO) total JAK2 protein was difficult to detect, if not completely absent. This is consistent with a number of emerging reports using *CALR*-mutated models of myelofibrosis<sup>55-57</sup> suggesting fundamental differences in the nature of JAK activation in myelofibrosis patients with mutated *CALR* (Figure 1).

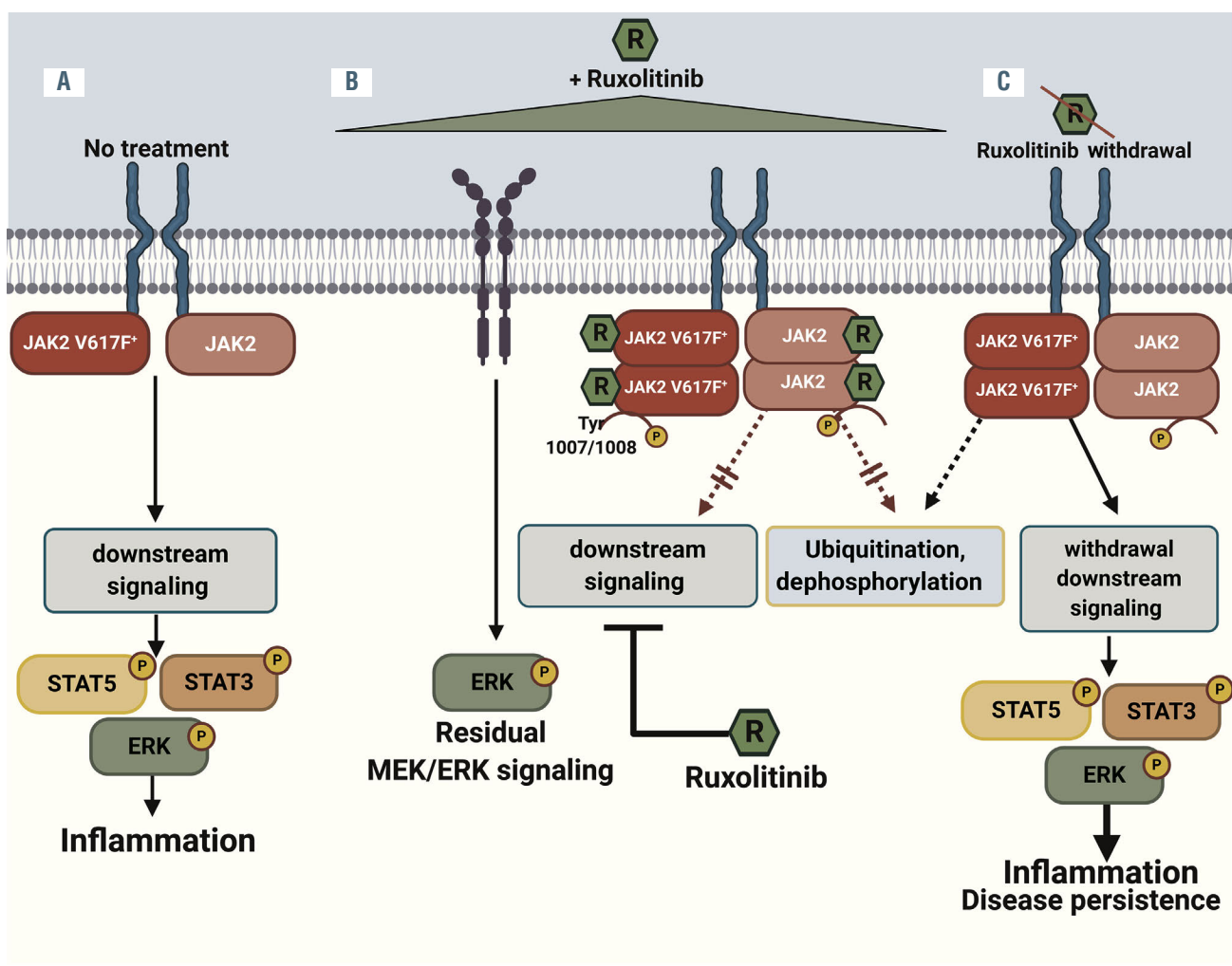


Figure 2. Signaling in JAK2 V617F cells before, during, and after discontinuation of ruxolitinib. Schematic representation of JAK-STAT activation in JAK2 V617F myelo-proliferative neoplastic cells. (A) JAK2 V617F leads to increased signaling through STAT5/STAT3/ERK leading to proliferation and inflammation. (B) In the presence of a type 1 JAK2 inhibitor, such as ruxolitinib, signaling through the JAK-STAT pathway is abrogated, but so too is ubiquitination and degradation of JAK2, leading to accumulation of p-JAK2. Signaling through MEK/ERK remains activated and contributes to disease persistence. (C) Ruxolitinib discontinuation leads to transiently increased signaling through the accumulated pool of p-JAK2 with inflammatory symptoms.

## Lessons from the clinic

### Ruxolitinib discontinuation syndrome

Interruption of ruxolitinib treatment typically leads to recrudescence of cytokine-mediated symptoms within a week,<sup>38</sup> often accompanied by increasing spleen size, highlighting the persistence of the underlying disease. The accumulation of p-JAK2 likely accounts for the prompt onset of withdrawal symptoms when ruxolitinib is stopped, since the increased p-JAK2 will rapidly lead to downstream signaling in the absence of the drug. Ruxolitinib has a short half-life of approximately 3 h,<sup>58</sup> so the drug is washed out rapidly if dosing is interrupted. Although the accumulation of p-JAK2 is likely to be a class effect of type 1 JAK inhibitors, we are not aware of any reports of discontinuation syndrome with fedratinib, momelotinib, or pacritinib. Both fedratinib and pacritinib have very long half-lives of more than 24 h.<sup>59,60</sup> Delayed clearance of these drugs may reduce the risk of withdrawal phenomena. Momelotinib has a half-life of 4–6 h,<sup>61</sup> similar to that of ruxolitinib, so withdrawal phenomena might be predicted to occur with abrupt discontinuation of momelotinib.

