(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 December 2010 (09.12.2010)

PCT

(10) International Publication Number
WO 2010/141062 A1

(51) International Patent Classification:
G01N 33/68 (2006.01)

(21) International Application Number:
PCT/US2010/001538

(22) International Filing Date:
26 May 2010 (26.05.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/184,160 4 June 2009 (04.06.2009) US

(71) Applicant *(for all designated States except US)*: **LUDWIG INSTITUTE FOR CANCER RESEARCH LTD.** [CH/US]; 605 Third Avenue, 33rd Floor, New York, NY 10158 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CONSTANTINESCU, Stefan N.** [BE/BE]; Ludwig Institute for Cancer Research, Avenue Hippocrate 74; Ucl 75-4, B-1200 Brussels (BE). **DUSA, Alexandra** [US/BE]; Ludwig Institute for Cancer Research, Avenue Hippocrate 74; Ucl 75-4, B-1200 Brussels (BE).

(74) Agent: **BAKER, C., Hunter**; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210-2206 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

[Continued on next page]

(54) Title: INHIBITORS OF CONSTITUTIVELY ACTIVE JANUS KINASES AND USES THEREOF

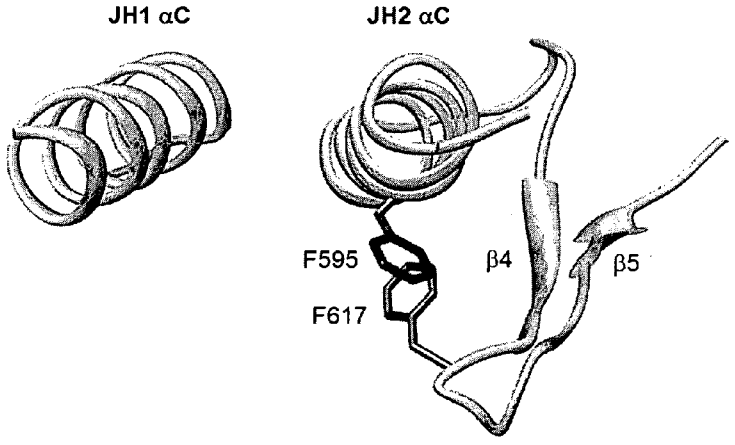

FIGURE 1C

(57) Abstract: Certain mutations in JAK1 and JAK2 result in constitutive activation of the respective kinase. For example, the JAK1 V658F mutation, which renders the JAK1 kinase constitutively active, is implicated in lymphoblastic leukemia. Similarly, the JAK2 V617F mutation, which renders the JAK2 kinase constitutively active, is present in more than 95% of polycythemia vera (PV) patients and 50-60% of patients with essential thrombocytopenia (ET) and primitive myelofibrosis (PMF). Up to now the mechanism of constitutive activation of JAK1 V658F and JAK2 V617F has remained elusive. The present invention stems from the identification of a hydrophobic interaction between residues F658 and F636 in JAK1 or between the homologous residues F617 and F595 in JAK2, both of which are located in the pseudokinase domain. Intramolecular interaction of the two phenylalanine residues leads to constitutive activation of JAK1 V658F or JAK2 V617F. Agents such as small molecules, peptides, or aptamers may be screened for their ability to interfere with this interaction, thereby reducing the activity of the kinase. Once identified, such compounds may be useful in treating myeloproliferative disorders such as polycythemia vera, essential thrombocythemia, primitive myelofibrosis, and lymphoblastic leukemia, particularly those in subjects expressing JAK1 V658F and JAK2 V617F.

WO 2010/141062 A1

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

INHIBITORS OF CONSTITUTIVELY ACTIVE JANUS KINASES AND USES THEREOF

Priority Information

[0001] The present application claims priority under 35 U.S.C. § 119(e) to U.S. provisional patent application, U.S.S.N. 61/184,160, filed June 4, 2009, which is incorporated herein by reference.

Background of the Invention

[0002] JAK 1 and JAK2 are members of the Janus kinase (JAKs) family of non-receptor tyrosine kinases, crucial to blood formation and immune responses. JAK 1 and JAK2 play a role in downstream signaling pathways such as the JAK/STAT pathway, involved in cytokine signaling. Members of the JAK family possess seven defined regions of conserved homology denoted JAK homology (JH) domains 1-7 (1). JH5-7 make up the amino terminus of JAKs and contain a predicted FERM (Band-4.1, ezrin, radixin, and moesin)-like motif (2), which are important in associating JAKs with their receptors and in some cases receptor cell-surface expression (3, 4, 5). Although the JH3-4 domains display some homology to SH2 domains, their function remains ambiguous (6). The carboxyl terminus is composed of JH1 and JH2 and contains the kinase and pseudokinase domains, respectively (7). The JAK2 JH1 domain includes all the features of a catalytic tyrosine kinase, while the JH2 domain, though highly sequence-homologous to JH1, lacks several elements conferring catalytic activity.

[0003] While currently there is no three-dimensional structure for any full-length Janus kinase, the crystal structure of JAK1, JAK2, and JAK3 kinase domains in isolation have been solved in complex with specific inhibitors (8, 9, 10). The JAK2 kinase domain exhibits a typical bilobar arrangement, with a secondary structure profile very similar to other solved kinase domains. (11, 12). The N-terminal lobe of JAK2 is composed of β-strands and includes a single helix, αC, while the C-terminal lobe is mostly helical (8, 10).

[0004] In 2005, several groups independently reported a single acquired somatic mutation in the pseudokinase domain of JAK2, in the form of a substitution of valine for phenylalanine at position 617, in >95% polycythemia vera (PV) patients and 50-60% of patients with essential thrombocytopenia (ET) and primitive myelofibrosis (PMF) (13, 14, 15, 16). The V617F mutation induces constitutive tyrosine phosphorylation of JAK2 and STAT5 and renders Ba/F3-EpoR cells cytokine-independent. Despite a plethora of recent reports describing detailed involvement of V617F in different pathologies, a comprehensive

mechanism of activation of this mutation has yet to be proposed. Based on a hypothesis for the mechanism of constitutive activation of JAK2 V617F, agents that reduce or inhibit the activation of mutant forms of JAK2 may be designed or identified for use in the treatment of myeloproliferative disorders.

Summary of the Invention

[0005] A key functional interaction between residues F617 and F595 of Janus kinase family member JAK2 V617F has been found to result in the constitutive activation of the kinase. This interaction changes the interface between the JH2 and JH1 domains such that the latter is in a conformation able to drive activation in the absence of ligand binding. Similarly, the interaction of the bases F658 and F636 in JAK1 V658F, which are homologous to F617 and F595, respectively, in JAK2 V617F, has been found to result in constitutive activation of JAK1. Based on these discoveries, agents such as small molecules or peptides may be identified or designed that modulate the activity of JAK1 or JAK2, for example, mutant JAK1 or mutant JAK2. For example, agents may be identified that interfere with the binding of F617 with F595 of JAK2, or the binding of F658 with F636 of JAK1, or masking/interacting with F595 of JAK2 or F636 of JAK1, thereby inactivating or reducing the activity of the constitutively active kinase. Further, agents may be identified that stimulate the binding of the amino acid residue at position 617 with F595 of JAK2, or the binding of the amino acid at position 658 with F636 of JAK1 thereby activating or stimulating the activity of the kinase. Agents that interfere with the interaction of F617 with F595 of JAK2 V617F or with the interaction of F658 with F636 of JAK1 may be useful in the treatment of myeloproliferative disorders, such as polycythemia vera, essential thrombocythemia, primitive myelofibrosis, and lymphoblastic leukemia. Based on the homology between JAK 1 and JAK2 and the discovery that constitutional activation of JAK1 and JAK2 results from similar intramolecular interaction of homologous residues in both kinases, the inventive concepts, methods and compositions provided herein in relation to JAK2 are also applicable for JAK1 and vice versa.

[0006] In one aspect, the invention provides methods of identifying agents that modulate, for example, interfere with, the interaction of F617 with F595 of JAK2 V617F. Agents that may be tested for their ability to modulate this interaction include small molecules, proteins, peptides, aptamers, *etc.* Agents may be identified by contacting a test agent with JAK2 V617F under suitable conditions for the test agent to interfere with the interaction of F617 with F595. Modulation, for example, interference with the F617-F595 interaction may be

determined by assaying the activity of the kinase. An agent that interferes with the interaction between these two amino acids should reduce the constitutive activity of the kinase. Similarly, an agent that stimulates the interaction between these two amino acids should increase the constitutive activity of the kinase. A plurality of agents (*e.g.*, a library of chemical compounds) may be screened using the inventive methods. In certain embodiments, the method may be used in high-throughput screening of a large number of test agents.

[0007] In another aspect, the invention encompasses agents found to modulate, with the interaction of F617 with F595 of JAK2 V617F, for example, agents that interfere with this interaction. Such agents may be identified using the inventive assay methods described herein. In some embodiments, the modulating agent may be designed *in silico* based on the three-dimensional structure of the binding pocket. Agents designed *in silico* may then be tested for their ability to inhibit the constitutive activity of JAK2 V617F.

[0008] Agents found to inhibit the constitutive activity of JAK2 V617F may be used alone or in pharmaceutical compositions to treat proliferative diseases. In certain embodiments, the proliferative diseases may be myeloproliferative disorders, such as, but not limited to, polycythemia vera, essential thrombocythemia, and primitive myelofibrosis.

[0009] Some aspects of this invention relate to the discovery that the F595 residue of JAK2 also functionally interacts with additional mutated residues implicated in constitutive or otherwise aberrant activation of JAK2, for example, with mutated residues K539L, T875N, or R683G, and that inhibition of this interaction inhibits or eliminates the constitutive or aberrant activation of the mutant JAK2. Accordingly, some aspects of the invention provide methods for the identification of agents blocking or inhibiting the interaction of the amino acid F595 and an amino acid K539L, T875N, or R683G in a mutated JAK2. Some aspects of this invention provide methods to identify agents inhibiting constitutive or aberrant K539L, T875N, or R683G mutant JAK2 activity, for example, kinase activity. Some aspects of this invention provide methods of inhibiting K539L, T875N, or R683G mutant JAK2 activity in a cell or a subject. Some aspects of this invention provide methods to use agents found to inhibit K539L, T875N, or R683G mutant JAK2 kinase activity to treat a myeloproliferative disease, a deep venous thrombosis or a leukemia.

[0010] Some aspects of this invention relate to the discovery that the F636 residue of JAK1 interacts with mutated residues implicated in constitutive or otherwise aberrant activation of mutant JAK1, for example, with mutated residue V658F and that inhibition of this interaction inhibits or eliminates the constitutive or aberrant activation of the mutant

JAK1. Accordingly, some aspects of the invention provide methods for the identification of agents inhibiting the interaction of the amino acid F636 and amino acid V658F in a mutant JAK1. Some aspects of this invention provide methods to identify agents inhibiting constitutive or aberrant V658F mutant JAK1 activity, for example, kinase activity. Some aspects of this invention provide methods of inhibiting V658F mutant JAK1 activity in a cell or a subject. Some aspects of this invention provide methods to use agents found to inhibit V658F mutant JAK1 kinase activity to treat a myeloproliferative disease or a hematological malignancy, for example, T-ALL (T-adult lymphoblastic leukemia).

Brief Description of the Drawings

[0011] **Figure 1**. Sequence homology and distances between homologous V617 and F595 positions in other kinases and a model of the relative positions of the two residues in JAK2. (A) Alignment of part of the pseudokinase domain of JAK2 with other kinases containing a GVCV-like motif. (B) Distances between the nearest atoms of homologous F595 and V617 residues in the other kinases. Distances were estimated based on the PDB coordinates with UCSF Chimera program (See ref. 32). (C) Predicted relative position of the JAK2 JH1 and JH2 αC helices based on the homology model of Lindauer *et al.* (20). In this predicted arrangement residue F595 in the JH2 αC helix is in the proximity of the mutated V617F residue, also located in the pseudokinase domain, and could make a pi-stacking interaction with the mutated V617F residue, contributing to a change in relative conformations of the JH1 and JH2 αC helices.

