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Transforming JAK1 mutations exhibit differential signalling, FERM domain requirements and growth responses to interferon- γ

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Abstract

Recent work has highlighted roles for JAK (Janus kinase) family members in haemopoietic diseases. Although sequencing efforts have uncovered transforming JAK1 mutations in acute leukaemia, they have also identified non-transforming JAK1 mutations. Thus with limited knowledge of the mechanisms of JAK1 activation by mutation, sequencing may not readily identify transforming mutations. Therefore we sought to further understand the repertoire of transforming mutations of JAK1. We identified seven randomly generated transforming JAK1 mutations, including V658L and a deletion of amino acids 629–630 in the pseudokinase domain, as well as L910P, F938S, P960S, K1026E and Y1035C within the kinase domain. These mutations led to differential signalling activation, but exhibited similar transforming abilities, in BaF3 cells. Interestingly, these properties did not always correlate with JAK1 activation-loop phosphorylation. We also identified a JAK1 mutant that did not require a functional FERM (4.1/ezrin/radixin/moesin) domain for transformation. Although we isolated a mutation of JAK1 at residue Val⁶⁵⁸, which is found mutated in acute leukaemia patients, most of the mutations we identified are within the kinase domain and have yet to be identified in patients. Interestingly, compared with cells expressing JAK1-V658F, cells expressing these mutants had higher STAT1 (signal transducer and activator of transcription 1) phosphorylation and were more sensitive to interferon- γ -mediated growth inhibition. The differential STAT1 activation and interferon-sensitivity of JAK1 mutants may contribute to the determination of which specific JAK1 mutations ultimately contribute to disease and thus are identified in patients. Our characterization of these novel mutations contributes to a better understanding of mutational activation of JAK1.

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AUTHOR CONTRIBUTION

Geoff Gordon, Que Lambert and Gary Reuther designed and performed the experiments. Kenyon Daniel assisted with rendering the structural images, and derived and interpreted the molecular measurements. Geoff Gordon, Kenyon Daniel and Gary Reuther wrote the manuscript. Gary Reuther supervised the studies.

Keywords

acute leukaemia; 4.1/ezrin/radixin/moesin (FERM) domain; interferon- γ ; Janus kinase 1 (JAK1); transformation; tyrosine kinase

INTRODUCTION

Members of the JAK (Janus kinase) family, JAK1, JAK2, JAK3 and Tyk2, play important roles in signalling downstream of cytokine receptor activation [1,2]. As non-receptor tyrosine kinases, JAKs interact with the cytoplasmic tail of cytokine receptors located in the plasma membrane. The JAKs share a common modular architecture consisting of a kinase domain, a pseudokinase domain, an SH2 (Src homology 2)-like domain and a divergent FERM (4.1/ezrin/radixin/moesin) domain (see Figure 1B). The FERM domain, which is required for receptor interaction, is located in the N-terminal portion of the proteins along with a SH2-like domain [1]. The C-terminal region of the JAKs contains the kinase and pseudokinase domains, often referred to as the JH1 (JAK homology 1) and JH2 domains respectively. Although the pseudokinase domain has homology with a tyrosine kinase domain, it lacks several critical residues essential for catalytic activity [3]. Experimentally, however, the pseudokinase domain has been demonstrated to exert an important negative regulatory influence on the kinase domain [4]. An auto-inhibitory role of the pseudokinase domain is also supported by a homology model of JAK2, which predicts the direct interface of the JH2 and JH1 domains, presumably by stabilizing the activation loop in an inactive conformation [5].

Members of the JAK family are mutated, in the form of chromosomal translocations, as well as point mutations, in various haematological diseases, including cancer [6]. Most notably, in 2005, several groups independently identified the JAK2-V617F mutation in various myeloproliferative disorders, which are now referred to as MPNs (myeloproliferative neoplasms) [7–11]. The V617F mutation lies in the pseudokinase domain and is predicted, from molecular dynamic simulations, to disrupt the JH1–JH2 interface causing constitutive activation of the protein [12]. Lu et al. [13] demonstrated that JAK2-V617F requires co-expression of a homodimeric receptor to transform cells, but overcame this requirement if JAK2-V617F was expressed at high levels. However, the FERM domain, which is necessary for receptor binding, must be intact in order for the mutant JAK2 to transform cells [13]. This suggested that the ability of JAK2-V617F to interact with a receptor is essential for its transforming activity.

The discovery of the V617F mutation of JAK2 in MPNs renewed interest in the JAK family and has prompted further studies to determine whether additional mutations in JAK family members are present in human disease [14]. Subsequent sequencing studies revealed that somatic mutations of JAK1 are present in acute leukaemia [15–18]. Importantly, certain JAK1 mutations identified by sequencing do not confer an increase in transforming abilities compared with wild-type JAK1 (JAK1-WT), although some of these mutations may slightly increase growth-factor-hypersensitivity [15,17]. Thus it is unknown how they might contribute to the disease process. The lack of a successful mouse model of mutationally

activated JAK1-induced disease has hindered progress in understanding the significance of JAK1 in disease pathogenesis. Despite the unknown role of these mutants, two studies indicated that patients harbouring mutations in JAK1 had a poor outcome [15,18]. This suggests that mutant JAK1 proteins may contribute to disease formation and thus could provide a potential therapeutic target for the management of disease in patients with such mutations. In support of this notion, an RNAi (RNA interference)-based screen in acute leukaemia revealed that JAK1 might be a viable target for some patients [19]. The exact incidence of JAK1 mutations in haemopoietic cancers is not completely clear, as there have been conflicting reports regarding their incidence in T-cell ALL (acute lymphoblastic leukaemia) [20]. Thus although activating mutations of JAK1 occur in various diseases including acute leukaemia, the specific functional characteristics of these mutations remain unknown.

In the present study, we sought to further understand the properties of JAK1 mutations that confer an increased transforming potential of the tyrosine kinase. We screened libraries of randomly mutated JAK1 cDNAs to specifically isolate transforming JAK1 mutations and identified seven mutants that promote transformation of haemopoietic cells. These mutations primarily localized to the tyrosine kinase domain of JAK1. Interestingly, several of these mutants exhibit only modest phosphorylation of their activation loop. These mutants, despite all being transforming, differentially activate downstream signalling pathways. We also determined that activating JAK1 mutants possess differential requirements for a functional FERM domain to induce transformation. Finally, we observed that activating kinase domain mutations in JAK1 led to signalling that included enhanced STAT1 (signal transducer and activator of transcription 1) phosphorylation, as well as increased sensitivity to the growth inhibitory properties of IFN- γ (interferon- γ), compared with the JAK1-V658F mutation, which has been identified in acute leukaemia patients.