In most patients who discontinue ruxolitinib there is a return to near baseline severity of symptoms, but in rare cases there are life-threatening manifestations thought to be due to an exaggerated inflammatory response. Ruxolitinib discontinuation syndrome is a diagnosis of exclusion based on a temporal relationship between drug withdrawal and onset of clinical manifestations, which can appear from less than 24 h to up to 3 weeks after discontinuation. In the original phase I/II trial, three of 47 patients who discontinued ruxolitinib developed acute respiratory distress syndrome.<sup>62</sup> In the phase III COMFORT-I study, one patient developed acute respiratory distress, pyrexia and splenic infarction following ruxolitinib discontinuation.<sup>38</sup> Coltro and co-workers described a case of JAK2 V617F essential thrombocythemia evolving to acute myeloid leukemia with acute respiratory distress syndrome developing after ruxolitinib was discontinued, and improving within 48 h of re-introduction of the drug.<sup>63</sup> Other reports include a patient who developed tumor lysis-like syndrome,<sup>64</sup> a case of acute respiratory failure,<sup>65</sup> and a case of acute respiratory distress syndrome that twice resolved after ruxolitinib re-introduction.<sup>66</sup>

Of note, of the nine published cases of life-threatening ruxolitinib discontinuation syndrome reported in the literature, all were reported to have a JAK2 mutation. This is a disproportionate enrichment compared to the prevalence of the CALR mutation (~35% of cases of myelofibrosis). Further research into mutation-specific side effects of kinase inhibitors is warranted.

Some experts recommend tapering the ruxolitinib dose rather than abrupt discontinuation, especially when the reason for the therapy interruption is an adverse event other than cytopenia.<sup>67,68</sup> One of the commonest causes of ruxolitinib discontinuation is infection.<sup>69</sup> By stabilizing the active conformation of JAK2 in a manner that prevents dephosphorylation and degradation, type I inhibitors may rarely induce a signaling state that heuristically resembles a cytokine storm (Figure 2C).<sup>51</sup> Experimental co-culture of JAK2 V617F cells with increased levels of cytokines in the medium promoted the accumulation of p-JAK2, since signaling through cytokine receptors is still capable of inducing p-JAK2 in the presence of ruxolitinib. Intriguingly, acti-

vation of JAK1 by interleukin-3 also led to phosphorylation of JAK2, suggesting that both JAK1 and JAK2 may be involved in withdrawal signaling.<sup>51</sup> These findings provide a biological rationale for why inflammation and discontinuation of ruxolitinib could interact to cause more severe withdrawal phenomena.