[0012] **Figure 2**. Mutation of residue F595 to Ala inhibits the constitutive activity of the JAK2 V617F mutant. Luciferase assay in JAK2-deficient γ-2A individually transfected with each JAK2 mutant and WT shows a marked decrease in the STAT5 transcriptional activity in the absence of ligand stimulation of the F595A/V617F double mutant when compared to V617F. The F595A single mutant exhibits an activity level and ligand response similar to WT JAK2.

[0013] **Figure 3**. Effect of the substitution of residue F595 in the JH2 αC helix on the constitutive activity of JAK2 V617F and on the ligand-induced activation of JAK2 wild-type measured by luciferase assays. (A) Mutation of F595 to some residues induces up to an 86% decrease in the constitutive activity of JAK2 V617F, depending on the particular substitution, while mutation of F595 to aromatic residues rescues the constitutive activity of JAK2 V617F. (B) The single substitution mutants at position 595 display Epo responses similar to JAK2

wild-type at various concentrations of Epo and do not induce constitutive activation of JAK2 wild-type. (C) The JAK2 F595X/V617F double mutants also respond to Epo in a manner analogous to JAK2 wild-type.

[0014] **Figure 4**. Role of JH2 αC helix residue F595 in the proliferation and activation of Ba/F3 cells stably expressing the Epo receptor and individual JAK2 mutants. (A) Proliferation assay of sorted Ba/F3-EpoR cells stably expressing each mutant and wild-type, in medium without growth factors for 7 days. JAK2 V617F is able to proliferate constitutively in this minimal medium starting from the first day (black triangles). The F595A/V617F double mutant lost most of its proliferative advantage (green stars), while the Ba/F3-EpoR parental cell line, or expressing either wild-type or F595A alone could not proliferate in the absence of growth factors. Substitution of F595 to an aromatic residue (F595W) restored autonomous growth (red circles). (B) Luciferase assay in sorted Ba/F3 cells stably expressing the Epo receptor and each individual JAK2 mutant or wild-type. Each cell line was individually electroporated with the pGRR5-Luc and pRLTK-Luc reporters and the STAT5 transcriptional activity was measured. The Ba/F3-EpoR cells expressing JAK2 V617F previously selected for autonomous growth show a typical increase in STAT5 transcriptional activity both in the presence and absence of Epo. (C) Immunoblot analysis of sorted Ba/F3-EpoR cells expressing individual mutants or wild-type, in presence or absence of Epo stimulation. pJAK2, pSTAT5 and pErk1/2 levels of JAK2 V617F, probed with specific antibodies revealed a constitutive signal in the absence of stimulation. This signal was absent in the cells expressing F595A/V617F but partially rescued in cells expressing F595W/V617F.

[0015] **Figure 5**. Selected cytokine-independent murine JAK2 (mJAK2) V617X mutants display high levels of STAT5 transcriptional activity. (A) JAK2-deficient γ-2A cells, which are also deficient in STAT5, were co-transfected with cDNAs coding either for JAK2 WT or for each JAK2 V617X mutant individually, along with STAT5A, and the pGRR5-Luc and pRLTK-Luc reporters. The mutants that were able to proliferate in the absence of cytokines when expressed in Ba/F3-EpoR cells displayed a STAT5-dependent transcriptional activity higher than that of the WT (average of three replicates ± S.D.). Shown is one representative experiment out of four independent experiments. (B) selected Ba/F3-EpoR cells expressing each murine active mutant were starved overnight in RPMI medium with 1mg/ml bovine serum albumin and electroporated with pGRR5-Luc and pRLTK-Luc the next day. The cells were lysed, and their luminescence was recorded. All five mutants, although to a lesser

degree V617C, generated a high STAT5 activity. One of three independent experiments is depicted.

[0016] **Figure 6.** Two other JAK2 mutants previously described to be constitutively active, V617W and V617M, show a large decrease in the constitutive activity in the presence of the F595A mutation. (A) Luciferase assay in γ-2A cells shows that JAK2 V617W alone can induce a constitutive transcriptional activation of STAT5 in the absence of cytokines. When coupled with F595A, the F595A/V617W double mutant loses most of this constitutive advantage, similar to the effect of F595A/V617F. (B) Transient transfection in γ-2A cells of JAK2 V617M mutant previously shown to be constitutively active, displays a marked decrease in its constitutive STAT5 activation in the presence of the F595A mutation. All Epo stimulations were carried out at 50 U/ml.

[0017] **Figure 7.** The JAK2 V617F mutation can induce constitutive activation regardless of the dimeric conformation of the receptor it is bound to, while JAK2 wild-type requires a ligand-activated EpoR dimer in order to signal. (A) The coiled-coil EpoR constructs were made by replacing the extracellular domain of EpoR with the dimeric coiled-coil domain of the yeast transcription factor Put3. (B) Luciferase assay in γ-2A cells transfected with each JAK2 and with previously engineered EpoR dimers containing coiled-coil replacements of their extracellular domains. JAK2 wild-type signals best from the EpoR dimeric conformations imposed by cc-EpoR-III and cc-EpoR-VI, previously shown to correspond to activated dimers. V617F has acquired the ability to signal constitutively from both active (cc-EpoR-III, cc-EpoR-VI) and inactive (cc-EpoR-II, cc-EpoR-V) dimeric interfaces. JAK2 F595A/V617F and F595V/V617F have lost the ability to signal constitutively from active and inactive dimeric conformations, suggesting that F595 may play a role in the constitutive activity of JAK2 V617F. Mutating F595 to Ala or Val forces the double mutant to prefer signaling from the active dimeric EpoR orientations (cc-EpoR-III and VI), akin to wild-type. (C) Activation model for cytokine-activated JAK2 versus JAK2 autoactivation caused by the V617F mutation. Only the pseudokinase domain is shown for simplicity. In wild-type JAK2, residues V617 (grey circle) and F595 (red circle in pseudokinase domain helix C, depicted as grey cylinder) do not play a key role in activation that is brought about by rotation of receptors and scissors-like movements, bringing JAK2 molecules in close proximity, and leading to activation of the kinase domain. In the case of the JAK2 V617F mutant, residues F617 (red circle) and F595 (red circle in pseudokinase domain helix C, depicted as a grey cylinder) are crucial for initiation activation, which is

transmitted to the kinase domain of JAK2, in the absence of the rotation and scissors-like movement of the receptors.

[0018] **Figure 8**. JAK2 V617F and V617W can signal comparatively well from all interfaces of an Epo receptor dimer, while WT can only signal from dimeric interfaces corresponding to an activated EpoR dimer. Eliminating the F at position 595 by mutation to Ala, causes V617F to lose its signaling advantage and to also be constrained to the dimeric interfaces characteristic of an activated EpoR. (A) Luciferase assay in γ-2A cells transfected with JAK2 WT or V617F and with previously engineered EpoR dimers containing coiled-coil replacements of their extracellular domains imposing all seven possible dimeric orientations. JAK2 WT signals best from the EpoR dimeric conformations imposed by cc-EpoR-III and cc-EpoR-VI, previously shown to correspond to activated dimers (See ref. 23). V617F has acquired the ability to signal constitutively regardless of the particular dimeric interface. (B) Similarly to V617F, JAK2 V617W also displays a comparatively high constitutive STAT5 signal constitutively from all conformations of an EpoR dimer, in a luciferase assay in γ-2A cells. (C) JAK2 F595A/V617F has lost the ability to signal constitutively from all seven dimeric conformations, suggesting that F595 may play a role in constitutive activity of JAK2, in collaboration with F617. Mutating F595 to Ala forces the double mutant to prefer signaling from the active dimeric EpoR orientations (cc-EpoR-III and VI), akin to WT.

[0019] **Figure 9**. In the absence of ligand, the F595-F617 stacking interaction affects the relative orientation of the JH1 and JH2 αC helices, pushing the JH1 αC-helix into a conformation suitable for signaling. (A) Predicted arrangement of the JH1 and JH2 αC helices in an inactive conformation (in grey, model coordinates from ref. 20), showing two predicted pairs of salt bridges between residues E890-R588 and R893-E592. In the JH1 kinase domain active structure (in cyan; PDB code 2B7A, ref. 8), residues E890 and R893 were observed to form a salt bridge with each other. (B) Luciferase assay in γ-2A cells shows that mutating R588 and E592 to Ala considerably increases the STAT5 activity of JAK2 WT in the presence of Epo and the constitutive activity of JAK2 V617F in the absence of the Epo ligand, suggesting a role of the JH1-JH2 salt bridges in maintaining the JH1 domain inactive in the absence of ligand. Addition of the F595A mutation to the R588A/E592A/V617F mutant, decreases the constitutive activity back to the level of F595A/V617F. Epo stimulations were carried out at 50U/ml. (C) Structural alignment of the αC helices of different kinases whose structures have been solved in both inactive (grey) and active (cyan) conformations. The view is down the helix axis, from the start to the end of the

helix. All structures show a shift in the position of their αC helices when going from an inactive to an active state.

[0020] **Figure 10**. The F595-F617 interaction is specific to activation of JAK2 V617F and does not affect the JAK2 K539L mutation in the same manner. Predicted arrangement of the JH1 and JH2 αC helices in an inactive conformation (model coordinates from ref. 20), showing the predicted arrangement of residues F595, V617, K539, and D620.

[0021] **Figure 11**. Substitution of F595 to Ala has an inhibitory effect on the constitutive activity of other JAK2 oncogenic mutants. (A) JAK2 K539L, T875N and R683G all induce constitutive activation of JAK2, however all three mutants display a marked decrease in STAT5 transcriptional activity in γ-2A cells when the F595A mutation is also introduced. (B) Proliferation assay of sorted Ba/F3-EpoR cells stably expressing each constitutive mutant and wild-type, in medium without growth factors for 7 days. Cells expressing JAK2 V617F (black triangles), K539L (blue lines), T875N (green stars), and R683G (yellow circles) can proliferate constitutively in this minimal medium starting from the first day. Substitution of F595 to Ala individually in the context of each active mutant causes a marked decrease in autonomous growth. The JAK2 K539L/F595A mutant (red lines) lost most of its proliferative advantage during the first 4 days, but by day 7 had regained the same level of autonomous growth as its single counterpart (blue lines). (C) Predicted locations (red spheres) of mutants K539L, R683G, V617F (left panel) and T875N (right panel) within the model structure of JAK2, based on coordinates of the homology model proposed by Lindauer *et al.*. The right panel is rotated 180° relative to the left panel. The kinase domain is depicted in yellow and the pseudokinase domain in blue. The location of F595 is shown as a green sphere. Circular arrows placed between helices C of JH2 and JH1 indicate potential conformational changes originating in JH2 and transmitted to JH1. Solid double-headed arrow placed near the R683G mutation suggests increased flexibility induced by this JH2 hinge mutant. Green star (right panel) suggests T875N mutation changes JH1-JH2 linker segment conformation, which is transmitted to JH2 helix C F595.

[0022] **Figure 12**. The F595A homolog in JAK1, F636A, blocks constitutive activity of JAK1 V658F. Transient transfection in the JAK1-deficient U4C cell line indicates a decrease in the STAT3 transcriptional activity in the JAK1 F636A/V658F, as compared to JAK1 V658F.