EXPERIMENTAL

Cell culture, retrovirus production, retroviral infection and growth curves

HEK-293T [HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40)] cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS (fetal bovine serum) and penicillin/streptomycin. BaF3 and 32D cells were grown in RPMI 1640 supplemented with 10% (v/v) FBS (RPMI/FBS), penicillin/streptomycin and 5% (v/v) WEHI-3B-conditioned medium, to provide IL (interleukin)-3. Retroviral production and infections using pEYK3.1 [21] and pBabepuro vectors were performed as described previously [22]. To generate stable cell lines following infection with pBabepuro-generated virus, cells were selected with 1.0 μ g/ml puromycin at 2 days after infection. To assay for cell growth after cytokine removal, cell lines were washed twice with RPMI/FBS. A total of 1.5×10^6 cells were plated in 10 ml of RPMI/FBS and total viable cell numbers were determined on alternate days by Trypan Blue exclusion.

Random and site-directed mutagenesis

A full-length human JAK1 cDNA (Thermo Scientific Open Biosystems) was cloned into the pEYK3.1 retroviral vector [21] and transformed into XL-1 Red bacteria (Agilent Technologies) according to the manufacturer's instructions. Plasmid DNA was prepared and used to generate retroviruses to screen for transforming JAK1 mutations in 32D cells. JAK1 mutations identified in the pEYK3.1 plasmid from the screen were site-directed into pBabepuro-hJAK1 using standard PCR techniques and PrimeSTAR polymerase (Takara Bio). The FERM domain mutations (L80A and Y81A) were introduced by site-directed mutagenesis into pBabepuro-hJAK1 mutant constructs. The integrity of the complete cDNA for all site-directed mutations was confirmed by DNA sequencing.

Screen for transforming JAK1 mutants

For mutagenesis screens, at 48 h after infection cells were washed twice with RPMI/FBS, and plated in 96-well plates at a density of approx. 2×10^5 per ml. Individual wells with cytokine-independent growth of 32D cells were expanded, genomic DNA was isolated and the pro-viral pEYK3.1-hJAK1 plasmid was recovered essentially as described previously [21]. Isolated proviral plasmid DNA was used to re-generate retroviruses and confirm transformation by infection of 32D cells. The entire cDNA for each rescued transforming JAK1 cDNA clone was sequenced in order to identify mutations.

Immunoblot analysis

Cell lysis and immunoblotting were performed as described previously [22]. Primary antibodies for immunoblotting were against: phospho-STAT1-(Tyr⁷⁰¹), phospho-STAT3-(Tyr⁷⁰⁵), phospho-ERK (extracellular-signal-regulated kinase)-(Thr²⁰²/Tyr²⁰⁴), JAK1 (catalogue numbers 9171, 9138, 4370 and 3344 respectively; Cell Signaling), phospho-JAK1-(Tyr¹⁰²²/Tyr¹⁰²³) (catalogue number 44-422G; Invitrogen), phospho-STAT5-(Tyr⁶⁹⁴) (catalogue number 611964; BD Biosciences), STAT1, STAT3, STAT5, ERK1, IRF (interferon regulatory factor)-1 (catalogue numbers sc-346, sc-483, sc-835, sc-93 and sc-640 respectively; Santa Cruz Biotechnology) and actin (catalogue number A5316; Sigma-Aldrich). Immunoprecipitations were performed with anti-JAK1 antibodies (catalogue number sc-277; Santa Cruz Biotechnology), collected with Protein A-agarose (Thermo Fisher Scientific) and immunoblotted with a mix of anti-phosphotyrosine antibodies PY20 (catalogue number sc-508; Santa Cruz Biotechnology) and 4G10 (catalogue number 05-321; Millipore) or phospho-JAK1-(Tyr¹⁰²²/Tyr¹⁰²³). Secondary antibodies were from Thermo Fisher Scientific. Blots were developed using standard or West Pico chemiluminescence (Thermo Fisher Scientific). Note that the activation-loop tyrosine residues of human JAK1 (Tyr¹⁰³⁴ and Tyr¹⁰³⁵) are sometimes listed as Tyr¹⁰²² and Tyr¹⁰²³ for the commercially available antibodies.

IFN- γ treatment of BaF3 cells

A total of 4×10^6 cells were washed twice with RPMI/FBS and resuspended in 4 ml of RPMI/FBS in the presence of 0, 0.1, 0.5 or 2.5 ng/ml IFN- γ (Peprotech) for 4 h at 37°C. Then, 8 ml of cold PBS was added and cell pellets were washed once with cold PBS. Cell pellets were snap-frozen and stored at -80°C until analysis. For growth analysis in response

to IFN- γ , 1×10^5 BaF3 cells were washed twice with RPMI/FBS and resuspended in 2 ml of RPMI/FBS supplemented with 0.5% WEHI-3B-conditioned medium, and 0 or 5 ng/ml IFN- γ . Total viable cell numbers were determined by Trypan Blue exclusion over time. Cells were diluted as appropriate, supplementing with IFN- γ to maintain a constant IFN- γ concentration.

Molecular modelling

The structure of activated JAK1 was analysed and rendered by PyMOL (DeLano Scientific; <http://www.pymol.org>) using PDB code 3EYH, deposited by Williams et al. [23]. The measurement of distances between amino acid residues of interest was performed utilizing Maestro (Schrödinger Software Suite; version 9.1).

RESULTS

Identification of transforming JAK1 mutations by functional screening

We used a functional screening approach to specifically assay for activating JAK1 mutations that induce cellular transformation of haemopoietic cells. For this screen, we utilized the 32D cell line, which has been extensively used to study mutationally activated tyrosine kinases. Such kinases can render 32D cells independent of cytokine for survival and proliferation. We also chose the 32D cell line as the basis for our screen since overexpression of wild-type JAK1 (JAK1-WT) does not promote cytokine-independent proliferation in these cells. In contrast, we and others have observed that exogenously expressed JAK1-WT mediated the transformation of cytokine-dependent BaF3 cells following long-term cytokine-free culture (G. M. Gordon, Q. T. Lambert and G. W. Reuther, unpublished work and [17,25]), and thus this could potentially interfere with identifying activating JAK1 mutations if this cell line were utilized in such a screen. To generate a library of mutant JAK1 cDNAs, we transformed the pEYK3.1-hJAK1 retroviral plasmid into XL-1 Red bacteria, an *Escherichia coli* strain that is defective in DNA repair and thus will randomly mutate the JAK1 cDNA. We pooled bacteria containing the mutated plasmid DNA, purified the DNA from the bacteria, made retroviruses and infected this library of retrovirus-containing randomly mutated JAK1 cDNAs into 32D cells. Cells were then washed free of IL-3 and plated in 96-well dishes. After approx. 1 week, surviving clones were expanded in culture in order to isolate the mutated JAK1 cDNA. A summary of the workflow of the screen is shown in Figure 1(A). Using this technique we uncovered seven mutations in JAK1, all of which lie in either the kinase or the pseudokinase domains (Figure 1B). Two mutations localize to the pseudokinase domain, a deletion of amino acids 629–630, as well as a point mutation V658L. The remaining mutations are located in the tyrosine kinase domain and include L910P, F958S, P960S, K1026E and Y1035C point mutations.

