A novel activating, germline JAK2 mutation, JAK2R564Q, causes familial essential thrombocytosis

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KEY POINTS

- JAK2R_{564}Q is the first germline JAK2 mutation found to contribute to a familial MPN that involves a residue other than V_{617}.

- The kinase activity of JAK2R_{564}Q and JAK2V_{617}F are the same, but only V_{617}F is able to escape regulation by SOCS3 and p27.
ABSTRACT

Along with the most common mutation, JAK2V_{617}F, several other acquired JAK2 mutations have now been shown to contribute to the pathogenesis of myeloproliferative neoplasms (MPNs). However, here we describe for the first time a germline mutation that leads to familial thrombocytosis that involves a residue other than Val_{617}. The novel mutation JAK2R_{564}Q, identified in a family with autosomal dominant essential thrombocythemia (ET), increased cell growth due to suppression of apoptosis in Ba/F3-MPL cells. Although JAK2R_{564}Q and JAK2V_{617}F have similar levels of increased kinase activity, the growth-promoting effects of JAK2R_{564}Q are much milder than those of JAK2V_{617}F, due to at least two counter-regulatory mechanisms. Whereas JAK2V_{617}F can escape regulation by SOCS3 and p27/Kip1, JAK2R_{564}Q-expressing cells cannot. Moreover, JAK2R_{564}Q-expressing cells are much more sensitive to the JAK inhibitor, ruxolitinib, than JAK2V_{617}F-expressers, suggesting that lower doses of this drug may be effective in treating patients with MPNs associated with alternative JAK2 mutations, allowing many undesirable side effects to be avoided. This work provides a greater understanding of the cellular effects of a non-JAK2V_{617}F, MPN-associated JAK2 mutation, provides insights into new treatment strategies for such patients, and describes the first case of familial thrombosis caused by a JAK2 residue other than Val_{617}. 
INTRODUCTION

The discovery that the acquired JAK2 mutation, $JAK2^{V617F}$, contributes to the Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) significantly advanced our understanding of these diseases\textsuperscript{1-3}. We now know that this mutation in the pseudokinase domain of $JAK2$ contributes to the origins of about half of all patients with acquired essential thrombocythemia (ET) and primary myelofibrosis (PMF) and nearly all patients with acquired polycythemia vera (PV)\textsuperscript{4-6}. More recently, other mutations, deletions or insertions in $JAK2^{7,9}$, $MPL^{10,11}$, $KIT^{12}$, $TET2^{13,14}$ and $SH2B3$, which encodes LNK\textsuperscript{15} have all been shown to contribute to the development of MPNs.

The JAK2 protein is comprised of a FERM (4-point, Erzin, Radixin, Moesin) domain at the N-terminus, followed by a SRC homology 2 (SH2)-like domain, a JAK homology-2 (JH2) pseudokinase domain and a JH1 active tyrosine kinase domain (Figure 1E). Occurring in the pseudokinase domain, the crystal structure of which has recently been published\textsuperscript{16}, the $JAK2^{V617F}$ mutation is commonly thought to interrupt autoinhibitory interactions that would normally facilitate inhibition of the JH1 kinase activity by the JH2 pseudokinase domain. This acquired somatic mutation occurs at the level of the hematopoietic stem cell, giving rise to lineage-specific cells that are hypersensitive to cytokine stimulation. Several mechanisms have been reported to be responsible for mediating these effects. $JAK2^{V617F}$ down-regulates p27/Kip1, a cell cycle inhibitor at the G1 to S transition. Direct phosphorylation of p27/Kip1 by $JAK2^{V617F}$ impairs its ability to inhibit the growth promoting cell cycle kinase Cdk, and marks it for proteasomal degradation\textsuperscript{17}. Activation of STAT5 by $JAK2^{V617F}$ also leads to increased transcription of $Skp2$, a subunit of ubiquitin E3 ligase, which further promotes p27/Kip1 degradation\textsuperscript{18,19}. Moreover, the normal control on over-exuberant cell growth, mediated by the suppressor of cytokine signaling 3 (SOCS3), that acts to regulate JAK2 activity, is also abrogated by $JAK2^{V617F}$ in BaF/3 cells\textsuperscript{20}. SOCS3 expression, induced several hours after the
onset of JAK/STAT signaling, inhibits JAK2 activity, either through direct binding to the JH1 catalytic loop, or through generation of an E3 ligase that ubiquitinates JAK2 and targets it for degradation. However, in BaF/3 cells, SOCS3 is unable to regulate JAK2V617F, and paradoxically enhances its activity20. This effect may be context-dependent though, since it has also been reported that SOCS3 can inhibit JAK2V617F signaling through proteasomal degradation in HEK cells21.