[0023] **Figure 13.** Effect of mutating residues in the JH2 helix C on the constitutive and ligand induced activity of JAK2 V617F. Transient transfection of the JAK2-deficient g2A

cell line was performed with cDNAs coding for the indicated JAK2 mutants, EpoR, STAT5 and luciferase reporters. Measurement of STAT5 transcriptional activity indicated that residues R588, E592 are not required for constitutive activity of JAK2 V617F. Like in the case of JAK2 V617F, mutation of F595 to alanine blocks constitutive activation of the triple JAK2 V617F R588A,E592A. JH2 residues M600, M601 and S602, Q603 are required for constitutive but not Epo-induced activation of JAK2 V617F

Definitions

[0024] As used herein and in the claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly indicates otherwise. Thus, for example, a reference to "an agent" includes a plurality of such agents.

[0025] As used herein, the term "agent" or "test agent" refers to any compound or molecule that is to be tested. Examples of agents of the present invention include but are not limited to peptides, small molecules, and antibodies. Agents can be randomly selected or rationally selected or designed. As used herein, an agent is said to be "randomly selected" when the agent is chosen randomly without considering the specific interaction between the agent and the target compound or site. As used herein, an agent is said to be "rationally selected or designed", when the agent is chosen on a non-random basis which takes into account the specific interaction between the agent and the target compound or site and/or the conformation in connection with the agent's action.

[0026] As used herein, the term "animal" refers to any member of the animal kingdom. In some embodiments, "animal" refers to a human, at any stage of development. In some embodiments, "animal" refers to a non-human animal, at any stage of development. In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and/or worms. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, an animal may be a transgenic animal, genetically-engineered animal, and/or clone.

[0027] The term "antibody" refers to an immunoglobulin, whether natural or wholly or partially synthetically produced. All derivatives thereof which maintain specific binding ability are also included in the term. The term also covers any protein having a binding domain which is homologous or largely homologous to an immunoglobulin binding domain. These proteins may be derived from natural sources, or partly or wholly synthetically produced. An antibody may be monoclonal or polyclonal. The antibody may be a member

of any immunoglobulin class, including any of the human classes: IgG, IgM, IgA, IgD, and IgE. Derivatives of the IgG class, however, are preferred in the present invention.

[0028] The term "aptamer" refers to single stranded oligonucleotides and peptides designed to bind a molecular target with high affinity and/or interfere with the function of a molecular target. The molecular target may be a protein, a nucleic acid, or virtually any other molecule. Aptamers may comprise a variable peptide or nucleotide loop attached at both ends to a scaffold, for example, a protein scaffold.

[0029] The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient of a pharmaceutical composition is combined to facilitate the application or administration. The characteristics of the carrier will depend on the route of administration. Examples of physiologically and pharmaceutically acceptable carriers include, without being limited to, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

[0030] As used herein, the term "contacting" refers to bringing a JAK2 molecule and a test compound together in a manner that the test agent can affect JAK2 activity. Contacting may be accomplished in a cell-free system, for example, by adding a test agent to a solution comprising an active JAK2 molecule under physiological conditions. Contacting may also be accomplished in a cell, for example, by adding a test compound able to permeate a cell membrane to a culture comprising a cell expressing a JAK2 molecule.

[0031] The term "effective amount" of an agent refers to an amount sufficient to elicit the desired biological response, for example, a reduction of proliferation of cells in a subject. As will be appreciated by those of ordinary skill in this art, the effective amount of an agent may vary depending on such factors as the desired biological endpoint, the pharmacokinetics of the compound, the disease being treated, the mode of administration, and the patient.

[0032] The term "*in vitro*" refers to an artificial environment and to reactions or processes that occur within an artificial environment. *In vitro* environments include, but are not limited to, test tubes and cell cultures. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reaction that occur within a natural environment.

[0033] The term "JAK1," as used herein, refers to the Janus Kinase 1 protein. The sequences of the JAK1 protein of several species, including human, are well known in the art and exemplary sequences are provided herein. If not further qualified, the term *JAK1*, is meant to include both wild type and mutant forms, for example, JAK1 V658F, of the JAK1 protein.

[0034] The term "JAK2," as used herein, refers to the Janus Kinase 2 protein. The sequences of the JAK2 protein of several species, including human, are well known in the art and exemplary sequences are provided herein. If not further qualified, the term *JAK2*, is meant to include both wild type and mutant forms, for example, JAK2 V617F, of the JAK2 protein.

[0035] The term "JAK2 activity-dependent transcription factor" refers to any transcription factor that is affected by JAK2 signaling. The term encompasses transcription factors that are direct phosphorylation substrates of JAK2, for example, some members of the STAT family of transcription factors. The term further encompasses transcription factors activated or inhibited as a result of JAK2 activity, for example, transcription factors that are encoded by target genes of STAT family transcription factors. JAK2 activity-dependent transcription factors are well known in the art and include STAT family transcription factors, for example, STAT5.

[0036] The term "minimal promoter" refers to a promoter comprising the binding sites for the base transcriptional machinery, but lacks binding sites for transcriptional transactivators and, therefore, does not exhibit significant transcriptional activity when not bound by a transactivating transcription factor, for example, phosphorylated STAT5.

[0037] The terms "modulating," as used herein, for example, in the context of intramolecular interactions or kinase activity, refers to a measurable increase or a decrease in the interaction or activity. For example, an agent identified to modulate mutant JAK2 activity may be either an agent that inhibits (*e.g.*, decreases) the activity of JAK2 or an agent that stimulates (*e.g.*, increases) the activity of JAK2. Similarly, an agent identified to modulate the activity of JAK1 may be an agent that inhibits the activity of JAK1 or an agent that stimulates the activity of JAK1.

[0038] As used herein, the term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients.

[0039] The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism.

[0040] A "protein" comprises a polymer of amino acid residues linked together by peptide bonds. The term, as used herein, refers to proteins, polypeptides, and peptide of any size, structure, or function. Typically, a protein will be at least three amino acids long. A protein may refer to an individual protein or a collection of proteins. Inventive proteins preferably contain only natural amino acids, although non-natural amino acids (*i.e.*,

compounds that do not occur in nature but that can be incorporated into a polypeptide chain; see, for example, www.cco.caltech.edu/~dadgrp/Unnatstruct.gif, which displays structures of non-natural amino acids that have been successfully incorporated into functional ion channels) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in an inventive protein may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, *etc.* A protein may also be a single molecule or may be a multi-molecular complex. A protein may be just a fragment of a naturally occurring protein or peptide. A protein may be naturally occurring, recombinant, or synthetic, or any combination of these.

[0041] The term "proliferative disease" as used herein refers to any disease associated with an undesired and/or abnormal proliferation of cells. The cells may be any type of cell found in the subject. The proliferation may be due to any cause (*e.g.*, any genetic mutation, any signal). Examples of proliferative diseases include cancer, neoplasms, inflammatory diseases, autoimmune diseases, graft-vs.-host disease, diabetic retinopathy, and benign tumors.

[0042] As used herein, the term "small molecule" is used to refer to molecules, whether naturally-occurring or artificially created (*e.g.*, via chemical synthesis) that have a relatively low molecular weight. Typically, a small molecule is an organic compound (*i.e.*, it contains carbon). The small molecule may contain multiple carbon-carbon bonds, stereocenters, and other functional groups (*e.g.*, amines, hydroxyl, carbonyls, heterocyclic rings, *etc.*). In some embodiments, small molecules are monomeric and have a molecular weight of less than about 1500 g/mol. In certain embodiments, the molecular weight of the small molecule is less than about 1000 g/mol or less than about 500 g/mol. Preferred small molecules are biologically active in that they produce a biological effect, for example, a kinase inhibitor produces inhibition of a kinase, in animals, preferably mammals, more preferably humans. In certain embodiments, the small molecule is a drug. Preferably, though not necessarily, the drug is one that has already been deemed safe and effective for use in humans or animals by the appropriate governmental agency or regulatory body. For example, drugs approved for human use are listed by the FDA under 21 C.F.R. §§ 330.5, 331 through 361, and 440 through 460, incorporated herein by reference; drugs for veterinary use are listed by the FDA under 21 C.F.R. §§ 500 through 589, incorporated herein by reference. All listed drugs are considered acceptable for use in accordance with the present invention.

[0043] The term "suitable conditions," as used herein, refers to conditions that allow and/or support JAK2 enzymatic activity. Suitable conditions, therefore, allow for a JAK2 molecule to exist in non-denatured form, allow a JAK2 molecule to bind a substrate, and allow a JAK2 molecule to carry out a phosphorylation reaction involving a substrate molecule.

[0044] The terms "therapy," "therapeutic," "treat," or "treatment" refer to, but are not limited to, one or more clinical intervention with an intent to prevent, ameliorate, or cure a condition or symptoms of the condition in a subject. In some embodiments, a treatment is aimed to reduce a level of hematopoietic cell proliferation in a subject having a myeloproliferative disorder.

Detailed Description of Certain Embodiments

[0045] JAK1 and JAK2 belong to the Janus kinases (JAKs) family of non-receptor tyrosine kinases, crucial to blood formation and immune responses. JAK1 and JAK2 play a role in downstream signaling pathways such as the JAK/STAT pathway, involved in cytokine signaling. Members of the JAK family possess seven defined regions of conserved homology denoted JAK homology (JH) domains 1-7 (1). JH5-7 make up the amino terminus of JAKs and contain a predicted FERM (Band-4.1, ezrin, radixin and moesin)-like motif (2), important in association of JAKs to their receptors and in some cases in receptor cell-surface expression (3, 4, 5). Although the JH3-4 domains display some homology to SH2 domains, their function remains ambiguous (6). The carboxyl terminus is composed of JH1 and JH2 and contains the kinase and pseudokinase domains, respectively (7). The JAK2 JH1 domain includes all the features of a catalytic tyrosine kinase, while the JH2 domain, though highly sequence-homologous to JH1, lacks several elements conferring catalytic activity.

[0046] While currently there is no three-dimensional structure for any full-length Janus kinase, the crystal structures of JAK1, JAK2 and JAK3 kinase domains in isolation have been solved in complex with specific inhibitors (8, 9, 10). The JAK2 kinase domain exhibits a typical bilobar arrangement, with a secondary structure profile very similar to other solved kinase domains (11, 12). The N-terminal lobe of JAK2 is composed of β-strands and includes a single helix, αC, while the C-terminal lobe is mostly helical (8, 10).

[0047] A single acquired somatic mutation in the pseudokinase domain of JAK2, in the form of a substitution of Val for Phe at position 617 (V617F), is at the base of >95% Polycythemia Vera (PV) patients and 50-60% of patients with Essential Thrombocythemia

(ET) and Primitive Myelofibrosis (PMF) (13, 14, 15, 16). The V617F mutation induces constitutive tyrosine phosphorylation of JAK2 and STAT5 and renders Ba/F3 cells that express the erythropoietin receptor (EpoR) cytokine-independent. Similarly, a single substitution of Val for Phe at position 658 (V658F) in JAK1, a position homologous to V617 of JAK2, is implicated to cause lymphoblastic leukemia and other myeloproliferative diseases.

[0048] Despite a plethora of recent reports describing the contribution of V617F to different pathologies, a comprehensive mechanism of activation of this mutation has yet to be proposed. In this work we explore the role of the pseudokinase domain helix C in the constitutive activation of JAK2 V617F by focusing on residue F595, predicted to be located in the middle of the helix.

[0049] The present invention is based, at least in part, on the elucidation that the molecular event rendering JAK2 V617F and JAK1 V658Fconstitutively active is a pi-stacking interaction of the phenylalanine residue at position 617 of the mutant protein with the phenylalanine residue at position 595 in JAK2, or an interaction of the phenylalanine residue at position 658 of the mutant protein with the phenylalanine residue at position 636 in JAK1. This interaction induces a conformational change resulting in ligand-independent activation of JAK2 V617F or JAK1 V658F. Ligand-independent, constitutive activation of JAK1 or JAK2 is associated with a number of myeloproliferative disorders, for example, polycythemia vera, essential thrombocythemia, primitive myelofibrosis, and lymphoblastic leukemia. Some aspects of the current invention provide methods to identify an agent that inhibits JAK2 V617F activity and/or JAK1 V658F activity. Some aspects of the present invention provide methods to identify an agent that inhibits JAK2 V617F activity and/or JAK1 V658F activity but not wild type JAK2 activity and/or wild type JAK1 activity, respectively. Agents found to inhibit JAK2 V617F and/or JAK1 V658F activity may be useful in the inhibition of aberrant JAK1 and/or JAK2 activity in subjects diagnosed with a myeloproliferative disorder.