Transforming JAK1 mutations exhibit enhanced tyrosine phosphorylation and activate downstream signalling

We selected a subset of the mutations isolated from the screens to undergo signalling and growth studies. Additionally, we included the patient-derived JAK1-V658F, which is a mutation analogous to the MPN-associated JAK2-V617F, as both a positive control and as a standard for comparison of our mutants to a patient-derived transforming mutation described

previously [16,26]. We assessed activation and downstream signalling of these transforming JAK1 mutants by expressing them in HEK-293T cells. Expression of JAK1-WT causes very little activation-loop phosphorylation (Figure 2A, lane 2), as measured using an antibody that is specific for JAK1 when phosphorylated at the activation-loop tyrosine residues Tyr¹⁰³⁴ and Tyr¹⁰³⁵. However, in addition to JAK1-V658F (Figure 2A, lane 3), the JAK1-F958S and L910P mutants had dramatically higher levels of basal phosphorylation (Figure 2A, lanes 4 and 7). In contrast, the K1026E and Y1035C mutants displayed levels of phosphorylation comparable with that of JAK1-WT (Figure 2A, lanes 5 and 6). Since the dual tyrosine site in the activation loop is considered the principal readout for activation of JAKs, a lack of significantly increased basal phosphorylation is a surprising finding given that these mutants were isolated based upon their transforming abilities.

Tyr¹⁰³⁵ is the second tyrosine residue in the activation loop. Therefore we reasoned that mutation of this residue into a cysteine might alter the epitope recognized by the antibody, which recognizes dual tyrosine phosphorylation of the activation loop, enough that it interferes with our ability to observe increased phosphorylation. Thus we immunoblotted JAK1 immunoprecipitates with a total phosphotyrosine antibody. We observed increased tyrosine-phosphorylated JAK1 with the Y1035C mutation when compared with JAK1-WT (Figure 2B, lanes 1 and 3). A similar increase in tyrosine phosphorylation was observed with JAK1-V658F (Figure 2B, lane 2). Unlike with Tyr¹⁰³⁵, Lys¹⁰²⁶ was not present in the immunogen used to generate the antibodies that recognize tyrosine phosphorylation of the activation loop of JAK1. Therefore the lack of phosphorylation of the activation loop tyrosine residues in the JAK1-K1026E mutant cannot be explained by the same reasoning.

Next, we analysed the activation of signalling proteins known to play a role downstream of JAK1. The activating mutants we identified from our library screen induce higher STAT3 phosphorylation compared with JAK1-WT expression. Like the V658F mutant, the F958S, Y1035C and L910P mutants activated other downstream signalling molecules, such as STAT5, STAT1 and ERK, to variable extents (Figure 2A, lanes 4, 6 and 7). However, the K1026E mutant seemed to cause only subtle activation of these targets (lane 5). Together these results suggest that expression of transforming JAK1 mutants leads to differentially increased signalling of important downstream effector molecules, but that significant increases in JAK1 activation-loop phosphorylation may not be required for transformation.

JAK1 mutants induce activation of signalling pathways leading to cytokine-independent proliferation of BaF3 cells

We next compared the ability of activating JAK1 mutants to induce signalling that could promote cellular transformation. Since our studies revealed differences in the signalling properties of activating JAK1 mutants, we compared their relative transforming abilities utilizing cytokine-dependent BaF3 cells. Although we knew the JAK1 mutants isolated from our screen could transform cytokine-dependent 32D myeloid cells, we wanted to study the transforming properties of these mutants in more detail in a lymphocytic cell line, since JAK1 mutants have been predominantly found in ALL patients [15,16,18]. BaF3 cells are a lymphocytic progenitor line that requires IL-3 for growth and viability and are commonly used to study the cellular effects of activated JAK mutations. BaF3 cells were generated to

stably express JAK1-WT, JAK1 mutants or empty vector. Each of the library-derived mutants analysed in Figure 2 induced rapid cytokine-independent proliferation in BaF3 cells in contrast with JAK1-WT overexpression (Figure 3A). We did not detect any significant difference in the rate of cytokine-independent transformation induced by these mutants. Since it has been reported that mutation of Val⁶¹⁷ of JAK2 into various amino acids has differential effects on kinase activity, we wanted to determine how the JAK1-V658L mutation from our screen compared with the JAK1-V658F patient-derived mutation [27]; the JAK1-V658L library-derived mutant exhibited identical transforming capacity in BaF3 cells with the JAK1-V658F patient-derived mutant (Figure 3C).

It was surprising that cells expressing the K1026E mutation transformed BaF3 cells at a rate comparable with that of other mutations since we did not observe strong activation of downstream signalling in HEK-293T cells (Figure 2A). Thus we wanted to assess how these mutants activated downstream signalling in BaF3 cells. After starving IL-3-dependent cells of cytokine, we were only able to detect prominent phosphorylation of JAK1 in V658F-expressing cells. Downstream activation of STAT5 was significantly higher in all JAK1-mutant-expressing cells when compared with those containing empty vector or expressing JAK1-WT (Figure 3B). Interestingly, in our library-derived mutants we observed enhanced STAT1 phosphorylation compared with JAK1-WT expression (Figure 3B). This was not observed with JAK1-V658F expression (Figure 3B, compare lane 3 with lanes 4–7). ERK activation was evident in cells expressing JAK1-V658F, whereas cells expressing the other JAK1 mutants did not exhibit higher than basal activation of ERK. Activation of STAT3 was not observed in any of these JAK1-expressing lines (results not shown). Finally, the library-derived JAK1-V658L mutant showed similar JAK1 phosphorylation and activation of STAT5 in BaF3 cells as the patient-expressed JAK1-V658F (Figure 3D). Identical results were obtained in transient transfection of HEK-293T cells (results not shown). Thus it appears that transforming JAK1 mutants have differential abilities to activate downstream signalling pathways, and do not necessarily require detectable activation-loop tyrosine residue phosphorylation in order to transform cells to cytokine independence.