### Ruxolitinib wash-out and rechallenge

A number of cases have been reported in which a 'drug holiday' led to restoration of ruxolitinib response upon rechallenge,<sup>70,71</sup> as predicted by the experimental models of ruxolitinib persistence and wash-out. When ruxolitinib is withdrawn the accumulated p-JAK2 becomes susceptible again to ubiquitination and degradation, so that after a wash-out period the untreated cell biology is restored for a variable period of time. Understanding this phenomenon has important implications for the design of clinical trials of second-line agents after ruxolitinib. It is often in the patient's interest to minimize the interval between stopping ruxolitinib and starting a second-line agent, because of the increase in symptom burden that commonly occurs. However, if baseline response assessments for the second-line agent are undertaken too early there may be further deterioration after the 'baseline' assessment. In the randomized FREEDOM-2 study, wash-out and resumption of ruxolitinib will be compared to wash-out of ruxolitinib followed by institution of fedratinib (NCT03952039). This study will provide important new data on the effects of ruxolitinib rechallenge.

### Inhibition of JAK1 versus JAK2 versus both

Many of the symptoms of myelofibrosis are related to increased levels of pro-inflammatory cytokines, including interleukin-6 and tumor necrosis factor- $\alpha$ . Symptomatic improvement in response to ruxolitinib is correlated with a reduction in cytokine levels.<sup>72</sup> Importantly, many cytokines signal through JAK1 and suppression of JAK1 signaling by dual inhibitors, such as ruxolitinib and momelotinib, may contribute to the symptomatic benefit observed with these treatments. A selective JAK1 inhibitor, itacitinib (INCB039110), has been tested in a phase II clinical trial involving 87 patients with myelofibrosis.<sup>73</sup> Symptomatic improvement was comparable to that seen with ruxolitinib (total symptom score reduced by 50% at 24 weeks in 49% of patients *versus* 46% in the COMFORT-I study) whereas the splenic responses were less ( $\geq 35\%$  reduction in spleen volume in 17% of patients at 24 weeks *versus* 32–42% in the COMFORT studies). Conversely, a 50% reduction in total symptom score was seen in 36% of patients treated with the JAK2-selective inhibitor, fedratinib.<sup>74</sup>

It may be an oversimplification that JAK1 inhibition mediates improvement in inflammatory symptoms and JAK2 inhibition mediates cytoreductive effects. There may be some crosstalk between JAK1 and JAK2, or splenic responses could perhaps be due to inhibition of cytokine-mediated recruitment of cells to sites of extramedullary hematopoiesis.

### Secondary resistance to ruxolitinib: comparison with chronic myeloid leukemia

Secondary resistance to TKI therapy is perhaps best understood in chronic myeloid leukemia. Resistance often

occurs relatively abruptly and leads to near-complete loss of therapeutic benefit, typically over a number of months. In contrast, loss of response to ruxolitinib in myelofibrosis is characterized by a gradual waning of the initial therapeutic benefit, sometimes associated with late-emerging cytopenia that is likely to represent progression of the underlying disease rather than direct toxicity of the TKI.

In chronic myeloid leukemia, around half of cases of secondary resistance are associated with the emergence of single amino acid changes in the BCR-ABL1 kinase domain which impair binding of the TKI or stabilize the active conformation of the protein.<sup>75</sup> Ruxolitinib-resistant kinase domain mutations have been isolated using *in vitro* mutagenesis screening, and such mutations have also been observed clinically in patients with acute lymphoblastic leukemia treated with ruxolitinib.<sup>76</sup> However, fewer than 50% of resistant clones after saturation mutagenesis in the presence of ruxolitinib harbor additional mutations in JAK2.<sup>49</sup> Fedratinib, another ATP-competitive JAK2 inhibitor in clinical use, appears to be less susceptible to this pattern of resistance because it inhibits not only ATP binding, but also the binding of peptide substrates to JAK2.<sup>77</sup> However, JAK2 kinase domain mutations have not yet been observed in clinical samples from myelofibrosis patients treated with JAK inhibitors, which could indicate either incomplete inhibition of JAK2 or that myelofibrosis is not critically dependent on the kinase activity of JAK2 in the way that chronic myeloid leukemia is addicted to a fusion oncogene.