[0050] Accordingly, methods to identify an agent that modulates JAK2 V617F or JAK1 V658F activity are described herein. For example, in some embodiments, methods to identify an agent that inhibits JAK2 V617F or JAK1 V658F activity are described herein. Further, methods to identify an agent that inhibits JAK2 V617F or JAK1 V658F activity by interfering with the interaction of F595 and F617 in JAK2 V617F or with the interaction of F636 and F658 in JAK1 V658F, respectively, are described herein. Also described herein are methods to identify an agent that modulates JAK2 V617F activity but does not modulate wild

type (wt) JAK2 activity. Also described herein are methods to identify an agent that modulates JAK1 V658F activity but does not modulate wild type JAK1 activity. For example, some embodiments of this invention provide methods to identify an agent that inhibits JAK2 V617F activity but does not inhibit wild type (wt) JAK2 activity. Similarly, some embodiments of this invention provide methods to identify an agent that inhibits JAK1 V658F activity but does not inhibit wild type JAK1 activity. Methods of administering a JAK2 V617F inhibitor or a JAK1 V658F inhibitor to a subject with a myeloproliferative disorder, for example, polycythemia vera, essential thrombocythemia, primitive myelofibrosis, or lymphoblastic leukemia are also provided herein. Such inhibitors are particularly useful for subjects expressing a mutated form of JAK1 or JAK2, for example, JAK1 V658F or JAK2 V617F.

Janus kinases

[0051] Janus kinases (JAKs) are a family of intracellular non-receptor tyrosine kinases that transduce cytokine mediated signals via the JAK-STAT pathway. JAK family member proteins range from 120-140 kDa in size and have seven defined regions of homology called Janus homology domain 1–7 (JH1-7). JH1 is the kinase domain mediating the enzymatic activity, containing typical features of tyrosine kinases, for example, conserved tyrosines that are essential for JAK activation (e.g. Y1038/Y1039 in JAK1, Y1007/Y1008 in JAK2, Y980/Y981 in JAK3, and Y1054/Y1055 in Tyk2). Phosphorylation of these tyrosines leads to conformational changes in the JAK protein that are associated with the active state of the enzyme and facilitate substrate binding and phosphorylation. JH2 is a pseudokinase domain structurally similar to a tyrosine kinase and essential for normal kinase activity, but lacking enzymatic activity. This domain may be involved in regulating the activity of JH1. JH3 and JH4 share homology with Src-homology-2 (SH2) domains. The N-terminal JH4-JH7 are called FERM domain (short for band 4.1 ezrin, radixin and moesin) and are involved in association of JAKs with cytokine receptors and/or other kinases.

[0052] Janus kinase 2 (JAK2) has been implicated, for example, in signal transduction of type II cytokine receptor family members (for example, interferon receptors), GM-CSF receptor family members (for example, IL-3R, IL-5R, and GM-CSF-R), the gp130 receptor family (for example, IL-6R), and the single chain receptors (for example, Epo-R, Tpo-R, GH-R, PRL-R). JAK2 further transduces signals downstream of the prolactin receptor. Mutations in the JAK2 gene leading to aberrant expression or activity patterns of JAK2 are associated with proliferative diseases, for example, leukemia, polycythemia vera, essential thrombocythemia, and other myeloproliferative disorders. A mutation of valine to

phenylalanine at the 617 position in JAK2 V617F has been associated with hypersensitivity of hematopoietic cells to growth factors such as erythropoietin and thrombopoietin. The discovery of the elusive molecular mechanism underlying the constitutive activation of JAK2 V617F, as described herein, translates into methods of identifying a JAK2 V617F inhibitor provided by some aspects of this invention. In some embodiments, the invention provides methods to reduce the proliferation of hematopoietic cells in a subject having a myeloproliferative disease. In some embodiments, the invention provides methods for treating a proliferative disorder in a subject expressing a mutated form of JAK2, for example, JAK2 V617F.

[0053] The amino acid sequence of human JAK2 is well known in the art. Human JAK2 sequences are, for example, represented in the NCBI database (www.ncbi.orgwww.ncbi.nlm.nih.gov/), for example, under accession number NP_004963.

[0054] >gi|4826776|ref|NP_004963.1| Janus kinase 2 [Homo sapiens]

[0055] MGMACLTMTEMEGTSTSSIYQNGDISGNANSMKQIDPVLQVYLYHSLGKSEADYL TFPSGEYVAEEICIAASKACGITPVYHNMFALMSETERIWYPPNHVFHIDESTRHNVLYRIR FYFPRWYCSGSNRAYRHGISRGAEAPLLDDFVMSYLFAQWRHDFVHGWIKVPVTHETQEECL GMAVLDMMRIAKENDQTPLAIYNSISYKTFLPKCIRAKIQDYHILTRKRIRYRFRRFIQQFS QCKATARNLKLKYLINLETLQSAFYTEKFEVKEPGSGPSGEEIFATIIITGNGGIQWSRGKH KESETLTEQDLQLYCDFPNIIDVSIKQANQEGSNESRVVTIHKQDGKNLEIELSSLREALSF VSLIDGYYRLTADAHHYLCKEVAPPAVLENIQSNCHGPISMDFAISKLKKAGNQTGLYVLRC SPKDFNKYFLTFAVERENVIEYKHCLITKNENEEYNLSGTKKNFSSLKDLLNCYQMETVRSD NIIFQFTKCCPPKPKDKSNLLVFRTNGVSDVPTSPTLQRPTHMNQMVFHKIRNEDLIFNESL GQGTFTKIFKGVRREVGDYGQLHETEVLLKVLDKAHRNYSESFFEAASMMSKLSHKHLVLNY GVCVCGDENILVQEFVKFGSLDTYLKKNKNCINILWKLEVAKQLAWAMHFLEENTLIHGNVC AKNILLIREEDRKTGNPPFIKLSDPGISITVLPKDILQERIPWVPPECIENPKNLNLATDKW SFGTTLWEICSGGDKPLSALDSQRKLQFYEDRHQLPAPKWAELANLINNCMDYEPDFRPSFR AIIRDLNSLFTPDYELLTENDMLPNMRIGALGFSGAFEDRDPTQFEERHLKFLQQLGKGNFG SVEMCRYDPLQDNTGEVVAVKKLQHSTEEHLRDFEREIEILKSLQHDNIVKYKGVCYSAGRR NLKLIMEYLPYGSLRDYLQKHKERIDHIKLLQYTSQICKGMEYLGTKRYIHRDLATRNILVE NENRVKIGDFGLTKVLPQDKEYYKVKEPGESPIFWYAPESLTESKFSVASDVWSFGVVLYEL FTYIEKSKSPPAEFMRMIGNDKQGQMIVFHLIELLKNNGRLPRPDGCPDEIYMIMTECWNNN VNQRPSFRDLALRVDQIRDNMAG (SEQ ID NO: 1)

[0056] Accordingly, the sequence of JAK2 V617F is as follows:

[0057] MGMACLTMTEMEGTSTSSIYQNGDISGNANSMKQIDPVLQVYLYHSLGKSEADYL TFPSGEYVAEEICIAASKACGITPVYHNMFALMSETERIWYPPNHVFHIDESTRHNVLYRIR FYFPRWYCSGSNRAYRHGISRGAEAPLLDDFVMSYLFAQWRHDFVHGWIKVPVTHETQEECL GMAVLDMMRIAKENDQTPLAIYNSISYKTFLPKCIRAKIQDYHILTRKRIRYRFRRFIQQFS QCKATARNLKLKYLINLETLQSAFYTEKFEVKEPGSGPSGEEIFATIIITGNGGIQWSRGKH KESETLTEQDLQLYCDFPNIIDVSIKQANQEGSNESRVVTIHKQDGKNLEIELSSLREALSF VSLIDGYYRLTADAHHYLCKEVAPPAVLENIQSNCHGPISMDFAISKLKKAGNQTGLYVLRC SPKDFNKYFLTFAVERENVIEYKHCLITKNENEEYNLSGTKKNFSSLKDLLNCYQMETVRSD NIIFQFTKCCPPKPKDKSNLLVFRTNGVSDVPTSPTLQRPTHMNQMVFHKIRNEDLIFNESL GQGTFTKIFKGVRREVGDYGQLHETEVLLKVLDKAHRNYSESFFEAASMMSKLSHKHLVLNY GVCFCGDENILVQEFVKFGSLDTYLKKNKNCINILWKLEVAKQLAWAMHFLEENTLIHGNVC AKNILLIREEDRKTGNPPFIKLSDPGISITVLPKDILQERIPWVPPECIENPKNLNLATDKW SFGTTLWEICSGGDKPLSALDSQRKLQFYEDRHQLPAPKWAELANLINNCMDYEPDFRPSFR AIIRDLNSLFTPDYELLTENDMLPNMRIGALGFSGAFEDRDPTQFEERHLKFLQQLGKGNFG SVEMCRYDPLQDNTGEVVAVKKLQHSTEEHLRDFEREIEILKSLQHDNIVKYKGVCYSAGRR NLKLIMEYLPYGSLRDYLQKHKERIDHIKLLQYTSQICKGMEYLGTKRYIHRDLATRNILVE NENRVKIGDFGLTKVLPQDKEYYKVKEPGESPIFWYAPESLTESKFSVASDVWSFGVVLYEL FTYIEKSKSPPAEFMRMIGNDKQGQMIVFHLIELLKNNGRLPRPDGCPDEIYMIMTECWNNN VNQRPSFRDLALRVDQIRDNMAG (SEQ ID NO: 2, V617F mutation site bold and underlined).

[0058] The amino acid sequence of human JAK1 is well known in the art. Human JAK1 sequences are, for example, represented in the NCBI database (www.ncbi.orgwww.ncbi.nlm.nih.gov/), for example, under accession number NP_002218.

[0059] >gi|102469034|ref|NP_002218.2| tyrosine-protein kinase JAK1 [Homo sapiens] MQYLNIKEDCNAMAFCAKMRSSKKTEVNLEAPEPGVEVIFYLSDREPLRLGSGEYTAEELCI RAAQACRISPLCHNLFALYDENTKLWYAPNRTITVDDKMSLRLHYRMRFYFTNWHGTNDNEQ SVWRHSPKKQKNGYEKKKIPDATPLLDASSLEYLFAQGQYDLVKCLAPIRDPKTEQDGHDIE NECLGMAVLAISHYAMMKKMQLPELPKDISYKRYIPETLNKSIRQRNLLTRMRINNVFKDFL KEFNNKTICDSSVSTHDLKVKYLATLETLTKHYGAEIFETSMLLISSENEMNWFHSNDGGNV LYYEVMVTGNLGIQWRHKPNVVSVEKEKNKLKRKKLENKHKKDEEKNKIREEWNNFSYFPEI THIVIKESVVSINKQDNKKMELKLSSHEEALSFVSLVDGYFRLTADAHHYLCTDVAPPLIVH NIQNGCHGPICTEYAINKLRQEGSEEGMYVLRWSCTDFDNILMTVTCFEKSEQVQGAQKQFK NFQIEVQKGRYSLHGSDRSFPSLGDLMSHLKKQILRTDNISFMLKRCCQPKPREISNLLVAT KKAQEWQPVYPMSQLSFDRILKKDLVQGEHLGRGTRTHIYSGTLMDYKDDEGTSEEKKIKVI

LKVLDPSHRDISLAFFEAASMMRQVSHKHIVYLYGVCVRDVENIMVEEFVEGGPLDLFMHRK
SDVLTTPWKFKVAKQLASALSYLEDKDLVHGNVCTKNLLLAREGIDSECGPFIKLSDPGIPI
TVLSRQECIERIPWIAPECVEDSKNLSVAADKWSFGTTLWEICYNGEIPLKDKTLIEKERFY
ESRCRPVTPSCKELADLMTRCMNYDPNQRPFFRAIMRDINKLEEQNPDIVSEKKPATEVDPT
HFEKRFLKRIRDLGEGHFGKVELCRYDPEGDNTGEQVAVKSLKPESGGNHIADLKKEIEILR
NLYHENIVKYKGICTEDGGNGIKLIMEFLPSGSLKEYLPKNKNKINLKQQLKYAVQICKGMD
YLGSRQYVHRDLAARNVLVESEHQVKIGDFGLTKAIETDKEYYTVKDDRDSPVFWYAPECLM
QSKFYIASDVWSFGVTLHELLTYCDSDSSPMALFLKMIGPTHGQMTVTRLVNTLKEGKRLPC
PPNCPDEVYQLMRKCWEFQPSNRTSFQNLIEGFEALLK (SEQ ID NO: 3, Amino acid residues V658 and F636 underlined).