Differential requirement for a functional FERM domain in activating JAK1 mutant-induced signalling and transforming activity

Whereas JAK2-V617F confers hypersensitivity to cytokine signalling in cells that express endogenous or exogenous homodimeric receptors [10,11], it also requires co-expression of an exogenous homodimeric receptor to mediate transformation of cells lacking such receptors [13,28]. Thus it is noteworthy that the JAK1 mutants in the present study were able to transform cells without expression of an exogenous receptor. It is possible that the expression level of JAK1 was high enough to overcome the need for an exogenous receptor, as is the case for high expression of mutant JAK2 [13]. Alternatively, JAK1 mutants may not require a functional interaction with a receptor to transform cells. To evaluate whether JAK1 mutations required a receptor to mediate activation of signalling and transformation, we mutated Leu⁸⁰ and Tyr⁸¹ each to alanine in several of the activating JAK1 mutants; this double-mutation within the FERM domain has been shown to abrogate JAK/receptor binding [29]. We focused on the V658F mutation, the library-derived L910P mutation and also included a JAK1 S646P mutation found recently in ALL patients, in these studies [18].

Following expression in HEK-293T cells, these three JAK1 mutants exhibited increased activation-loop phosphorylation, as well as activation of downstream signalling proteins, including STAT1 and ERK, compared with expression of JAK1-WT (Figure 4, compare lanes 3, 5 and 7 with lane 1). In the experiment, STAT5 was phosphorylated in response to JAK1-L910P expression, but not JAK1-V658F. This result, however, is consistent with our observations that the L910P mutant of JAK1 is a stronger activator of STAT5 than the V658F mutant of JAK1, which only weakly activates STAT5 in the experimental system used in the present study (Figure 2). When we introduced the FERM mutations into the JAK1-V658F mutant there was a complete loss of enhanced JAK1 phosphorylation as well as loss of the phosphorylation of the downstream signalling proteins STAT1 and ERK (Figure 4, lanes 3 and 4). The FERM mutations did not affect JAK1 protein expression. Identical results were obtained when the FERM mutations were placed into the patient-derived JAK1-S646F mutant (Figure 4, lanes 5 and 6). Interestingly, a decreased yet detectable amount of JAK1 phosphorylation was present following mutation of the FERM domain within the context of the JAK1-L910P mutant (Figure 4, lanes 7 and 8). Whereas the FERM mutations in JAK1-L910P blocked the enhanced phosphorylation of downstream signalling proteins, such as STAT1 and ERK, a detectable level of phosphorylated STAT5 remained (Figure 4, lane 8).

To assess their transforming abilities we expressed versions of each of these JAK1 mutants with either a wild-type or mutant FERM domain in BaF3 cells. Since we did not see significant phosphorylation of JAK1-L910P in IL-3-dependent BAF3 cells washed of cytokine, as described above, we utilized cells prior to cytokine removal to analyse JAK1 phosphorylation. In this experiment, the V658F, S646F and L910P mutations exhibited increased phosphorylation of JAK1 compared with cells expressing JAK1-WT (Figure 5A, compare lanes 3, 5 and 7 with lane 1). JAK1-V658F and S646F containing a mutated FERM domain did not exhibit any JAK1 phosphorylation (Figure 5A, lanes 4 and 6). However, mutation of the FERM domain within the context of the JAK1-L910P mutant did not completely eliminate its phosphorylation (Figure 5A, lane 8). This is consistent with the HEK-293T studies in Figure 4. Finally, we assessed the requirement for the FERM domain on the ability of these activating JAK1 mutants to transform cells to cytokine independence. As expected, we observed clear induction of cytokine-independent growth with the V658F and L910P mutations of JAK1, as well as with JAK1-S646F, in agreement with a previous study [18]. In contrast, mutation of the FERM domain in the context of the JAK1-V658F and -S646F activating mutants completely abolished their transforming ability (Figure 5B), which was not due to differential JAK1 protein expression (Figure 5A). However, whereas mutation of the FERM domain within JAK1-L910P decreased the transforming potential of the activating mutant, JAK1-L910P containing the FERM domain mutations continued to display a dramatic ability to induce cytokine-independent growth. This is consistent with the biochemical signalling studies (Figures 4 and 5A) that suggest that JAK1-L910P containing a mutated FERM domain is still competent to induce detectable levels of activation and downstream signalling.

Differential growth response of cells expressing mutationally activated JAK1 proteins following IFN- γ treatment

Since JAK1 plays a role in interferon signalling, we next determined whether cells expressing activating JAK1 mutants exhibited hypersensitivity to interferon stimulation. Treatment of BaF3 cells stably expressing activating JAK1 mutants with increasing doses of IFN- γ activated STAT1, as measured by STAT1 phosphorylation (Figure 6A). No significant difference in STAT1 phosphorylation was seen in cells expressing the various activating JAK1 mutants. STAT1 activation was observed with IFN- γ treatment of 4 h (Figure 6A), as well as rapid responses following 15 min of IFN- γ treatment (results not shown). Likewise, stimulation of the expression of IRF-1, an IFN- γ -responsive gene requiring STAT1 for induction, demonstrated no significant differences in the sensitivity of the activating JAK1 mutants to induce IFN- γ signalling (Figure 6A) [30]. Although there were no apparent differences in the ability of activating JAK1 mutants to transduce signals in response to IFN- γ , there were differences in growth in response to IFN- γ treatment. Cytokine-dependent BaF3 cells expressing JAK1 proteins were cultured in low cytokine (IL-3) conditions and treated with 5 ng/ml IFN- γ . Cells expressing JAK1-V658F were significantly more resistant to the growth inhibitory effects of IFN- γ (Figure 6B, lower panel). The activating mutants analysed, including F958S, K1026E, Y1035C and L910P, displayed only 15–20% of the total number of viable cells observed for JAK1-V658F-expressing cells after culturing the cells in the presence of IFN- γ . In the same experiment, untreated cells expressing activating forms of JAK1 exhibited similar growth to each other in the same time period (Figure 6B, upper panel). Similar results were obtained with higher levels of IFN- γ (50 ng/ml) and in cytokine-free conditions (results not shown).