Although JAK2V617F in exon 14 is the most common mutation of JAK2 associated with MPNs, insertion/deletion events in JAK2 exon 12 are also known contributors8,9. A screening of blood samples from suspected MPN patients revealed further point mutations in exons 12-157, although functional studies to confirm their contribution to disease pathogenesis have yet not been performed for many of these mutant kinases. Thus far, nearly every previously identified JAK2 alteration, found in patients with MPNs, including JAK2V617F, are somatic, acquired mutations. The single exception is JAK2V617I, recently proposed as a germline mutation associated with hereditary thrombocytosis22,23. Here we describe for the first time a JAK2 mutation associated with a familial MPN that involves a residue other than Val617. This novel mutation in exon 13 of JAK2 is a single nucleotide substitution, g1691a, which results in an arginine to glutamine change at position 564 (JAK2R564Q). This mutation, present in three out of four of the studied family members, is associated with hereditary thrombocytosis and increased JAK2 activation in platelets. We have further shown that in cell lines the mutation leads to JAK2 hypersensitivity and increased cell growth due to suppression of apoptosis.

Despite being localized in the same pseudokinase domain as V617, and generating similar levels of increased JAK2 activity, the effects of JAK2R564Q are distinct from those of JAK2V617F. Like the wild-type kinase, proliferation of JAK2R564Q-Ba/F3-MPL cells is regulated by the cell cycle inhibitor, p27/Kip1, whereas JAK2V617F is able to decrease p27/Kip1 levels and escape this regulation. Furthermore, SOCS3 negatively regulates JAK2R564Q, as for wild-type kinase, but is
unable to inhibit JAK2V_{617}F. Finally, we show that the JAK inhibitor, ruxolitinib, is able to effectively reduce the cell growth associated with JAK2R_{564}Q-expressing cells, and much lower concentrations are required than those needed to generate the same effect in JAK2V_{617}F-expressing cells. This work describes the first reported case of familial thrombocytosis caused by a JAK2 mutation other than at position V_{617}, provides valuable insights into the cellular effects of alternative MPN-associated JAK2 mutations, and has clear clinical implications for the treatment of the MPNs arising in mutations other than JAK2V_{617}F.
MATERIALS AND METHODS

Patients and Samples

Written informed consent was obtained from patients in accordance with the Declaration of Helsinki and with approval from the Stony Brook University ethics committee. XCIP experiments were performed on patient myeloid cells according to\textsuperscript{24} and analyzed on 3% agarose gels. Platelet lysate collection and all other methods are described in supplemental data.
RESULTS

Case presentation and generation of $JAK2_{R564Q}$ cell lines

A 6 year old male with a prolonged, elevated platelet count (800-1300 $x10^3/\mu L$, monitored over three years), negative for secondary thrombocytosis such as iron deficiency and inflammatory diseases, was diagnosed with ET (C1, Table 1). A blood smear further confirmed increased platelet number (Figure 1A). Evaluation of the patient for MPN revealed a normal BCR-ABL PCR test, a normal karyotype, 46XY, and assessments for $JAK2_{V617F}$, $MPL_{W515}$ and $S_{505}$ mutations were negative. However, sequence analysis revealed a novel $JAK2$ G-to-A mutation at nucleotide 1691 in exon 13 (Figure 1B), resulting in an amino acid substitution of arginine to glutamine at position 564 ($JAK2_{R564Q}$, Figure 1C). In addition, the patient’s sister (C2) and mother (C3) were also thrombocytotic (500-600 $x10^3/\mu L$ platelet counts) and both tested positive for the $JAK2_{R564Q}$ mutation (Figure 1D, Table 1). The father (C4), however, had platelet counts in the normal range and was negative for $JAK2_{R564Q}$.

XCIP analysis was performed on myeloid cells from the two $JAK2_{R564Q}$-positive female patients (C2 and C3) in order to assess clonality in hematopoietic cells. A polyclonal pattern with 57% expression of one allele was seen in C2, indicating that the $JAK2_{R564Q}$ mutation may be sufficient to drive the thrombocytosis, without the acquisition of additional somatic mutations. C3 showed 90% expression of the predominant allele, suggesting myeloid clonality and the possibility that an additional somatic mutation may be contributing to the mild thrombocytosis of this patient. Given the limitations of the assay, and without determining clonality in T-cell controls, it is difficult to accurately interpret this imbalanced pattern.