[0060] Residues F595 of JAK2 and F636 of JAK1 are homologous, as are residues V617 of JAK2 and V658 of JAK1. Those of skill in the art will be able to identify additional homologous amino acid residues and sequences of JAK1 and JAK2 by methods well known in the art, for example, by sequence alignment. Accordingly, it will be apparent to those of skill in the art that the concepts, methods, and compositions disclosed herein in relation to mutant JAK2 are also applicable in relation to JAK1 having the same or similar mutations in homologous residues and vice versa.

[0061] Those of skill in the art will be able to delineate the sequences of other mutant JAK2 molecules as provided herein, for example K539L, T875N, and R683G mutant JAK2.

[0062] It is well known in the art, that other substitutions at position 617 of JAK2, for example, substitution of the original valine (V) residue with an aromatic amino acid, for example, phenylalanine or tryptophan, are also possible and such substitutions are encompassed within the scope of this invention. Substitution of valine at position 617 with any different naturally occurring amino acid residue are also envisioned and similarly encompassed within the scope of this invention.

[0063] It will be apparent to those of skill in the related arts that homologous sequences exist, which, while overall similar, exhibit mutations of one or more amino acid residues. Typically, such homologous sequences are at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical with the JAK2 or JAK1 sequences provided herein and are encompassed within the scope of this invention.

Screening

[0064] Methods for generating, isolating, purifying, and/or formulating test agents, for example, small organic molecules, peptides, or proteins, are well known to those of skill in

the related arts. A test agent can be synthesized or isolated from a natural source. In some embodiments, the test agent is purified. In some embodiments, a single test agent is characterized by the methods provided herein. In some embodiments, a plurality of test agents are characterized, or screened, for example, in parallel, or sequentially. A test agents may be an agent selected, randomly or rationally, from a list of known agents, for example, a list of agents previously synthesized, a list of agents previously administered to a subject, for example, a human subject or a mammalian subject, a list of FDA approved agents, a historical list of agents, for example, a historical list of a pharmaceutical company, *etc.* Suitable lists of known agents are well known to those of skill in the art and include, but are not limited to, the Merck Index and the FDA Orange Book.

[0065] Small molecules and libraries of small molecules can be obtained from commercial and academic sources, for example, from Sigma-Aldrich (www.sigmaaldrich.com), ChemDiv (www.chemdiv.com), Evotec (www.evotec.com), or ICCB (iccb.med.harvard.edu/screening/compound_libraries/index.htm). Combinatorial libraries of small molecules are also well known to those of skill in the art and may be screened for agent that inhibit JAK2 V617F by methods provided herein.

[0066] In some embodiments, a library of randomly selected and/or randomly synthesized test agents is screened. In some embodiments, a library of rationally selected or designed test agents is screened. In some embodiments, a library of kinase inhibitors is screened. In some embodiments, JAK inhibitors and closely structurally related test agents are screened.

[0067] In some embodiments, test agents are chosen based on 3D modeling of interactions between the F617 and/or the F595 residue of JAK2 V617F and a test agent. In some embodiments, test agents are chosen based on 3D modeling of interactions between the F658 and/or the F636 residue of JAK1 V658F and a test agent In some embodiments, a test agent is designed in silico based on structural data or interaction data of the target protein, for example, JAK2 V617F or wild type JAK2. Methods of rational design of test agents are well known to those of skill in the related art. Structure-based rational design methods allow for the design of small molecules predicted to bind a specific site, for example, a site including F617 in JAK2 V617F, with high affinity and/or selectivity. In some embodiments, test agents are rationally designed de novo. In some embodiments, binding affinity and/or selectivity of a test agent is scored *in silico*, for example, based on an algorithm well known to those of skill in the art, for example, as published in Boehm, 1994, J. Comput Aided Mol Des, 8(3):243-256. Software suitable for various aspects of rational design of test agents is

available from commercial and academic sources and is well known to those in the art. Non-limiting examples of software for rational design of test agents are AutoDock (autodock.scripps.edu), Flexidock, SYBYL (by Tripos, tripos.com) SITUS, DOCK, 3D-Dock-Suite (www.bmm.icnet.uk/docking/), ClusPro (structure.bu.edu/Projects/PPDocking/cluspro.html), DOT, SPROUT (www.simbiosys.ca/sprout/index.html), and GrowMol (Ripka et al., Org Lett 2001 26, 3:2309-2312). In some embodiments, test agents are chosen based on 3D modeling of interactions between the 617, 539, 875, or 683 residue and/or the F595 residue of JAK2 or mutated JAK2 and a test agent. In some embodiments, test agents are chosen based on 3D modeling of interactions between the 658 residue and/or the F636 residue of JAK1 or mutated JAK1 and a test agent. Methods of synthesizing rationally designed small molecules are well known to those in the relevant chemical arts.

[0068] In some embodiments, the present invention provides a method for the design and identification of a potential binding compound for V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1, comprising the steps of: (a) using a three-dimensional structure of V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1; (b) employing the three-dimensional structure to design and/or select the potential binding compound; and (c) synthesizing and/or choosing the potential binding compound.

[0069] Suitable computer programs which may be used in the design and selection of potential binding compounds (*e.g.*, by selecting suitable chemical fragments) include, but are not limited to, GRID (Goodford (1985) *J. Med. Chem.* 28:849 857); *MCSS* (Miranker, A. and M. Karplus, (1991) *Proteins: Structure. Function and Genetics*, 11:29-34); AUTODOCK (Goodsell, D. S, and A. J. Olsen (1990) *Proteins: Structure. Function, and Genetics* 8:195 202); and DOCK (Kuntz, I. D. *et al.* (1982) *J. Mol. Biol.* 161:269-288), the entirety of each of which is incorporated herein by reference.

[0070] Suitable computer programs which may be used in connecting the individual chemical entities or fragments include, but are not limited to, CAVEAT (Bartlett, (1989) *Molecular Recognition in Chemical and Biological Problems*, Special Pub., Royal Chem. Soc. 78:182-196); and *3D Database systems such as MACCS-3D* by MDL Information Systems, San Leandro, Calif.), HOOK (Molecular Simulations, Burlington, Mass.) and as reviewed in Martin, Y. C., (1992) *J. Med. Chem.* 35:2145 2154), the entirety of each of which is hereby incorporated herein by reference.

[0071] In addition to the method of building or identifying a potential binding compound in a step-wise fashion (*e.g.*, one fragment or chemical entity at a time as described above),

potential binding compounds may be designed as a whole or "*de novo*" using either an empty active site or, optionally, including some portion(s) of a known inhibitor(s), activator(s) or stabilizer(s). Suitable computer programs include, but are not limited to, LUDI (Bohm, (1992) *J. Comp. Aid. Molec. Design* 6:61-78); LEGEND (Nishibata, Y. and A. Itai, (1991) *Tetrahedron* 47:8985); and LEAPFROG (Tripos Associates, St. Louis, Mo.). Other molecular modeling techniques may also be employed in accordance with this invention; see, for example, Cohen, N. C. *et al.* (1990) *J. Med. Chem.* 33: 883-894, and Navia (1992) *Current Opinions in Structural Biology* 2:202-210, the entirety of each of which is hereby incorporated herein by reference.

[0072] Once a potential binding compound has been designed, selected, identified, synthesized, or chosen by the methods described herein, the affinity with which that compound binds to V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1 may be tested and optimized by computational evaluation. A compound designed, or selected, or synthesized, or chosen as potential binding compound or may be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the target site. Such non-complementary (*e.g.*, electrostatic) interactions include repulsive charge-charge, dipole-dipole and charge-dipole interactions. Specifically, the sum of all electrostatic interactions between the potential binding compound and the site at which it is bound to V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1, in certain embodiments, make a neutral or favorable contribution to the enthalpy of binding. Suitable computer software which may be used to evaluate compound deformation energy and electrostatic interactions, includes, but is not limited to, *Gaussian 92, revision C* by M. J. Frisch, Gaussian, Inc., (1992) Pittsburgh, Pa.; *AMBER, version 4.0* by P. A. Kollman, (1994) University of California at San Francisco; *QUANTA/CHARMM* by Molecular Simulations, Inc., (1994) Burlington, Mass.; and *Insight II/Discover* by Biosysm Technologies Inc., (1994) San Diego, Calif. These programs may be implemented, for example, using a Silicon Graphics workstation, IRIS 4D/35 or IBM RISC/6000 workstation model 550. Hardware systems, such as an IBM thinkpad with LINUX operating system or a DELL latitude D630 with WINDOWS operating system, may be used. Other hardware systems and software packages will be known to those skilled in the art of which the speed and capacity are continually modified.

[0073] In certain embodiments, binding compounds may be specifically designed and/or selected and/or synthesized and/or chosen by the above methods to induce non-complementary (*e.g.*, electrostatic) interactions, such as repulsive charge-charge, dipole-

dipole and charge-dipole interactions. In certain embodiments, the sum of all electrostatic interactions between the potential binding compound and the site at which it is bound to V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1 make a contribution to the enthalpy of binding that is not neutral.

[0074] In certain embodiments, the above method comprises using a suitable computer program in designing and/or selecting a potential binding compound.

[0075] Additionally, in certain embodiments, the above method comprises using a suitable computer program in conjunction with synthesizing and/or choosing the potential binding compound.

[0076] Furthermore, in certain embodiments, the above method further comprises the steps of using a suitable assay, as described herein, to characterize the potential binding compound's influence on V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1 activity, stability, folding, and/or intracellular localization. In certain embodiments, the above method further comprises: (d) contacting the potential binding compound with V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1 in the presence of a substrate; and (e) determining the amount of substrate conversion of the V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1 compared to a wild-type JAK2 or JAK1, respectively, to determine the effect of the potential binding compound on mutant JAK2 or mutant JAK1 enzymatic activity.

[0077] Alternatively, in certain embodiments, the above method further comprises the steps of: (d) contacting the potential binding compound with a cell that expresses V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1, for example a cancer cell; and (e) determining the effect of the binding compound on V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1 activity in the cell. In certain embodiments, step (e) may comprise determining the effect of the binding compound on the proliferation rate or cell survival.