DISCUSSION

The JAKs have emerged as potentially critical players in a variety of human diseases [6]. Whereas JAK1 has been found mutated in acute leukaemia, it is not immediately clear whether this protein is a significant contributor to the disease pathogenesis [16–18,20]. The lack of a successful mouse model for JAK1-induced disease and the fact that although many of the JAK1 mutants isolated thus far are activating, but only some have been shown to be transforming, makes it difficult to determine a role for JAK1 in tumour progression. This may stem from an unappreciated subtlety in the biology of malignancies containing these mutations. Indeed, it has been proposed that JAK1 mutations may work in concert with other molecular lesions, although the precise nature of these events is speculative at this time [15]. One possibility is that an aberrantly expressed cytokine receptor associates with mutated JAK1, analogous to observations in B-progenitor ALL where mutant JAK2 associates with overexpressed CRLF2 (cytokine-receptor-like factor 2) [31]. Despite the gaps in the current understanding of a role for JAK1 in neoplastic haemopoietic disease, a recent siRNA (small interfering RNA) screen identified JAK1 as a drug target in acute leukaemia, further supporting the potential significance of JAK1 activation in leukaemia [19].

In the present study, we focused on identifying novel JAK1 mutants that are capable of inducing haemopoietic cell transformation. To that end, we screened randomly mutated

JAK1 cDNAs to isolate transforming mutations. Among the mutations recovered, two mutations altered residues in the pseudokinase domain: a deletion of amino acids 629–630, as well as a V658L point mutation. Five other mutations were point mutations in the tyrosine kinase domain and include: L910P, F958S, P960S, K1026E and Y1035C. The structure of the JAK1 kinase domain was recently solved and deposited in the PDB (code 3EYH) [23]. Therefore we used this structure as a basis for understanding the kinase domain mutations that we isolated in our screen. Figures 7(A) and 7(B) show the structure of the JAK1 kinase domain with the residues mutated in the present study highlighted.

Most of the JAK1 mutations we identified in the present screen are of conserved residues among JAK family members [14]. Some of the mutations are similar to those identified from leukaemia patients, thus validating our approach. Val⁶⁵⁸ of JAK1, located in the pseudokinase domain, is found mutated to phenylalanine in ALL and, thus, mutation of this residue is clinically significant. This residue of JAK1 is analogous to Val⁶¹⁷ of JAK2, which is commonly mutated in MPNs. This mutation is believed to relieve the ability of the pseudokinase domain to inhibit the kinase domain [12]. The JAK1 residues 629–630 are located at the beginning of the α C-helix of the pseudokinase domain [14]. The deletion mutation we identified involving these residues is similar to the L624/629W mutation of JAK1 identified previously in paediatric ALL patients [18]. This mutation consists of a deletion as well as a missense mutation at residue 629. Our identification of the deletion of residues 629–630 as a transforming JAK1 mutation suggests that this similar patient-identified deletion mutation, that also alters residue 629, is likely transforming.

Leu⁹¹⁰ of JAK1 is located just after the β 3-strand of the JAK1 kinase domain (Figures 7B) [23]. A leucine residue at this position is conserved among all four members of the human JAK family, as well as additional tyrosine kinases, suggesting it plays a potentially important role in the structure/function of these kinases [14]. Given the rigidity of a proline, the substitution of L910P may cause a significant conformational change to the N-terminal lobe of the kinase, thereby stabilizing an active conformation of the kinase domain. Leu⁹¹⁰ contacts two hydrophobic residues, Ile⁹¹⁹ and Leu⁹²², in the α -helix of the N-terminal lobe. These interactions may provide an anchoring point for this helix, the orientation of which is believed to be critical in kinases [32]. Disrupting this anchoring point with a proline may re-orient this helix leading to activation of the kinase.

Phe⁹⁵⁸ of JAK1 is located just after the β 5-strand in the hinge region of the kinase domain (Figure 7B) [23]. The corresponding residue in all other JAK family members is tyrosine, thus a large aromatic residue is conserved at this position [14]. It is possible that substituting the bulky side chain of the phenylalanine for a more compact serine residue could relieve steric constraints essential for maintaining the kinase in an inactive form, thus facilitating the transition toward the active conformation. Pro⁹⁶⁰ is also located within the hinge region, between the β 5-strand and the α D-helix, of the JAK1 kinase domain and is also conserved in all JAK family members [14,23]. Since the hinge region connects the N-terminal lobe of the kinase domain with the C-terminal lobe of the kinase domain, its structural integrity is likely to be crucial for proper regulation of JAK1. Interestingly, and in support of this idea, Arg⁸⁷⁹ of JAK1 is structurally in close proximity to Phe⁹⁵⁸ and Pro⁹⁶⁰, and Arg⁸⁷⁹ has been found mutated in T-cell ALL [15]. In addition, Pro⁹⁶⁰ of JAK1 corresponds to Pro⁹³³ of JAK2 and

JAK2-P933R is a transforming mutation identified in paediatric B-cell ALL [14,18]. Thus it appears that amino acid side chains in and near the hinge region of JAK proteins play important roles in maintaining the integrity of the regulation of kinase activity. In addition, this region is in close proximity to the ATP-binding site in the catalytic cleft, making it logical to surmise mutations near this region could mediate constitutive activation of the kinase [14].

Lys¹⁰²⁶ of JAK1 is located within the β -sheet, which is within the activation loop of the kinase domain (Figure 7B), and a lysine residue at this position is conserved in all members of the JAK family [14]. From the activated JAK1 crystal structure, it appears Lys¹⁰²⁶ (Figure 7C, green residue) coordinates with phosphorylated Tyr¹⁰³⁵ (Figure 7C, yellow residue) in the activation loop. To examine this possibility we measured the distance from the side chain nitrogen of Lys¹⁰²⁶ to the proximal oxygen on the phosphate group of phosphorylated Tyr¹⁰³⁵. Utilizing Maestro software, this distance was found to be approx. 3.5 Å (1 Å = 0.1 nm). We then performed a constrained energy minimization on the crystal structure using the Protein Preparation workflow in Maestro in order to ensure the structure was at an energetic minimum. Measuring the Lys¹⁰²⁶ to phosphorylated Tyr¹⁰³⁵ distance again resulted in a decreased distance (3.0 Å). That these two groups would move together in this lower energy conformation supports the notion that these residues exhibit an electrostatic interaction. A similar conclusion was made with the analogous residues in the JAK3 crystal structure [33]. Thus the activating K1026E mutation, where the non-conservative substitution would dramatically alter the local electrostatic environment, could lead to activation of JAK1 by potentially altering the stabilization/positioning of the activation loop. In addition, the presence of the negative charge (via the glutamate residue) in the region could prevent, or functionally replace, the negative charge induced by phosphorylation of Tyr¹⁰³⁵. This could explain our inability to detect a significant increase in activation-loop phosphorylation in the K1026E mutant (Figures 2A and 3B).