$R_{564}$ is very highly conserved across species (Figure 1E). It is located in the JH2 pseudokinase domain (Figure 1F), along with the well-characterized $JAK2_{V617F}$ mutation. In order to determine the functional consequences of $JAK2_{R564Q}$ and compare them to those of $JAK2_{V617F}$, we
generated 4 cell lines expressing WT and mutated forms of human JAK2 (Figure 2A). We previously generated a Ba/F3 cell line, which stably expresses the human TPO receptor c-MPL (Ba/F3-MPL)\textsuperscript{26}. A single Ba/F3-MPL clone was further transfected with one of four forms of JAK2: WT JAK2, JAK2\textsubscript{R564Q}, JAK2\textsubscript{V617F} and finally a double mutant, JAK2\textsubscript{R564Q} and V\textsubscript{617F}, to generate stably-expressing pools. The double mutant was studied alongside the two single mutants to test whether the effects of the mutations were additive, in order to determine whether distinct or similar mechanisms are utilized by each mutant. Since no commercially available antibodies can distinguish between human and murine JAK2, expression of human JAK2 mRNA was verified by real time PCR using human specific primers. Levels of human JAK2 cDNA, normalized by endogenous mouse Jak2 cDNA levels, were quantified and no significant difference was observed between the four cell lines (Figure 2B). Similar levels of both total JAK2 and MPL protein were determined in all four cell lines by western blot analysis (Figure 2C). Cell surface expression of MPL was also measured by flow cytometry (Figure 2D) and no significant difference in cell surface MPL expression was found between the cell lines (Figure 2E).

**Expression of JAK2\textsubscript{R564Q} causes increased intracellular signaling**

To begin to understand the contribution of JAK2\textsubscript{R564Q} to the pathogenesis of familial thrombocytosis, we determined the effects of the mutation on JAK2 activity and signaling. The kinase activity of JAK2, JAK2\textsubscript{R564Q}, JAK2\textsubscript{V617F} and the combined mutant kinase were assessed using an \textit{in vitro} kinase assay (Figure 3A). All three mutant forms of JAK2 showed a similar, approximate 3 fold, increase in activity compared to WT. However, there were no significant differences in kinase activity between each of the JAK2 mutants. To confirm the ability of the mutant JAK2s to phosphorylate MPL, cell lysates were immunoprecipitated with an anti-MPL antibody, before probing with an anti-phospho-Tyrosine antibody (Figure 3B). In the absence of TPO, no phosphorylated MPL was observed in WT JAK2 cells, although MPL was
phosphorylated in JAK2R564Q, JAK2V617F and the double mutant cells. As expected from the kinase activity results, we confirmed an increase in the levels of the positive regulator, phosphorylated JAK2Tyr1007/8 in both JAK2R564Q- and JAK2V617F-expressing mutants compared to WT in the absence of, and at 1ng/ml and 10ng/ml TPO (Figure 3C). However, we also observed increased phosphorylation of the negative regulatory site JAK2Tyr570, suggesting a general global increase in JAK2 tyrosine phosphorylation. Phosphorylated levels of the downstream signaling proteins STAT1, 3 and 5 were elevated in each of the mutant JAK2 cell lines, especially in the absence of TPO (Figure 3C). We also found higher levels of pSTAT1 and pSTAT3 in cells expressing JAK2V617F, compared to JAK2R564Q. Total STAT1 levels were also increased with JAK2R564Q expression, compared to WTJAK2 and this effect was even more prominent with JAK2V617F. An overall increase in downstream signaling in mutant JAK2 cells under starved conditions was further demonstrated (Figure 3D). Tyrosine-phosphorylation of proteins was also up-regulated in JAK2R564Q-expressing cells, compared to WTJAK2, and this was even more robust in the JAK2V617F-expressing mutants (Figure 3D). Furthermore, similar increased signaling was observed in JAK2R564Q-positive patients (Figure 3E). Platelets were isolated from 3 members of the family with the JAK2R564Q mutation and subject to western blot analysis. Phosphorylation of JAK2 was increased in the JAK2R564Q-positive family members (R564Q#1 and #2), compared to the father, who is negative for the mutation (WT).

**JAK2R564Q-expressing cells exhibit reduced cell growth compared to JAK2V617F-expressing cells in the absence of cytokine**

The growth characteristics of the JAK2-expressing cell lines in response to TPO-treatment were then determined using MTT assays. Cells expressing JAK2V617F, either with or without JAK2R564Q, were factor independent and proliferation was significantly increased from WTJAK2-expressing cells in the absence of, and at all concentrations of TPO (Figure 4A). JAK2R564Q-expressing cells also showed significantly increased proliferation, compared to
WTJAK2 cells, although cell proliferation was much less striking than with JAK2V617F, and was still responsive to cytokine stimulation (Figure 4A). We hypothesized that the mild hyperproliferative phenotype of JAK2R564Q cells compared to WT controls, was possibly due to a decrease in apoptosis in the absence of, or at low concentrations of TPO.

To test this hypothesis, cells were grown in the absence of cytokine and viable cells were counted every 24hrs (Figure 4B). Viable cell number dropped significantly even after only 24hrs in WTJAK2-expressing cells, whereas a significant increase was seen in the number of cells expressing JAK2V617F, confirming their factor independence. However, the JAK2R564Q-expressing cells exhibited no significant change in cell number over the 72 hour period. These data indicate that while the JAK2R564Q mutation may not stimulate cell proliferation in the absence of cytokine, it inhibits apoptosis.