[0078] Some aspects of this invention relate to methods for identifying an agent that inhibits JAK2 V617F or JAK1 V658F activity. Accordingly, methods are provided herein to determine JAK2 V617F of JAK1 V658F activity in the presence of a test agent. Kinase activity assays, both cell-based and in cell-free systems, are well known to those of skill in the art. The type of assay is dependent on the specific kinase to be assayed. In some embodiments, a kinase activity assay is used to determine the activity of JAK2 V617F or JAK1 V658F in the presence of a test agent. JAK1 and JAK2 kinase assays are well known to those of skill in the art (see, for some non-limiting examples, Robers et al., Assay Drug

Dev Technol 2008, 6:519-529; Lee et al., J Biomed Sci 2006, 13:773-786; EL-Adawi et al., Cardiovasc Res 2003, 57:129-138).

[0079] In general, JAK1 or JAK2 kinase activity may be determined by measuring JAK1 or JAK2-mediated phosphorylation of a JAK1 or JAK2 substrate, typically a protein or peptide, over time. In some embodiments, the kinase activity assay comprises contacting a JAK1 or JAK2 molecule with a suitable substrate, for example, a synthetic substrate peptide, and a labeled phosphate group source, for example, a radiolabeled nucleotide, for example, ^{33}P-ATP or ^{32}P-ATP, under conditions suitable for JAK1 or JAK2 to phosphorylate the substrate, and measuring the transfer of labeled phosphate groups to the substrate over a given time interval. Substrate peptides and proteins as well as suitable sources of phosphate groups and suitable labels are well known in the art and JAK1 and JAK2 kinase activity assays can be purchased from commercial sources for single assays or for use in high-throughput screening methods as provided by some embodiments of this invention (for example, HTScan JAK2 kinase assay, Cell Signaling Technology; JAK2 total ELISA kit, Invitrogen; JAK2 Kinase assay, Perkin Elmer).

[0080] In some embodiments, the substrate may be an enzyme the activity of which changes after phosphorylation by JAK1 or JAK2 and may be measured *in vitro*. In some embodiments, JAK2 knockout cells, for example, γ-2A cells, are used and JAK2 activity is measured in these cells after artificially expressing JAK2 V617F or wild type JAK2 in these cells. In some embodiments, JAK1 or JAK2 activity is assayed by measuring the activity of a JAK1 or JAK2 substrate, for example, a STAT transcription factor, for example, STAT5, in a cell. In some embodiments, the activity of a JAK1 or JAK2 transcription factor phosphorylated by JAK1 or JAK 2 is measured with a reporter construct comprising a promoter with a binding site for the JAK1 or JAK2 phosphorylated transcription factor and a reporter gene, for example, encoding a luciferase, a fluorescent protein, or an antibiotic resistance marker. In some embodiments, the reporter construct comprises an artificial promoter, for example, comprising a minimal promoter, for example, a minimal CMV promoter, and a binding site for the JAK1 or JAK2 activity-dependent transcription factor, for example, a STAT transcription factor, for example, STAT5. In some embodiments, the reporter construct comprises a fragment of a naturally occurring promoter, for example, a promoter bound by a JAK2 activity-dependent transcription factor, operably linked to a reporter gene. In some embodiments the expression of a target gene of a JAK1 or JAK2 activity-dependent transcription factor, for example, a STAT transcription factor target gene,

is assayed by methods known in the art to measure JAK1 or JAK2 activity. Suitable reporter constructs other than those provided herein will be apparent to those of skill in the art.

[0081] In some embodiments, methods are provided to identify an agent that inhibits JAK2 V617F activity and/or JAK1 V658F activity. In some embodiments, methods are provided to identify an agent that inhibits JAK2 V617F activity, but does not inhibit wild type JAK2 activity. For example, an agent that interferes with the pi-stacking interaction of F595 and F617 in JAK2 V617F, but does not bind to, or interfere with the induction of the active conformation of wild type JAK2. In some embodiments, methods are provided to identify an agent that inhibits JAK1 V658F activity, but does not inhibit wild type JAK1 activity. For example, an agent that interferes with the pi-stacking interaction of F636 and F658 in JAK1 V658F, but does not bind to, or interfere with the induction of the active conformation of wild type JAK1. In some embodiments, wild type JAK1 or JAK2 activity is determined in the presence of agents identified to inhibit JAK2 V617F activity and/or JAK1 V658F activity. In some embodiments, JAK1 or JAK2 activity is determined with a JAK1 or JAK2 kinase assay. JAK1 kinase assays are well known in the art and the same assay useful for determining JAK1 V658F activity may be used to determine wild type JAK1 activity. Similarly, JAK2 kinase assays are well known in the art and the same assay useful for determining JAK2 V617F activity may be used to determine wild type JAK2 activity.

[0082] In some embodiments, JAK1 or JAK2 molecules are contacted with a test agent under suitable conditions or the activity of JAK1 or JAK2 is determined under suitable conditions. In some embodiments, suitable conditions allow a test agent to interfere with the interaction of F595 and F617 in JAK2 V617F, or with the interaction of F636 and F658 in JAK1 V658F. In some embodiments, suitable conditions allow a test agent to interfere with the interaction, if any, of amino acid residue 595 and amino acid residue 617 in wild type JAK2, or with the interaction, if any, of amino residue 636 and amino acid residue 658 in wild type JAK1. In some embodiments, suitable conditions are physiological conditions. In some embodiments, suitable conditions are conditions found in a cell in which JAK1 or JAK2 is naturally expressed as well as in cells of the same type in which the JAK1 or JAK2 gene has been knocked out. In some embodiments, suitable conditions are generated in cell-free assays by providing the necessary salts, co-factors, phosphate source, pH, temperature, reaction partners, and/or adjuvants (for example protease inhibitors, stabilizers *etc.*), for example, in aqueous solution. Suitable conditions for JAK1 and JAK2 kinase activity assays are well known in the art and kits containing buffers and descriptions on how to carry out various *in vitro* kinase assays can be obtained from commercial sources. Suitable salts and

reagents will be apparent to those of skill in the art. A non-limiting example of a commercially available suitable buffer is 50 mM HEPES, pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT, and 0.01% Tween 20. Another non-limiting example of a commercially available suitable buffer is 6.25 mM MOPS, pH 7.2, 3.125 mM β-glycerol phosphate, 6.25 mM $MgCl_2$, 1.25 mM EGTA, 0.5 mM EDTA. Suitable phosphate sources are nucleoside triphosphates, for example, ATP and analogs thereof. A suitable pH is a pH within the range of 7.2-7.5, 5.5-7.2, 7.5-8, 4-5.5, 0-4, or 8-12. Suitable temperatures typically are temperatures allowing for the protein to be active without denaturing the protein. Suitable temperatures typically range from 0 °C-4 °C, 4 °C-16 °C, 16 °C-25 °C, 25 °C-37 °C, 37 °C-39 °C, 39 °C-50 °C, or 50 °C-70 °C. Suitable osmolarities are typically within the range of 0.1-10 mM, 10-20 mM, 20-50 mM, 50-100 mM, 100-200 mM, 200-500 mM.

[0083] In some embodiments, the activity of JAK1 and/or JAK2 (JAK1/2) in the presence of a test agent is compared to the activity of JAK1/2 in the absence of the test agent. In some embodiments, the activity of JAK1/2 in the presence of a test agent is compared to the activity of JAK1/2 in the absence of a test agent. In some embodiments, the activity of JAK1/2 in the presence of a test agent is compared to a reference or control activity. Comparing JAK1/2 activities typically includes a quantification step. The methods used for the quantification of JAK1/2 activity assay results depend on the readout of the specific assay and may include, for example, quantification of radiation, fluorescence, luminescence, RNA expression, or protein expression. A reference or control activity may be the activity of JAK1/2 in a control sample. A control sample may be a sample comprising the same conditions and reagents as a sample used to determine JAK1/2 activity in the presence of a test agent, but not containing the test agent, or not containing a test agent. A reference or control activity may be the average activity of JAK1/2 in multiple control samples, for example, in multiple samples comprising the same conditions and reagents as the samples containing test agents, but without added test agents or containing test agents of known effect on JAK1/2 activity. Accordingly, a reference or control activity may be a positive or negative reference or control activity. For example, a negative control activity may be a JAK2 activity, for example wild type JAK2 or JAK2 V617F activity, measured under physiological conditions in the absence of a test agent. A test agent may be identified as an agent that inhibits JAK2 activity if the activity measured in the presence of the agent is at least 10%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 90%, at least 95%, at least 99%, at least 99.9%, or 100% less than JAK1/2 activity observed in the absence of the agent, for example, as a

negative reference or control activity. A positive reference or control activity may be the activity measured in the presence of a strong JAK1/2 inhibitor. Accordingly, a test agent may be identified as an agent that inhibits JAK1/2 activity, if the activity measured in the presence of the test agent is similar or less than the positive control activity. For example, a test agent may be identified as an agent that inhibits JAK1/2 activity, if the JAK1/2 activity in the presence of the test agent is about 300%, about 250%, about 200%, about 150%, about 100%, about 75%, about 50%, about 30%, about 25%, about 10%, or less than 10% the activity observed in a positive control sample, for example, a sample comprising a JAK1/2 activity inhibitor (for example US Patent Application 2006/0106020, SB1518 (S*Bio), TG101348 (TargeGen), WP1066 (Callisto Pharmaceuticals), AG490 (Tocris)). Further, a reference or control activity may be the average JAK1/2 activity in a plurality of samples comprising different test agents, for example, during a screen of a plurality of compounds. A reference or control activity can further be a theoretical or historical activity.

[0084] In some embodiments, the effect on JAK1/2 activity of a plurality of test agents is screened. In some embodiments, at least 50, at least 100, at least 500, at least 1000, at least 5000, at least 10000 , at least 20000, at least 50000, or at least 100000 test agents are screened. Screening of a plurality of test agents may be performed in parallel or sequentially.

[0085] In some embodiments, methods are provided to identify an agent that inhibits JAK2 V617F and/or JAK1 V658F and does not inhibit wild type JAK2 and/or wild type JAK1, respectively. In some embodiments, wild type JAK2 is contacted with an agent identified to inhibit JAK2 V617F under conditions allowing the agent to inhibit JAK2 V617F activity and wild type JAK2 activity is measured. If the agent does not significantly affect wild type JAK2 activity, then it is identified as an agent that inhibits JAK2 V617F and does not inhibit wild type JAK2. In some embodiments, wild type JAK1 is contacted with an agent identified to inhibit JAK1 V658F under conditions allowing the agent to inhibit JAK1 V658F activity and wild type JAK1 activity is measured. If the agent does not significantly affect wild type JAK1 activity, then it is identified as an agent that inhibits JAK1 V658F and does not inhibit wild type JAK1. An agent that inhibits the activity of the mutant but not the wild type JAK may be useful in decreasing the proliferation of hematopoietic cells in subjects having a myeloproliferative disorder.

[0086] Methods for the use of an agent that inhibits mutant JAK activity, for example, JAK2 V617F or JAK1 V658F activity, are provided by some aspects of this invention. In some embodiments, methods to administer an agent that inhibits mutant JAK, for example, JAK2 V617F or JAK1 V658F, to a subject having a myeloproliferative disorder are provided.

In some embodiments, methods of administering a JAK2 V617F inhibitor or a JAK1 V658F inhibitor to a subject having a myeloproliferative disorder or a hematological malignancy are provided. In some embodiments, the subject is identified as expressing JAK2 V617F or carrying a gene coding for JAK2 V617F, or as expressing JAK1 V658F or carrying a gene coding for JAK1 V658F.

[0087] A treatment according to some aspects of this invention can be a monotherapy, for example, treating a subject only by using one or more methods described herein, for example, to reduce hematopoietic cell proliferation. However, the treatment also can be an adjunct therapy to one or more other therapies, for example, immune therapies, radiation therapies, and/or chemotherapies.

[0088] Therapeutic compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary agents affecting JAK2 signaling and/or JAK1 signaling, such as cytokine inhibitors, and optionally other therapeutic agents.