### Strategies to improve inhibition of JAK-STAT signaling

Here we discuss various approaches that might be employed to achieve more complete inhibition of JAK-STAT signaling (summarized in Table 1) with the aim of improving treatment response, and ultimately finding

treatments with greater disease-modifying potential. Type 2 kinase inhibitors, such as the BCR-ABL1 inhibitor, imatinib, are ATP-competitive inhibitors that bind the inactive conformation of the kinase. All of the JAK2 inhibitors that have been tested clinically to date for myelofibrosis are type 1 inhibitors. Since JAK2 V617F is a weak activating mutation (relative to constitutive activation of fusion kinases, such as BCR-ABL1 and PCM1-JAK2) it does not strongly favor the active conformation. In contrast, the KIT D816V mutation that is commonly found in systemic mastocytosis leads to a bias in favor of the active conformation of the stem cell factor receptor (which KIT encodes). Midostaurin and avapritinib (both type 1 inhibitors) have significant clinical activity against KIT D816V: they show greater potency against KIT D816V than against wild-type KIT, whereas the converse is true for type 2 inhibitors, such as imatinib and sunitinib.<sup>78</sup> An experimental type 2 JAK inhibitor, CHZ868, did not result in accumulation of p-JAK2 and led to more potent suppression of myelofibrosis cells.<sup>49,51</sup> This compound is not being developed for clinical use, but these pre-clinical data suggest that the development of type 2 JAK inhibitors for clinical use might offer advantages over the available TKI for myelofibrosis. A potential risk of this approach is greater suppression of normal hematopoiesis.

The JH2 pseudokinase domain of JAK2 binds ATP, and mutation of certain residues abrogates ATP binding, as well as leading to loss of the activated JH1 tyrosine kinase activity in V617F.<sup>79</sup> In a mouse model of JAK2 V617F MPN, co-mutation with K581A prevented the development of polycythemia. Notably, mutation of the same residue had little or no effect on cytokine signaling through wild-type JAK2, potentially opening the opportunity for the development of mutation-specific allosteric inhibitors that target this site.

JAK2 is a client protein of Hsp90, and inhibition of Hsp90 leads to degradation of JAK2 (including p-JAK2). Combining ruxolitinib with an Hsp90 inhibitor was more

**Table 1. Possible strategies to inhibit JAK2 and downstream signaling in myeloproliferative neoplasms.**

	Mechanism of action	Examples	Notes
<b>Current standard of care</b>			
Type 1 inhibitors	ATP-competitive inhibitors that bind the active conformation of JAK2	Ruxolitinib Fedratinib Momelotinib Pacritinib	Lead to accumulation of p-JAK2 Limited disease-modifying potential
<b>Alternative JAK inhibitors</b>			
Type 2 inhibitors	ATP-competitive inhibitors that bind the active conformation of JAK2	CHZ868 BBT594	Do not lead to accumulation of p-JAK2
Allosteric inhibitors	Non-ATP-competitive inhibitors of the pseudokinase domain or substrate binding	LS104	Could confer greater specificity for JAK2 V617F over wild-type JAK2
<b>Drugs to degrade JAK2</b>			
Hsp90 inhibitors	Inhibit chaperone function to expose JAK2 to degradation	AUY922	Clinical development discontinued because of ocular toxicity
De-ubiquitinase inhibitors	Small molecules that promote ubiquitination without specificity for JAK2	WP1130	Could reduce accumulation of p-JAK2 in combination with a type 1 JAK2 inhibitor
Proteolysis-activating chimeras (PROTAC)	Designed to target specific proteins to degrade	-	Could reduce accumulation of p-JAK2 in combination with a type 1 JAK2 inhibitor
<b>Inhibitors of downstream signaling</b>			
MEK/ERK inhibitors	Inhibitors of bypass signaling in the presence of JAK inhibitor	Trametinib Binimetinib Ulixertinib	Synergistic with type 1 JAK2 inhibitor in experimental models Not tested clinically in MPN