To confirm this finding, following cytokine starvation we determined the percentage of apoptotic cells every 24hrs by annexin V staining (Figure 4C). The vast majority of WT cells were apoptotic by 48hrs of starvation (approximately 70%), whereas the number of apoptotic cells in JAK2V617F-expressing cell lines was significantly less (approximately 35%). Supporting our previous results, apoptosis was attenuated in JAK2R564Q-positive cells (approximately 42% after 48hrs). Concurrent with these findings, higher levels of uncleaved PARP with prolonged starvation was also observed in cells expressing JAK2R564Q, compared to WTJAK2, by western blot (Figure 4D). However, levels of uncleaved PARP were even higher in the JAK2V617F and double mutants.

Next, we determined whether any of the cell lines were able to proliferate in the absence of cytokine using BrdU assays (Figure 4E). JAK2R564Q exhibited only a modest increase in the number of cycling cells compared to control and only after 48hrs. Cell lines with the JAK2V617F mutation, however, continued to actively proliferate for the entire 48hrs, with only a slight
decrease compared to cells grown with cytokine. Expression of cell cycle regulators was further examined (Figure 4F). Levels of p27/Kip1, the negative regulator of the G1 to S phase transition, increased in both WTJAK2 and JAK2R564Q-expressing cells with prolonged starvation. However, p27/Kip1 levels remained low in the JAK2V617F-expressing cells throughout the 36hr period, suggesting a mechanism by which proliferation might be enhanced in these cells, compared to those expressing JAK2R564Q. p21/CIP/WAF1 levels remained constant in WTJAK2 and JAK2R564Q-expressing cells throughout the 36hr period, although they were increased after 12hrs in the JAK2V617F-expressing cells. As p21 is also a cyclin-dependent kinase inhibitor, it may be involved in negative feedback to inhibit JAK2V617F cell proliferation. However, if the effects of p21 are less potent than those of p27, this may explain the differences in proliferation. Therefore, we concluded that the differences in cell cycle behavior between JAK2V617F- and JAK2R564Q-expressing cells were accounted for by p27 and not p21. Taken together, this data strongly suggests that the JAK2R564Q mutation is able to inhibit apoptosis in the absence of cytokines, but does not stimulate proliferation.

To determine whether JAK2R564Q can also signal through EPOR, rather than MPL, we generated a Ba/F3 cell line that stably expresses the human EPOR (Ba/F3-EPOR), then further transfected a single Ba/F3-EPOR clone with WTJAK2, JAK2R564Q or JAK2V617F. Each of the cell lines showed similar levels of both total JAK2 and EPOR (supplemental Figure 1A). As shown by others, JAK2V617F-expression was still able to increase cell growth in Ba/F3-EPOR cells, compared to WTJAK2 (supplemental Figure 1B). However, JAK2R564Q expression had no effect on cell growth, compared to WTJAK2 despite causing a modest increase in the phosphorylation of signaling proteins (supplemental Figure 1C). Therefore, it appears that JAK2R564Q can only exert its proliferation-enhancing effects by signaling through MPL, and not EPOR.
**JAK2R564Q is negatively regulated by SOCS3 overexpression**

SOCS proteins negatively regulate JAK2 activity, either through direct inhibition, or by stimulating the ubiquitination and subsequent degradation of the kinase. To explore whether SOCS proteins might also account for the differences in cell proliferation between JAK2V617F and JAK2R564Q in BaF-MPL cells, we next determined the endogenous levels of SOCS1 and 3 in the JAK2-expressing cell lines by western blot analysis (Figure 5A). Although SOCS1 expression was largely unaltered between WT and the mutant JAK2-expressing cell lines, SOCS3 levels were increased in all cell lines expressing mutant JAK2, compared to WT JAK2, both with and without TPO stimulation. Therefore, we next investigated the effect of SOCS3 overexpression in these cells (Figure 5B). In JAK2R564Q-expressing cells, the increased concentration of SOCS3 was associated with a decrease in pJAK2. However, this negative feedback was not observed in the JAK2V617F-expressing cells, where pJAK2 levels remained unaltered. Furthermore, we observed more ubiquitinated JAK2 in starved JAK2R564Q-expressing cells compared to starved WTJAK2-expressing cells, as there are more cells still proliferating in the absence of cytokine, and therefore presumably more stimulation of the negative feedback mechanism to ubiquitin JAK2 and target it for proteasomal degradation (Figure 5C). Conversely, although JAK2V617F-expressing cells also proliferate more robustly than WTJAK2-expressing cells, in the absence of cytokine, ubiquitination levels of JAK2 were much lower in the JAK2V617F-bearing cells compared with those expressing JAK2R564Q. This may be due to an escape of the negative regulation by JAK2V617F, so that it is not ubiquitinylated for degradation in response to proliferation.