Tyr¹⁰³⁵ is in the JAK1 activation loop (Figures 7B) and is a conserved site of phosphorylation among JAK family members [14,23]. Alteration of the stability of the activation loop may also be responsible for the transforming abilities of the Y1035C mutation. Another possibility is that mutation of Tyr¹⁰³⁵, one of the activation loop tyrosine residues, may hinder interactions with negative regulators, including suppressors of cytokine signalling proteins and tyrosine phosphatases, which bind to or act on the activation-loop tyrosine residues of JAK proteins [2]. Thus such a mutation might alter inhibition of the kinase following activation, leading to enhanced steady-state activation.

Upon overexpression of library-derived JAK1 mutants we observed rapid cytokine-independent growth of BaF3 cells (Figure 3). Notably, among the set of mutations we isolated, several exhibited only minimal phosphorylation of activation-loop tyrosine residues, suggesting that a high level of phosphorylation of the JAK1 activation loop is not required for transformation (Figures 2A and 3B). The activating Y1035C mutation supports this notion, as this mutation changes an activation-loop tyrosine residue into a non-phosphorylatable residue. Although expression of JAK1 mutants in cells generally led to activation of signalling, JAK1 phosphorylation and downstream signalling were not completely consistent between the HEK-293T and BaF3 studies. In general, more consistent

activation of STAT5 was seen in BaF3 cells, whereas greater phosphorylation of JAK1, STAT3 and ERK was seen in HEK-293T cells. These differing results observed between cell types might be explained by differences in expression of endogenous cytokine receptors. This would presumably dictate the specificity and strength of signalling to downstream target proteins, since the number and motif context of phosphorylated tyrosine residues in receptors would affect the recruitment of STATs as well as negative regulators, thus affecting the steady-state activation of specific downstream signals. Differences in autocrine cytokine signalling between the two cell types could also contribute to the signalling differences in these assays. Nonetheless, these results suggest that specific activating JAK1 mutations differentially activate downstream signalling pathways. This is consistent with Flex et al. [15] who have also observed differential signalling by different JAK1 mutants. Differential activation of such pathways may dictate the extent to which such a mutation can contribute to disease pathogenesis.

The JAK1-V658L mutation that we identified in our screen is at the same residue (amino acid 658) that is found mutated into phenylalanine in acute leukaemia patients [16,18]. This is also the analogous residue to the JAK2-V617F mutation found in MPNs and shown previously to be an activating JAK1 mutation [26]. Dusa et al. [27] have shown that different amino acids at the 617 position of JAK2 have a differential effect on JAK2 activation, with large non-polar residues having an activating effect. This report also suggested that the constitutive activation of JAK2-V617L is less than its V617F counterpart. In the present study, a JAK1 protein that contains a V658L mutation appears equally as active as JAK1-V658F, as demonstrated by the similar phosphorylation profiles of JAK1 and STAT5 in cytokine-dependent JAK1 mutant-expressing BaF3 cells, as well as their transforming capacity in BaF3 cells (Figures 3C and 3D). Dusa et al. [27] also suggested that JAK1 and JAK2 were similar in that they could both be activated by a length-dependent hydrophobic side-chain amino acid substitutions, at residues 658 and 617 respectively, resulting in destabilization of the inhibitory interactions between the pseudokinase and kinase domains. Our results with JAK1-V658L provide further support for this proposal.

It is important to note that the present screen did not require expression of an exogenous cytokine receptor. The initial studies of JAK2-V617F revealed differences in the *in vitro* transformation properties with respect to the requirement for expression of an exogenous cytokine receptor. However, it was subsequently revealed that transformation was a function of the expression levels of mutated JAK2 [13]. The FERM domain of JAK proteins is required for binding to cytokine receptors, and JAK2-V617F requires a functional FERM domain in order to induce its activation and a transforming signal even when it is highly expressed [13]. In addition, a functional JAK1 FERM domain is required for an activated JAK1 protein to support signalling by type I interferon [34]. We observed a complete loss or reduction in the transforming abilities of activated JAK1 proteins that also contained a mutated FERM domain. In the case of the L910P activating mutation, the reduction in transforming abilities was not as dramatic as it was for the V658F or the S646F patient-derived JAK1 activating mutations. If residual receptor binding was responsible for the transforming abilities of the L910P-containing mutated FERM domain JAK1, then we would also expect to observe phosphorylation and transformation with the versions of

V658F and S646F with a mutant FERM domain. These proteins do not exhibit phosphorylation or transforming activity, thus it appears unlikely that residual binding is sufficient to account for the differential activity of these mutants in cells. Alternatively, studies have suggested a delicate interplay between the functionality of the FERM domain and JAK catalytic activity, such that mutations in the FERM domain not only block receptor binding, but also affect activity of the kinase domain [35–37]. Simultaneous mutations in each of these domains could have unexpected effects on JAK1 activity. It is possible that the divergent behaviours observed could be accounted for by the location of the activating JAK1 mutation. For example, both the V658F and S646F mutations lie in the pseudokinase domain, whereas the L910P mutation is located in the kinase domain. The destabilized pseudokinase domain/kinase domain interface induced by the pseudokinase domain mutations may prove detrimental to catalytic activity in the context of the FERM mutant. In contrast, a kinase domain mutant, which might not alter the domain interface, may still have some level of activation.