Approaches that have been used clinically (in any disease) are shown in bold.

effective than ruxolitinib monotherapy in a mouse model of myelofibrosis.<sup>60</sup> A clinical trial using the Hsp90 inhibitor AUY922 was terminated prematurely because of safety concerns, despite evidence of splenic responses.<sup>61</sup> Other studies using the same drug documented a high frequency of visual alterations, including night blindness.<sup>62</sup> An alternative approach to target the accumulation of p-JAK2 is to use a proteolysis-targeting chimera (PROTAC). These small-molecule drugs contain two functional domains: one binds the target protein and the second engages an E3 ubiquitin ligase, thereby triggering proteasomal degradation of the target protein. JAK family PROTAC have been developed that recruit inhibitor of apoptosis protein leading to proteasomal degradation.<sup>63</sup> An inhibitor of the de-ubiquitinase, USP9X, has been shown to accelerate the degradation of JAK2 *in vitro*.<sup>64</sup> Whether any of these approaches could selectively target pathological accumulation of p-JAK2 in patients with myelofibrosis remains to be tested.

One of the pathways activated downstream of JAK2 in MPN is RAS/RAF/MEK/ERK. Ruxolitinib treatment of a cell line expressing JAK2 V617F inhibited downstream phosphorylated ERK1 and ERK2 (p-ERK1/2), but ruxolitinib treatment of a V617F mouse MPN model did not, despite effective suppression of other proteins downstream of JAK2.<sup>46</sup> Persistence of MEK/ERK signaling *in vivo* was found to be due to activation of PDGFRA by PDGF-BB signaling as a bypass pathway that enables persistence of MPN cells despite effective inhibition of JAK2 kinase activity. Combined inhibition of JAK2 and MEK with ruxolitinib and binimetinib (an approved treatment for RAF-mutant melanoma) was more efficient than either TKI alone in achieving regression of splenomegaly, fibrosis and JAK2 V617F allele burden.<sup>46</sup>

## Conclusion

Despite the symptomatic benefits of currently available JAK2 inhibitors, there is a need for agents that have a greater effect on the disease clone and its natural history. JAK2 remains a crucial target in myelofibrosis, and deletion of JAK2 in a mouse model of myelofibrosis substan-

tially abrogates disease manifestations. Data from several groups have shown that a conformational change in JAK2 induced by the binding of ruxolitinib and other type 1 inhibitors may contribute to disease persistence by preventing JAK2 de-phosphorylation and proteasomal degradation, allowing heterodimerization of JAK2 with JAK1 or TYK2. Type 2 inhibitors of JAK2 do not cause this phenomenon and have greater impact on disease biology in a mouse model. The V617F mutation leads to a highly inflexible substitution, which may be indicative of a neomorphic change in function, rather than non-specific over-activation. Several lines of evidence suggest that it may be possible to exploit the unique properties of the mutant pseudokinase to develop mutation-specific JAK2 inhibitors that spare normal hematopoiesis. Whereas current clinical trials are mostly targeting cooperating pathways or pathways downstream of JAK2, these observations suggest that novel approaches to the targeting of JAK2 could lead to substantial benefits for patients with myelofibrosis.

## Disclosures

*DMR has received research funding and honoraria from Novartis and BMS/Celgene unrelated to the current work. JJB, DTv and DTh declare that they have no conflicts of interest.*

## Contributions

*DMR and DTh reviewed the literature and wrote the paper. JJB and DTv critically reviewed the content and wrote the paper.*

## Acknowledgments

*We thank Suraiya Onnesha for assistance with the figures.*

## Funding

*Research support for DMR was provided through the Medical Research Future Fund and The Hospital Research Foundation. Research support for JJB was provided by the National Health and Medical Research Council (APP1113577; APP1121755; APP1122999). Research support for DTh was provided through the National Health and Medical Research Council (APP1182564), Medical Research Future Fund, The Hospital Research Foundation and a CSL Centenary Fellowship.*

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