**JAK2R564Q is more sensitive to ruxolitinib than JAK2V617F**

Finally, to determine if any or all of these cellular differences might affect clinical responses to therapeutic JAK inhibition, we used a commercially available JAK inhibitor, ruxolitinib (Jakafi), to
observe the effects of inhibiting JAK activity in mutant JAK2-expressing cells (Figure 5D). Viable cell number was measured after incubation in increasing concentrations of ruxolitinib under starved conditions. The number of viable cells was significantly decreased by concentrations of 100nM ruxolitinib (p<0.05) and above in the JAK2V617F-expressing cell line. However, a significant decrease in cell viability, compared to DMSO controls was seen in the presence of only 0.1nM ruxolitinib (p<0.01) in JAK2R564Q-expressing cells and viability continued to decrease as the concentration of the JAK inhibitor was raised. The IC_{50} value for JAK2R564Q (15.2nM) was approximately 8x lower than the IC_{50} value for JAK2V617F (114.7nM).
DISCUSSION

Here we describe a novel, autosomal dominant mutation that causes familial ET resulting from a single nucleotide substitution, generating the mutant kinase $JAK2_{R564Q}$. MPNs are commonly associated with somatic mutations acquired by individuals, which disrupt regulation of JAK2 signaling. To date, only one other inherited $JAK2$ mutation has been suggested to be responsible for the development of MPN\[^{22,23}\] and this involved an Ile substitution of the well-studied $Val_{617}$ residue. Here we describe a novel germline mutation of $JAK2$ associated with familial ET that does not involve $Val_{617}$, but an alternative residue in the same JH2 domain, $Arg_{564}$. Our data indicates that this mutation may be sufficient to drive thrombocytosis. The phenotype appears highly penetrant and is observed in young family members, screening for other known MPN-associated mutations proved negative and mild thrombocytosis was observed despite myeloid polyclonality, shown by XCIP analysis in a female family member. Furthermore, increased activation of JAK2 was confirmed in the platelets of $JAK2_{R564Q}$-positive family members, compared to those without the mutation.

In order to elucidate the cellular effects of $JAK2_{R564Q}$, we generated Ba/F3-MPL cell lines that expressed either WT$JAK2$, $JAK2_{R564Q}$, $JAK2_{V617F}$, or both $JAK2_{R564Q}$ and $JAK2_{V617F}$ mutations. Interestingly, our results showed that despite $JAK2_{R564Q}$ and the well-described $JAK2_{V617F}$ mutation residing within the same region, and causing similar levels of increased JAK2 activity, these two mutations have differing effects on cell cycle and proliferation. The JAK2 kinase activity, pMPL and pJAK2 levels were all significantly increased compared to WT$JAK2$ controls, and were comparable in all of the $JAK2$ mutants. Downstream signaling was also increased in the $JAK2_{R564Q}$ mutant cells, but not to the same extent as the $JAK2_{V617F}$-expressing cells. Concurrent with these findings, $JAK2_{R564Q}$ offered a significant growth advantage over WT$JAK2$-expressing cells, mediated via a mild proliferative effect, and a much more pronounced anti-apoptotic effect. The proliferative effect of $JAK2_{V617F}$ was even
more robust and cells expressing this mutation were factor-independent. To establish if the weaker effects of JAK2R564Q, compared to JAK2V617F, were due to differences in the levels of JAK2Tyr570 phosphorylation, a negative regulator of JAK2 activity27,28, we determined the phosphorylation status of this residue in the four cell lines. However, Tyr570, like Tyr1007/8, was more phosphorylated in JAK2V617F-expressing cells, compared to JAK2R564Q, excluding this negative regulation as a possible explanation for the more subtle effects of JAK2R564Q on cell proliferation. However, we did find differences in the expression of cell cycle regulators between the mutant JAK2 cell lines. Although levels of p27/Kip1, the G1 to S transition cell cycle regulator, were decreased in JAK2V617F-expressing cells, consistent with previous reports19,29, p27/Kip1 was still present in starved Ba/F3-MPL-JAK2R564Q cells. The JAK2R564Q mutation was able to significantly inhibit apoptosis in the absence of cytokines, as shown by reduced Annexin V staining and decreased PARP cleavage, although it did not cause cytokine-independence, as seen in JAK2V617F-expressing mutants. Overall levels of PARP were increased in JAK2R564Q cells and to a much greater extent in JAK2V617F-expressing cells, which may be due to the increased cell division in the JAK2 mutants. Regulation of phosphorylated JAK2R564Q levels by SOCS3 was observed, as well as increased ubiquitination of JAK2 and the targeting of the kinase for proteasomal degradation, although this effect was absent in the JAK2V617F-expressing mutants, consistent with previous findings in BaF/3 cells20. However, it should be noted that SOCS3-regulation of JAK2V617F appears context-dependent and/or dependent on mutant JAK2 level, as SOCS3 has been shown to inhibit JAK2V617F signaling in HEK cells21. In all assays, the double mutant cell line, expressing both JAK2R564Q and JAK2V617F, was phenotypically very similar to the JAK2V617F cell line, indicating that the effects of the JAK2V617F mutation are dominant over those of JAK2R564Q. However, a mildly additive cell growth effect was seen in the double mutant cells, over the JAK2V617F only expressers (Figure 4A), suggesting there may be potential cellular mechanisms utilized by JAK2R564Q and not by JAK2V617F that we have not yet identified.
It is intriguing that despite the similar localization of the two MPN-causing mutations of JAK2, and the fact that they result in the same levels of increased kinase activity in biochemical assays, their effects in a cellular setting are very different; our results indicate this to be due to differences in the regulation of the kinase. p27/Kip1 levels are reduced in JAK2V617F-expressing cells, but not in JAK2R564Q-expressing cells, indicating that with JAK2V617F expression the cells can escape cell cycle regulation. In addition, JAK2R564Q is controlled by a negative feedback mechanism involving SOCS3, whereas the JAK2V617F mutant is not. It is conceivable that the patient with the JAK2R564Q mutation developed ET, rather than PV, due to the remaining presence of regulatory mechanisms that can somewhat control the hyperactivity caused by the JAK2 mutation. Recent reports have also shown allelic burden to have an important role in disease phenotype determination30,31. We predict that the index patient in this study is heterozygous for JAK2R564Q, since his father does not carry the mutation. Our preliminary results also suggest that JAK2R564Q has a much stronger effect on cell viability when signaling through MPL than through EPOR. The panoply of signaling proteins that EPOR recruits is undoubtedly different to those recruited by MPL and we speculate that the proteins involved in MPL signaling may be more likely to interact with JAK2R564Q. This preference to signal through MPL may also provide a possible explanation of why the patient developed ET, rather than PV, since MPL predominantly drives platelet production.