[0089] The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

[0090] Therapeutics according to some embodiments of the invention can be administered by any conventional route, for example, injection or infusion over time. The administration may, for example, be oral, intravenous, intratumoral, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal. Depending on the type of therapeutic, an agent inhibiting JAK1/2 activity may be administered by pulmonary aerosol. Whether or not an agent is suitable for aerosolic delivery can be readily determined by those of skill in the art. Techniques for preparing aerosol delivery systems containing therapeutic agents are well known to those of skill in the art. Generally, such systems should utilize components which will not significantly impair the biological properties of the agent, such as the capacity to inhibit the activity of JAK2 V617F and/or JAK1 V658F (see, for example, Sciarra and Cutie, "Aerosols," in *Remington's Pharmaceutical Sciences*, 18th edition, 1990, pp 1694-1712). Those of skill in the art can readily determine the various parameters and conditions for producing pharmaceutical compositions of the identified agents inhibiting JAK2 V617F and/or JAK1 V658F activity.

[0091] The compositions of some embodiments of the invention are administered in effective amounts. In some cases of administering an agent identified to inhibit JAK1/2 activity to a subject having a disorder characterized by the presence of aberrant, constitutively active JAK1/2 signaling, such as the myeloproliferative disorders described herein, the desired response may be inhibiting the progression of the disorder. This may involve slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. In some cases, the desired response is a permanent reduction of hematopoietic cell proliferation to levels comparable to those found in healthy individuals. In some cases, the desired response can be delaying or preventing the manifestation of clinical symptoms characteristic for the disease or condition.

[0092] The effect of administering an agent identified to inhibit JAK1/2 activity by a method provided herein can be monitored by routine methods well known to those of skill in the related medical arts.

[0093] The effective amount will depend on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health care professional treating the subject. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a lower dose or tolerable dose may be used for medical reasons, psychological reasons, or virtually any other reasons.

[0094] Typical dosages for agents identified by methods of this invention are well known to those of skill in the art or can be determined empirically. Typical dosage ranges are (given as weight of agent/body weight of the subject), for example, 1-100 ng/kg, 0.1-1 μg/kg, 1-100 μg/kg, 0.1-1 mg/kg, 1-10 mg/kg, 10-100 mg/kg, or 0.1-1 g/kg. It will be apparent to those of skill in the art that the dosage will depend on the structure and characteristics of the agent to be administered, the administration route, and other factors well known to those of skill in the pharmaceutical and medical arts. Administration may be as a single dose, or as multiple, subsequent doses. In some embodiments, four or more individual doses are administered per day, in some embodiments 1-3 individual doses are administered per day, in some embodiments, 1-7 individual doses are administered per week, in some embodiments, 1-5 individual doses are administered per month.

[0095] Pharmaceutical compositions according to some embodiments of this invention some of which are exemplified in the foregoing methods preferably are sterile and contain an effective amount of one or more therapeutic agents as described herein for producing the desired response in a unit of weight or volume suitable for administration to a patient. The response can, for example, be measured by determining the proliferation of hematopoietic cells in a subject after treatment by, for example, cell counting, flow cytometry, FACS, and other methods well known in the art to be suitable to determine cell proliferation.

[0096] The doses of one or more therapeutic agents as described herein (for example, small molecule, peptide, protein, antibody, or aptamer) administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired period of treatment. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

[0097] Administration of therapeutic compositions to mammals other than humans, for example, for testing purposes or veterinary therapeutic purposes, is also envisioned herein and may be carried out under substantially the same conditions as described above.

[0098] The pharmaceutical compositions may contain suitable buffering agents, for example, acetic acid in a salt form, citric acid in a salt form, boric acid in a salt form, and/or phosphoric acid in a salt form.

[0099] The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: ascorbic acid, benzalkonium chloride, chlorobutanol, parabens, EDTA, EGTA, and/or thimerosal.

[00100] The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy.

[00101] All methods may include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

[00102] Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other examples of compositions include suspensions in aqueous liquids or non-aqueous liquids, such as a syrup, elixir, or an emulsion. Examples of compositions for

parenteral administration include, without being limited to, sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Examples of aqueous carriers are water, alcoholic/aqueous solutions, emulsions or suspensions, for example, saline and buffered media. Examples of parenteral vehicles are sodium chloride solution, Ringers dextrose, dextrose and sodium chloride, and lactated Ringers or fixed oils. Examples for intravenous vehicles are fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringers dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases, and the like.

[00103] The pharmaceutical agents of some embodiments of the invention may be administered alone, in combination with each other, and/or in combination with other drug therapies and/or treatments. Examples of therapies and/or treatments may include, but are not limited to, surgical intervention, chemotherapy, radiotherapy, and adjuvant systemic therapies.

[00104] The entire contents of all references cited herein are hereby incorporated by reference.

[00105] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as illustrations of particular aspects of the invention and other functionally equivalent embodiments are well within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each and every embodiment of the invention.

Examples

JAK2 V617F Constitutive Activation Requires JH2 Residue F595: A Pseudokinase Domain Target for Specific Inhibitors

A predicted interaction between residue 617 and the JH2 αC helix

[00106] The structure of the pseudokinase domain has not been solved for any JAK family member. Since residue V617 is located in the JH2 domain of JAK2, this lack of information has hindered a detailed understanding of the mechanism of activation of JAK2 V617F. A homology model of the kinase and pseudokinase domains of JAK2, suggests an overall 3D structure of the JH2 domain, similar to that of JH1 and other solved kinase domains, as well as a potential face-to-face arrangement of the two domains (20). This model places residue V617 in a loop connecting β-strands 4 and 5 in the N-terminal lobe of JH2 and in close proximity to the JH2 αC helix. The β4/β5 loop as well as the αC-β4 loop that precedes β4, were previously shown to play regulatory roles in the mechanisms of Src and Abl tyrosine kinases through interactions with the αC helix in the N-terminal lobe (33, 34). A specific conformation of the αC helix is essential for kinase activation (18) and members of the kinase family have evolved diverse ways to influence the position of their αC helices as a means to affect activity (11, 12, 19).

[00107] Given the proximity of the V617F mutation to the JH2 αC helix, we sought to identify residues in the JH2 αC helix, which could have a potential effect on the overall position and conformation of this helix. Inspection of the crystal structures of several kinases revealed that the closest residue in space to the homologous V617 position frequently resided in the middle of the αC helix, on the face of the helix facing the homologous V617 residue (Figure 1A and B). In JAK2, this residue corresponds to F595 in the JH2 αC helix (Figure 1C). The homologous residues of position 595 are generally represented by large, hydrophobic residues pointing to preservation of the hydropathy profile at this position, and suggesting a mechanistic role for those residues in kinase function (Figure 1A).

[00108] Because of their predicted arrangement, we hypothesized that when V617 is mutated to F, this could create a strong F617-F595 hydrophobic interaction based on pi-stacking between the two phenyl rings (Figure 1C). This interaction would be able to affect the conformation of αC-helix of JH2 in a specific manner that would translate to the conformation of αC-helix of JH1, inducing activation in the absence of ligand. A similar

type of hydrophobic stacking interaction has been shown to play a role in the regulation of catalysis of c-Src kinase (21).

F595A decreases the constitutive activity of JAK2 V617F

[00109] When V617 is mutated to Phe, this could influence the neighboring F595, affecting the conformation of the αC helix of JH2 in a specific manner, that would promote activation in the absence of ligand (Figure 1C). Such Phe-Phe stacking interactions in regulatory domains were shown to play a role in the regulation of catalysis of c-Src (21) and ZAP-70 kinases (35).

[00110] The mutation of V617 to Ala does not support constitutive activation of JAK2 (22). In order to further test the effect of the predicted contact between F595 and F617, we mutated the Phe at position 595 of murine JAK2 to Ala and tested the activity of the F595A/V617F double mutant in a luciferase assay, in the presence of the erythropoietin (Epo) receptor and in the presence or absence of stimulation by Epo. We used the JAK2- and STAT5-deficient γ-2A fibrosarcoma cell line and quantified the STAT5 transcriptional activity induced by JAK2 F595A/V617F, as well as JAK2 WT and V617F as negative and positive controls, respectively. As the γ-2A cell line does not express any endogenous JAK2, the activity levels we elicited were due solely to our transfected mutants. We measured an approximately 70% decrease in STAT5 transcriptional activity of the F595A/V617F mutant compared to V617F (Figure 2). As seen in Figure 2, the F595A single mutant displayed an activity level comparable to WT and all mutants retained the ability to respond to Epo ligand normally. Similar results were obtained with another mutant, F595K. Neither F595A nor F595K induced constitutive activation of wild type JAK2.

[00111] We next wanted to observe the effect of the F595A/V617F mutation in Ba/F3 cells and compare its activity level to V617F alone and to WT. 293T-derived BOSC cells were used to produce retroviral supernatants of the JAK2 mutants cloned into the bicistronic vector pMX-IRES-GFP. Ba/F3 cells expressing the murine Epo receptor were infected with the supernatants and sorted for similar GFP levels 72 hours later. Cells were then washed, incubated without serum overnight, and electroporated with pGRR5 and pRLTK reporters. Similar to the γ-2A cells, we observed a marked decrease in STAT5 transcriptional activity of the sorted Ba/F3-EpoR cells expressing the F595A/V617F mutant as compared to cells expressing V617F alone. Both WT and F595A expressing Ba/F3-EpoR cells displayed a low activity in the absence of ligand but responded well upon addition of Epo (Figure 4b).

[00112] Using the same sorted Ba/F3-EpoR cells expressing JAK2 mutants we performed a proliferation assay in a medium without growth factors. JAK2 WT and JAK2 F595A were not able to support constitutive proliferation in this minimal medium, but as expected they could grow in the presence of Epo, while JAK2 V617F proliferated at a very high rate in the absence of any growth factors (Figure 4a). The cells expressing F595A/V617F lost most of their proliferative advantage, consistent with a specific interaction between F595 and F617 being responsible for the preservation of constitutive activity (Figure 4a).

[00113] Lastly, the sorted Ba/F3-EpoR cells expressing each mutant were stimulated or not with Epo, lysed and immunoblotted in the presence of phosphospecific antibodies (Figure 4c). The total lysate levels probed with anti-JAK2 revealed that all JAK2 proteins were expressed at the correct size of 130 kDa. When comparing their pSTAT5 levels (anti-phosphoY694), we were able to detect a pSTAT5 activity for V617F, in the absence of Epo stimulation. This activity level was decreased in the F595A/V617F mutant, similar to what we observed in the luciferase assays and was altogether not detectable in JAK2 WT or F595A. As expected, all mutants displayed strong pSTAT5 and pJAK2 activation upon Epo stimulation. Ba/F3-EpoR cells expressing JAK2 V617F which had previously been selected for autonomous growth exhibited the strongest pSTAT5 level, both in the presence and absence of Epo.

[00114] In order to further investigate the effect of the predicted contact between F595 and F617, we mutated the Phe at position 595 of murine JAK2 to several different amino acids in the context of V617F, and tested the activity of the double mutants in luciferase assays by quantifying the STAT5 transcriptional activity induced by each mutant in transient transfections in the JAK2-deficient fibrosarcoma cell line, γ-2A (36). As the γ-2A cell line does not express any endogenous JAK2, the activity levels we elicited were due solely to our transfected mutants. We observed up to an 85% decrease in the constitutive activity of JAK2 V617F, depending on the particular amino acid substitution at position 595 (Figure 3A). Mutation of F595 to Lys elicited the highest decrease in JAK2 V617F constitutive activity, followed by Ala, Val and Ile. Contrarily, mutation of F595 to Tyr or Trp, supported a level of constitutive activity of V617F similar to non-mutated F595, indicating the presence of an aromatic residue at position 595 is required to maintain the constitutive activity of JAK2 V617F (Figure 3A).