Given the role of JAK1 in the inhibitory interferon signalling pathway, it is not surprising that inactivating mutations have been found in cancer cells [38,39]. Evidence suggests that JAK1 signalling may constitute a barrier to *in vivo* transformation induced by some oncogenes. JAK1 deficiency did not have an appreciable effect on *in vitro* transformation induced by the *v-Abl* oncogene [40]. However, when *v-Abl* transformed cells were grafted into SCID (severe combined immunodeficiency) mice, JAK1 expression proved to be a hindrance to tumorigenesis, as *v-Abl* transformed cells deficient in JAK1 lost IFN- γ sensitivity [40]. Thus the acquisition of an activating JAK1 mutation may inadvertently attenuate tumour progression by effectively mirroring or enhancing interferon signalling. The conflicting roles of JAK1 in growth promotion and suppression could be at the heart of their poor transforming abilities in some cases and may account for the lack of a successful mouse model for JAK1-mediated transformation. If JAK1 mutations are not capable of promoting *in vivo* transformation on their own, perhaps disabling components of the interferon signalling network downstream of JAK1 will allow for transformation to occur. Such results would be important in understanding the molecular evolution underlying diseases containing JAK1 mutations. A recent report suggests that JAK1 mutations are capable of conferring hypersensitivity to type I interferon [34]. In this respect, it is interesting that the JAK1 kinase domain mutants we isolated in our screen exhibit elevated levels of STAT1 phosphorylation in BaF3 cells, whereas the JAK1-V658F mutant, which has been found in patients, did not enhance STAT1 activation compared expression of JAK1-WT (Figure 3B). Since STAT1 is essential to interferon signalling and has been suggested to have tumour-suppressive properties, the lower level of STAT1 activation found with the Val658F mutation may not be a hindrance to transformation in a physiological context. However, other JAK1 mutants, as we demonstrate in the present study, do activate STAT1 in lymphoid cells. Activation of this pathway could conceivably impede transformation *in vivo*. Indeed, Xiang et al. [17] analysed two JAK1 mutants, which activate STAT1 in BaF3 cells, in a mouse model and did not detect any disease formation. Perhaps, activating JAK1 mutations that contribute to disease formation may be biased for mutants that selectively activate growth-promoting pathways while minimizing tumour-suppressive pathways. Interestingly, we demonstrate in the present study that the growth of cells

expressing our library-derived activating JAK1 kinase domain point mutations is more sensitive to IFN- γ (type II interferon) treatment than cells expressing the patient-derived JAK1-V658F mutant (Figure 6B). This differential sensitivity to interferon treatment suggests that perhaps only certain activating JAK1 mutations can overcome the growth inhibitory properties of certain cytokines (e.g. IFN- γ) *in vivo* and this may explain why strongly activating and transforming kinase domain mutations, similar to those we identified in our screen, have yet to be identified in sequencing efforts in patients. The mechanism(s) by which JAK1-V658F confers enhanced resistance to IFN- γ treatment does not appear to involve decreased STAT1 phosphorylation in response to IFN- γ treatment. We observed no significant difference in the activation of this pathway, including IRF-1 induction, which is a STAT1-dependent event following IFN- γ stimulation [30], in cells expressing JAK1 mutants following IFN- γ stimulation (Figure 6A). Identification of this mechanism may uncover potential therapeutic targets that could be utilized in order to sensitize JAK1-V658F-expressing cells to the action of the immune system in patients. Finally, although our work suggests that expression of the patient-derived JAK1-V658F mutant displays more resistance to IFN- γ than the other JAK1 mutations tested, Hornakova et al. [34] demonstrated that JAK1 mutants from patients display hypersensitivity to type I interferon. Although the JAK1 mutations and the type of interferon utilized in these two studies differ, it is clear that mutationally activated JAK1 proteins probably participate in interferon signalling and the specific activating mutation may dictate the extent to which a cell's growth properties are sensitive to interferon signalling. Needless to say, the milieu of cytokines JAK1-mutant cells could be exposed to *in vivo* is more complex, introducing further variables affecting the response of JAK1-mutant expressing cells to cytokines.

In summary, we have identified novel JAK1 mutations that induce transformation of haemopoietic cells in culture. Although two of these mutations reside in the pseudokinase domain, a common theme for JAK mutations in human haemopoietic diseases, the majority are point mutations affecting the kinase domain. We demonstrate that certain JAK1 mutations found in patients, such as V658F and S646F, require a functional FERM domain for transforming potential. However, this was not consistent among all activating mutants, as a point mutation in the JAK1 kinase domain still retained activity and transforming potential even in the absence of a functional FERM domain. Interestingly, we observed that the kinase domain mutations we identified enhanced phosphorylation of STAT1 in haemopoietic cells, whereas JAK1-V658F, a patient-derived mutation located in the pseudokinase domain, did not. The anti-transforming properties associated with certain signalling proteins, such as STAT1, downstream of activated JAK1 may provide selective pressure in determining whether or not specific JAK1 mutations contribute to disease formation. We also determined that the activating kinase domain mutations we identified exhibited higher sensitivity to the growth inhibitory properties of IFN- γ than the patient-derived JAK1-V658F. Such differential properties of activating JAK1 mutations may contribute to the reason why certain activating mutations have not been observed in patients. Future studies regarding the interplay between STAT1 activation, interferon sensitivity, and activated JAK1-mediated signalling are needed to address such a possibility.

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Abbreviations used

ALL	acute lymphoblastic leukaemia
ERK	extracellular-signal-regulated kinase
FBS	fetal bovine serum
FERM	4.1/ezrin/radixin/moesin
HEK-293T	HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40)
IFN-γ	interferon- γ
IL	interleukin
IRF	interferon regulatory factor
JAK	Janus kinase
JH	JAK homology
MPN	myeloproliferative neoplasm
STAT	signal transducer and activator of transcription
WT	wild-type

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Figure 1. Isolation of mutations of JAK1 that induce haemopoietic cell transformation
(A) An outline of the screen used to identify transforming JAK1 mutations is shown. **(B)** A schematic diagram of JAK1; the location of transforming mutations isolated in the present study are depicted. The location of the patient-derived JAK1-V658F mutation is also shown for reference. Amino acid numbers are based on GenBank[®] accession number NP_002218.

Figure 2. JAK1 point mutations induce differential tyrosine phosphorylation of JAK1 and downstream signalling proteins

(A) HEK-293T cells were transfected with a control empty vector (V, lane 1), or expression vectors for JAK1-WT (WT, lane 2), JAK1-V658F (VF, lane 3), JAK1-F958S (FS, lane 4), JAK1-K1026E (KE, lane 5), JAK1-Y1035C (YC, lane 6) and JAK1-L910P (LP, lane 7). At 2 days after transfection, cells were lysed and lysates were analysed by immunoblotting for phospho-JAK1-(Tyr¹⁰²²/Tyr¹⁰²³) (pJAK1), JAK1, phospho-STAT5-(Tyr⁶⁹⁴) (pSTAT5), STAT5, phospho-STAT3-(Tyr⁷⁰⁵) (pSTAT3), STAT3, phospho-STAT1-(Tyr⁷⁰¹) (pSTAT1), STAT1, phospho-ERK-(Thr²⁰²/Tyr²⁰⁴) (pERK) and ERK, as indicated. (B) HEK-293T cells were transfected with expression vectors for JAK1-WT (lane 1), JAK1-V658F (lane 2) and JAK1-Y1035C (lane 3). At 2 days after transfection cells were lysed,

JAK1 was immunoprecipitated (IP) and immunoprecipitations were analysed by immunoblotting for total phosphotyrosine (pTyr) and JAK1, as indicated.