Our studies further showed that JAK2V617F-expressing cells were sensitive to similar concentrations of the JAK inhibitor, ruxolitinib to those recently described using SET-2/UKE-1 (JAK2V617F-positive leukemia) cells (100nM)32, with an IC₅₀ of 114.7nM, comparable with a previously reported IC₅₀ of 127nM for JAK2V617F-positive BaF/3 cells33. However, consistent with our findings demonstrating tighter control of JAK2 activity, JAK2R564Q-expressing cells were far more sensitive to ruxolitinib treatment, showing significant decreases in viable cell number at 1000-fold lower concentrations (0.1nM), with an 8-fold lower IC₅₀ value. These
results indicate that lower doses of ruxolitinib may be used to successfully treat patients with MPNs associated with JAK2 mutations other than JAK2V617F. Low doses are particularly desirable as ruxolitinib inhibits both mutant and wild type JAK2 non-discriminately, functioning as an ATP-competitive inhibitor of JAK2. Therefore, patients given standard doses of the drug commonly experience adverse effects, such as myelosuppression34. Given that these alternative JAK2 mutations may be germline, as in this case, possibly requiring patients to begin treatment at an early age, and that JAK2 inhibitor therapy does not eradicate the MPN clone, treatment is necessary throughout life, making a low dose additionally advantageous.

In addition to the JAK2V617F mutation in exon 14, insertion/deletion events in exon 12 have also been shown to contribute to MPNs8,9. Further mutations involving exons 12-15, including Arg564, were identified in a large screening of blood samples from suspected MPN patients7, although confirmation of their contribution to MPN is now required. As this region encodes the pseudokinase domain, these mutations, similar to JAK2V617F, may interrupt the regulation of JAK2 activity. As well as JAK2R564Q, the mutation JAK2R564L was also discovered in this study, highlighting the importance of Arg564 and suggesting that mutation of this residue has specific consequences. The recently published crystal structure of the JH2 domain16 revealed that the V617F mutation causes the stabilization of α-helix C in the N lobe of JH2, enabling trans-phosphorylation of the JH1 kinase domain and therefore hyperactivation of JAK2. However, the mechanism by which the JAK2R564Q mutation causes JAK2 hypersensitivity remains unclear. One possible explanation, based in the JH2 domain crystal structure16, is that JAK2R564 may form a hydrogen bond with JAK2H538 (Figure 6A). Both JAK2H538L and JAK2K539I have been described as activating mutations in the SH2-pseudokinase domain linker35, possibly due to H538 and K539 facilitating an inhibitory interaction with the SH2 domain. The R564-H538 hydrogen bond may position H538 for this inhibitory interaction and anchor the rest of the kinase domain relative to the SH2 domain (Figure 6Bi). This hydrogen bond would be broken in the JAK2R564Q mutant,
preventing the inhibitory interaction between H$_{538}$ and the SH2 domain, leading to JAK2 activation (Figure 6Bii). Further studies are now required to determine whether this is indeed the mechanism responsible.

The precise genetic foundations of familial MPNs have proven difficult to define (reviewed in$^{36}$). Recently, familial thrombocytosis has been associated with a germline $MPL$ mutation$^{37}$ and a $TET2$ truncation mutation$^{38}$, but evidence so far has shown that $JAK2$ mutations, particularly $JAK2V_{617}F$, are acquired independently by individuals. Although two recent reports have associated a germline $JAK2V_{617}I$ mutation with familial thrombocytosis$^{22,23}$, our study is the first to demonstrate the contribution of a novel germline mutation involving an alternative $JAK2$ residue to MPN development and outlines the potential mechanisms responsible for causing familial thrombocytosis. We hypothesize that the $JAK2R_{564}Q$ mutation prevents apoptosis in hematopoietic stem cells and megakaryocyte progenitors, giving rise to a moderate increase in platelets. Importantly, even though this mutation is localized to the same JH2 pseudokinase domain, its effects on cell survival and proliferation are significantly different to the $JAK2V_{617}F$ mutation. Our work provides an insight into the functionality of alternative, clinically relevant $JAK2$ mutations and may aid in the development or modification of therapeutic strategies.
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AUTHORSHIP CONTRIBUTIONS

All authors have substantially contributed to the content of the paper and have agreed to the submission in its current format. S.L.E. designed and performed research, analyzed data and wrote the manuscript. M.E.C. and V.S. contributed to study design, study implementation, and writing of the manuscript. L.C. and M.R. performed research. M.A.S. was responsible for interpreting location of the mutation within in 3D structure, predicting possible interactions, and contributed to the writing of the manuscript. E.L.C. completed the patient analysis and contributed to the writing of the manuscript. I.S.H. designed and performed experiments, interpreted data, supervised the project and revised the manuscript.

CONFLICT-OF-INTEREST DISCLOSURE

The authors declare no competing financial interests.
REFERENCES


### TABLES

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<th>Characteristics</th>
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<td>10.2</td>
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<td>275</td>
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<td>-</td>
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<td>-</td>
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Table 1. Clinical and laboratory characteristics of the ET family
FIGURE LEGENDS

Figure 1. Patient with JAK2R564Q mutation displays MPN

(A) Examination of the index patient’s blood smear revealed increased platelet number. Platelet morphology and granulation was normal and most platelets were of normal size (black arrows). Rarely, however, giant platelets were observed (open arrow). (B) Sequence analysis revealed a novel g-to-a single point mutation in JAK2 at nucleotide 1691, causing an amino acid substitution of arginine for glutamine at position 564 (C). (D) Pedigree of the studied ET family. Squares represent males, circles represent females. JAK2R564Q-positive family members are shown in black and the JAK2R564Q-negative member in white. Plts indicates platelet counts of each family member (x10^3/μL). (E) Alignment of JAK2 sequences from different species shows that the arginine at position 564 in the pseudokinase domain is highly conserved. (F) 3D model of the JAK2 JH2 pseudokinase domain based on16, with the N-lobe colored orange and the C-lobe colored blue. Arginine 564 (R564) is located in the 5 stranded β-sheet of the N-lobe.

Figure 2. JAK2 mutant Ba/F3-MPL cell line generation

(A) Ba/F3 cell lines that stably express Mpl (BaF-MPL) and one of four types of human JAK2: WTJAK2, JAK2R564Q, JAK2V617F and both JAK2R564Q and JAK2V617F, were generated. (B) No significant difference (n.s.d) is seen in levels of human/mouse JAK2 cDNA levels between the four cell lines, measured by real time PCR. (C) Western blot analysis of total JAK2 and total MPL levels in each of the four mutant cell lines, compared to Ba/F3-MPL parental cells (1st lane from left). (D & E) Cell surface expression of MPL in the Ba/F3-MPL-JAK2 cell lines, analyzed by flow cytometry. (D) Examples of overlays of the surface MPL flow cytometry of stained cells (black peak), compared to BaF3 parental controls (unfilled peak), for all four of the cell lines. (E) Histogram representing the combined flow cytometric data from all three repeats. No significant difference (n.s.d.) in surface MPL expression was found between the four cell lines.
Figure 3. Expression of JAK2R<sub>564Q</sub> causes increased intracellular signaling in cell lines and patients

(A) JAK2 activity determined by in vitro kinase assay, based on the ability of the kinase to phosphorylate an IkB-α substrate. (B) Following starvation overnight and treatment with/without 10ng/mL TPO for 5 minutes, cells were lysed and proteins subject to MPL pull down and p-Tyr probe to show levels of phosphorylated MPL in the cell lines. In the absence of TPO, MPL is not phosphorylated by WTJAK2, but is phosphorylated by each of the three JAK2 mutants. (C) Following starvation overnight, western blot analysis shows increased levels of phosphorylated JAK2 and STAT1, 3 and 5 in the mutant cell lines, in the absence of, and at low concentrations of TPO treatment for 5 minutes. (D) Western blot analysis of the phosphorylation status of signaling proteins downstream of JAK2 in starved conditions. (E) Platelets were isolated from 3 members of the family with JAK2R<sub>564Q</sub> mutation and subject to western blot analysis.