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Figure 3. Comparison of the transforming and signalling properties of mutant JAK1 proteins in BaF3 cells

(A and B) BaF3 cells were retrovirally infected with viruses that encode for an empty control vector (vector), JAK1-WT (WT), JAK1-V658F (V658F, VF), JAK1-F958S (F958S, FS), JAK1-K1026E (K1026E, KE), JAK1-Y1035C (Y1035C, YC) and JAK1-L910P (L910P, LP). (A) Following stable selection, cells were washed to remove cytokine and plated in the absence of cytokine on day 0. Total viable cell numbers were determined by Trypan Blue exclusion over time. The broken line indicates that the total viable cell number went below the limit of detection of the haemocytometer towards zero. (B) Cells were washed to remove cytokine and incubated without cytokine for 1 h. Lysates were prepared and analysed by immunoblotting as described in Figure 2, except JAK1 was immunoprecipitated for immunoblot analysis. Similar results were obtained with independently derived cell lines. (C) BaF3 cells stably expressing vector (V), JAK1-WT (WT), JAK1-V658F (V658F, VF) or JAK1-V658L (V658L, VL) were washed to remove cytokine and plated in the absence of cytokine on day 0. Total viable cell numbers were determined by Trypan Blue exclusion over time. (D) The BaF3 cell lines described in (C) were washed to remove cytokine and incubated without cytokine for 1 h. Cell lysates were prepared and analysed by immunoblotting with antibodies that recognize the indicated proteins.

Figure 4. Effect of a FERM domain mutation on the phosphorylation of JAK1 and activation of downstream signalling proteins

HEK-293T cells were transfected with expression vectors for unmutated FERM domains (lanes 1, 3, 5 and 7) and mutated FERM domain (-mt; lanes 2, 4, 6 and 8) versions of JAK1-WT (WT, lanes 1 and 2), JAK1-V658F (VF, lanes 3 and 4), JAK1-S646F (SF, lanes 5 and 6) and JAK1-L910P (LP, lanes 7 and 8). At 2 days after transfection cell lysates were prepared and analysed by immunoblotting as described in Figure 2.

Figure 5. Effect of a mutated FERM domain on the phosphorylation and transformation of BaF3 cells by transforming JAK1 mutants

(A) BaF3 cells expressing an unmutated FERM domain (lanes 1, 3, 5 and 7) or a mutated FERM domain (-mt, lanes 2, 4, 6 and 8) version of JAK1-WT (WT, lanes 1 and 2), JAK1-V658F (VF, lanes 3 and 4), JAK1-S646F (SF, lanes 5 and 6) and JAK1-L910P (LP, lanes 7 and 8) were lysed and analysed by immunoblotting for phospho-JAK1-(Tyr¹⁰²²/Tyr¹⁰²³) (pJAK1) and JAK1, as indicated. (B) The BaF3 cell lines described in (A) were washed of cytokine and plated in the absence of cytokine on day 0. Total viable cell numbers were determined by Trypan Blue exclusion over time. The broken lines indicate the total viable cell number went below the limit of detection of the haemocytometer toward zero. Similar results were obtained with independently derived cell lines.

Figure 6. BaF3 cells expressing mutant JAK1 proteins demonstrate similar phosphorylation of STAT1 and induction of IRF-1 but differential growth properties following IFN- γ treatment (A) BaF3 cells stably expressing JAK1-WT (WT), JAK1-V658F (V658F), JAK1-F958S (F958S), JAK1-K1026E (K1026E), JAK1-Y1035C (Y1035C) and JAK1-L910P (L910P) were washed of cytokine and left untreated (–) or treated with increasing concentrations of IFN- γ (0.1, 0.5 and 2.5 ng/ml) for 4 h. Cell lysates were prepared and immunoblotted with antibodies that recognize phospho-STAT1-(Tyr⁷⁰¹) (pSTAT1), STAT1, IRF-1 and actin, as indicated. (B) The BaF3 cell lines described in (A) were washed of cytokine, plated in low cytokine, and either left untreated (upper panel) or treated with 5 ng/ml IFN- γ (lower panel). Total viable cell numbers were determined by Trypan Blue exclusion over time. The total number of viable cells relative to the total number of viable JAK1-V658F cells after culturing for 6 days is shown. A representative experiment is shown (error bars are the S.D. for triplicate cell counts). Similar results were obtained with independently derived cell lines, with 50 ng/ml IFN- γ , as well as in cytokine (IL-3)-free conditions (not shown).

Figure 7. Locations of the transforming JAK1 mutations within the context of the JAK1 kinase domain crystal structure

(A) The N-terminal lobe of the JAK1 kinase domain is shown in dark blue and the C-terminal lobe is shown in light blue. The activation loop of the JAK1 tyrosine kinase domain is shown in red. The amino acid residues within the JAK1 kinase domain that were found to be mutated in activating JAK1 proteins are shown in yellow. (B) An enlarged version of the JAK1 kinase domain shown in (A). Phe⁹⁵⁸ and Pro⁹⁶⁰ are located in the hinge region in between the N- and C-terminal lobes. Leu⁹¹⁰ is located at the end of β -sheet in the N-terminal lobe. Lys¹⁰²⁶ and Tyr¹⁰³⁵ (shown phosphorylated) are in the activation loop of the JAK1 kinase domain. (C) A localized enlarged/rotated view of part of the JAK1 kinase domain activation loop. The phosphorylated activation-loop tyrosine residues 1034 and 1035 are depicted in yellow. Lys¹⁰²⁶ (green) is in close proximity to the phosphorylated Tyr¹⁰³⁵ in the activation loop. The distance between the nitrogen of the side chain of Lys¹⁰²⁶ and the proximal oxygen of the phosphate group of phosphorylated Tyr¹⁰³⁵ is approx. 3.5 Å, as measured using Maestro. This distance decreases to 3.0 Å following a constrained energy minimization of the structure, suggesting an electrostatic interaction between these residues. Structural images were rendered using PyMol based on the JAK1 crystal structure developed by Williams et al. (PDB code 3EYH) [23].