Figure 4. JAK2R<sub>564Q</sub>-expressing cells exhibit increased cell growth, with a pronounced anti-apoptotic effect and a mild proliferative effect

(A) MTT assay to measure proliferation in the four mutant JAK2 cell lines, with increasing TPO concentration under starved conditions. Each data point is expressed as a percentage of proliferation stimulated by a maximal dose of murine IL-3 and represents 6 repeats. All mutant JAK2-expressing cells show significantly increased proliferation, compared to WTJAK2-expressing cells in the absence of, and at all concentrations of TPO. **= p<0.01 and ***=p<0.001. (B) Viable cell counts every 24hrs under starved conditions, for a total of 72hrs. Data shown are from 3 independent repeats. JAK2V<sub>617F</sub> and double mutant cells are able to proliferate in the absence of cytokine and show a significant increase in cell number, compared to 0hrs, at both 48 and 72hrs (*=p<0.05). The number of viable WTJAK2-expressing cells, compared to starting number, was significantly decreased at all time points (*=p<0.05 and **= p<0.01). No significant difference was seen in the number of viable JAK2R<sub>564Q</sub> cells, compared
to starting number, for the 72 hour period. (C) Apoptosis measured by Annexin V staining of cells under starved conditions for 72hrs. By 48hrs, the number of apoptotic cells in the mutant JAK2 cell lines was significantly less than the number of WT JAK2-expressing apoptotic cells.

(D) Western blot analysis over 36hrs of starvation demonstrates increased levels of uncleaved PARP in all three of the JAK2 mutants, compared to WT JAK2. (E) BrdU assay to measure proliferation during 48 hours of starvation. Proliferation in both WT JAK2 and JAK2R564Q cells decreased over the 48 hour period, although proliferation in JAK2R564Q cells was significantly increased compared to WT JAK2 controls after 48 hours (*=p<0.05). Proliferation continued in the cell lines with the JAK2V617F mutation however, and the percentage of BrdU-positive cells in these cell lines was significantly more than in the WT JAK2 controls after both 24 and 48hrs (***=p<0.001). (F) Western blot analysis during starved conditions showed an increase in p27/Kip1 protein levels over the starvation period in the WT JAK2 and JAK2R564Q cell lines. p27/Kip1 levels were much reduced in the JAK2V617F-expressing mutants, however. p21CIP/WAF1 levels remained fairly constant in each cell line throughout the starvation period.

**Figure 5. JAK2R564Q is negatively regulated by SOCS3 over-expression and is more sensitive to the JAK2 inhibitor, ruxolitinib, than JAK2V617F**

(A) Endogenous levels of SOCS1 and SOCS3 in the JAK2 cell lines, with and without TPO stimulation, shown by western blot. (B) Western blot analysis of protein levels in the JAK2-expressing cells transiently transfected with increasing concentrations of SOCS3 for 24 hours, then starved for a following 4 hours. Numbers represent densitometric quantification of phosphorylated protein levels. (C) JAK2 immunoprecipitation and ubiquitin probe by western blot, following starvation. (D) Cells were grown under starved conditions in the presence of 0-1μM ruxolitinib, a JAK inhibitor. After 48 hours the number of viable cells was measured, as a percentage of the DMSO control. A significant decrease in cell viability, compared to DMSO control, was observed in JAK2R564Q-expressing cells at concentrations of 0.1nM ruxolitinib and
above, whereas cell viability only significantly dropped, compared to the control, in the cells expressing JAK2\textsubscript{V617F} with 100nM and 1\textmu M ruxolitinib treatment. The IC\textsubscript{50} value of JAK2\textsubscript{R564Q} (15.2nM) was almost 8x lower than that of JAK2\textsubscript{V617F} (114.7nM).

**Figure 6. Potential inhibitory role of Arg564**

(A) JAK2 pseudokinase domain structure\textsuperscript{16} showing position of Arg564 and a potential interaction with His538. Given the close proximity of these two residues, a hydrogen bond (dashed red line) may form between them. (B) Model to show a potential mechanism for an inhibitory role of Arg564. (i) Both K\textsubscript{539I} and H\textsubscript{538L} have been described as activating mutations\textsuperscript{37}, possibly due to His538 and Lys539 being involved in inhibitory interactions with the SH2 domain (pink). The Arg564-His538 H-bond (dashed red line) may position His538 for this inhibitory interaction and anchor the rest of the kinase domain relative to the SH2 domain, keeping JAK2 in an inactive state. (ii) Upon mutation of Arg to Gln (R\textsubscript{564Q}), this interaction would be broken, so that His538 is no longer held in place for the inhibitory interaction with the SH2 domain. Further experiments are now required to determine if Arg564 is indeed involved in this potential inhibitory mechanism.
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A novel activating, germline JAK2 mutation, JAK2R564Q, causes familial essential thrombocytosis

S. Leah Etheridge, Megan E. Cosgrove, Veena Sangkhae, Lana Corbo, Michelle Roh, Markus A. Seeliger, Edward L. Chan and Ian S. Hitchcock