Next, we asked whether mutation of the F595 residue to Ala, Lys, Val or Ile critically influenced the ability of wild-type JAK2 or of JAK2 V617F to respond to Epo-activated

EpoR. As depicted in Figure 3B, F595 mutations generally do not impair activation by Epo. The F595I mutant responds to Epo indistinguishably from JAK2 wild-type. Mutants F595A and F595V exhibit a small inhibition to high Epo doses (Figure 3B), while in the V617F context these mutants do not affect Epo response (Figure 3C). The F595K mutants in contrast, appear to reduce Epo activation of JAK2 V617F to a certain degree. Importantly, the single mutations at F595 did not induce constitutive activation of JAK2 (Figure 3B). Taken together, our results indicate that helix C residue F595 is critical for constitutive activation of JAK2 signaling by V617F, and that the nature of the amino acid substitution at F595 can alter the full response to ligand but is not required for initiation of activation of JAK2 in response to cytokine receptor activation.

Functional studies of the F595A mutation on the proliferation and transformation of Ba/F3 cells stably expressing JAK2 F595A/V617F

[00115] The effect of the F595 substitution on the constitutive activity of JAK2 V617F, in stably-expressing Ba/F3 cells and compared its activity level to the V617F single mutant and to wild-type. Ba/F3 are murine bone-marrow derived proB cells which depend on interleukin-3 (IL-3) for proliferation. Withdrawal of IL-3 leads to cell death, unless JAK2 V617F is expressed. We chose the F595 to Ala mutation as it induced a large decrease in the constitutive activity of V617F (Figure 3A). 293T-derived BOSC cells were used to produce retroviral supernatants of the JAK2 mutants cloned into the bicistronic vector pMX-IRES-GFP. Ba/F3 cells expressing the murine EpoR were infected with the supernatants and sorted for similar GFP levels 72 hours later. The sorted cells stably expressing each JAK2 mutant were washed and their proliferation in growth factor-free medium was followed for 7 days. Parental Ba/F3-EpoR cells and the sorted cells expressing JAK2 wild-type or JAK2 F595A were not able to support constitutive proliferation in this minimal medium, but could grow in the presence of Epo, while JAK2 V617F-expressing cells proliferated at a very high rate in the absence of any growth factors (Figure 4A). The cells expressing JAK2 F595A/V617F lost most of their proliferative advantage, consistent with the presence of F595 being key to the preservation of constitutive activity (Figure 4A). As an additional control, we included Ba/F3-EpoR cells expressing JAK2 F595W/V617F, a mutant which maintained a level of constitutive activity almost identical to V617F in luciferase assays (Figure 3A). As expected, the JAK2 F595W/V617F mutant could also support autonomous growth of sorted Ba/F3-EpoR cells to a level identical to V617F (Figure 4A), pointing to an aromaticity requirement at position 595 playing a role in the constitutive activity of V617F.

[00116] Aliquots from the sorted cells expressing JAK2 wild-type, V617F and F595A/V617F were separately washed, incubated without serum and cytokines overnight (starvation) and electroporated with pGRR5-Luc and pRLTK-Luc reporters the next day. The STAT5 transcriptional activity induced by each mutant was measured two hours later. Similar to the γ-2A cells (Figure 3A), we observed a marked decrease in STAT5 transcriptional activity of the sorted Ba/F3-EpoR cells expressing the F595A/V617F mutant as compared to cells expressing V617F alone (Figure 4B). Ba/F3-EpoR cells expressing JAK2 wild-type displayed a low activity in the absence of ligand, but responded well upon stimulation with 50 U/ml Epo (Figure 4B). As an additional positive control, we included JAK2 V617F-expressing cells that had previously been selected for autonomous growth. As expected, these cells induced a constitutive STAT5 activation in the absence of cytokines almost double that induced by their sorted counterparts (Figure 4B).

[00117] Lastly, the sorted Ba/F3-EpoR cells expressing each mutant were stimulated or not with Epo, lysed and immunoblotted in the presence of phosphospecific antibodies (Figure 4C). When comparing the pJAK2 and pSTAT5 levels in the total lysates, we were able to detect pJAK2 and pSTAT5 activities for V617F, in the absence of Epo stimulation. This activation was absent in the cells expressing JAK2 wild-type and JAK2 F595A/V617F, but could be detected to a lower degree in the cells expressing JAK2 F595W/V617F, consistent with the proliferation data (Figure 4A) and the transient transfection luciferase assay (Figure 3A). As expected, all mutants displayed strong pSTAT5 and pJAK2 activation upon Epo stimulation (Figure 4C). We also investigated how signaling via the Erk pathway was affected by the F595 mutations and detected that pErk1/2 levels were elevated in nonstimulated sorted cells expressing JAK2 V617F, but decreased once F595A mutation was additionally introduced. However, substitution of F595 to Trp, restored the autonomous pErk1/2 signaling (Figure 4C). Taken together, these results indicated that a Phe, or at least another aromatic residue, was required at position 595 to support JAK2 V617F constitutive signaling via the STAT and Erk pathways.

F595A also decreases activity of JAK2 V617W and V617M

[00118] We previously showed that substitution of V617 by large, non-polar amino acids induces constitutive activation of JAK2. Specifically, we observed that succeeding V617F, the next two strongest mutants were V617W and V617M (Figure 5, see also (22)). If the mechanism of constitutive activation indeed involves a specific hydrophobic interaction

between F595 and F617, the same mechanism should be responsible for activating JAK2 V617W and V617M, and eliminating the F595-W617 or F595-M617 interaction by the F595A mutation, would negatively affect the constitutive activity of these mutants. When we mutated F595 to Ala in the context of V617W and separately of V617M, both double mutants indeed lost most of their STAT5 constitutive activity in luciferase assays, consistent with our hypothesis (Figure 6a, b). The fact that JAK2 V617F and V617W displayed the strongest constitutive activity in our previous study, also suggested to us that the strength of constitutive activity may be proportional to the strength of hydrophobic contact between positions 595 and 617. What distinguishes Phe, Trp, and Tyr from Met, Ile and Leu is the presence of an aromatic ring which is capable of making a pi-stacking type of hydrophobic interaction with F595, stronger than a simple hydrophobic interaction such as V617M-F595 for example.

Orientation of the dimeric Epo receptor is essential for activation of JAK2 WT, but not for V617F or V617W

[00119] Given that JAKs are appended to the cytoplasmic domains of cytokine receptors, the hypothesis that V617F can induce activation of JAK2 in the absence of ligand through a specific interaction with the JH2 αC-helix which changes the position of the JH1 αC-helix, has one immediate implication. That is, JAK2 V617F can overcome the requirement for a ligand-induced conformational change and effectively be capable of signaling from all possible relative conformations of a dimeric cytokine receptor, while in the wild-type scenario the proper conformation of JH1 αC-helix can only be achieved by a ligand-induced conformational change in the receptor transmembrane and cytoplasmic domains leading to the proper active conformation and triggering catalytic activity. In order to test whether this implication holds true, we employed a system we previously used to determine the active orientations of the transmembrane and cytosolic domains of the Epo receptor (23). Briefly, the extracellular domain of the Epo receptor was replaced with a dimeric coiled-coil and by varying the junction between the coiled coil and transmembrane domain, all seven possible conformations were imposed on the TM-cytosolic domains of EpoR (Figure 7A). The predictions of dimeric orientation were confirmed by cysteine-scanning mutagenesis of the transmembrane domain of EpoR in fusion with the Put3 coiled coil (42). Using this system we identified two conformations corresponding to an activated EpoR dimer (denoted cc-

EpoR-III and cc-EpoR-VI) and two corresponding to an inactive EpoR dimer (denoted cc-EpoR-II and cc-EpoR-V) (Figure 7A) (23).

[00120] We co-expressed these previously engineered coiled-coil EpoR constructs with either JAK2 WT or V617F, along with STAT5, pGRR5-Luc and pRLTK-Luc, and measured their STAT5 transcriptional activities in luciferase assays in γ-2A cells. We observed that, as expected, JAK2 WT signals best from the two conformations corresponding to an active EpoR dimer (III and VI) (Figures 7B and 8a). On the other hand, consistent with our hypothesis, we observed that V617F was able to signal relatively well regardless of the particular dimeric conformation (Figures 7B and 8a). This is in line with reports showing that presence of a dimeric receptor, like EpoR, supports constitutive activation of JAK2 V617F (28, 29). Next we tested whether JAK2 V617W is also able to signal well from all interfaces of a dimeric EpoR similarly to V617F and found that indeed it is (Figure 8b), confirming that the mechanisms of activation of JAK2 V617F and V617W are similar.

[00121] We then compared the activities of two F595 mutants which decreased JAK2 V617F constitutive activity, F595A/V617F and F595V/V617F with V617F and wild-type in the same assay. We noticed that substituting the Phe at position 595, causes the F595A/V617F and F595V/V617F mutants to lose their ability to signal from both active and inactive dimeric conformations (Figures 3A and 8c) and to prefer the conformations corresponding to an activated EpoR dimer, similar to JAK2 wild-type (III and VI) (Figure 7B).This suggests that the F595- F617 interaction is responsible for conferring to the V617F mutant the ability to overcome the requirement for a particular ligand-induced active conformation.

F595-F617 interaction affects the relative position of the αC-helices of JH1 and JH2

[00122] The homology model of the combined structure of the JH1 and JH2 domains in an inactive conformation proposes an interaction interface between the αC-helices of both domains defined by two pairs of salt bridges between R588.E890 and E592.R893 (20). On the other hand, both available crystal structures of the active JH1 domain of JAK2 (8, 10) describe a E890.R893 salt bridge within the JH1 αC-helix itself. We speculated that the R588.E890 and E592.R893 salt bridges could be part of a network of interactions responsible for keeping the JH1 αC-helix in an inactive conformation in the absence of ligand. These predicted inactive state interactions could either simply prevent the formation of the E890.R893 salt bridge, which would further inhibit the proper arrangement of the critical salt

bridge between Glu898 in JH1 αC-helix and Lys882 in β3, one of the features of active kinases (24, 18, 25); or prevent a more complex conformational change of the JH1 αC-helix, both of which would play a role in kinase activation (Figure 9a).

[00123] In order to test this, we mutated both R588 and E592 to Ala simultaneously in the context of WT and V617F and checked the activities of the R588A/E592A and R588A/E592A/V617F mutants in a luciferase assay in γ-2A cells. As seen in Figure 9b, and in Figure 13, mutating R588 and E592 both to Ala, did not block constitutive signaling by JAK2 V617F. However, when we additionally introduced the F595A mutation in R588A/E592A/V617F, the activity level of this quadruple mutant dropped down to the level of F595A/V617F (Figure 9b, 13). This result suggested that the Lindauer model of interface 1 cannot alone explain the V617F activation and that it is not sufficient to eliminate the predicted salt bridges between the αC of JH1 and JH2 in the inactive conformation; the specific functional interaction between F595 and F617 is additionally required for induction of complete constitutive activity of V617F. We speculate that the pi/hydrophobic interaction can reposition JH2 αC-helix, causing a rearrangement of the JH1 αC into an active conformation.

[00124] This type of repositioning of the kinase αC-helix upon activation has been observed in other kinases. We compared the conformations of αC-helices from several kinases whose structures were solved in both active and inactive states and noticed that in all cases, the active αC-helix (cyan) is displaced relative to the inactive αC-helix (grey) (Figure 9c). This displacement probably contributes to the proper arrangement of a group key residues involved in catalysis, such as the salt bridge between a residue in the αC-helix and one in the β3-strand; the Tyr residues in the activation loop, or residues which play a role in ATP or substrate binding (24).

Pseudokinase domain F595 is required for activation of JAK2 K539L

[00125] In some rare polycythemia vera (PV) cases where the V617F mutation is not present, four somatic gain-of function mutations in exon 12 of JAK2 have recently been reported, out of which JAK2 K539L mutation was the most common (17). Position K539 is predicted to be located in a loop just below the loop containing V617 (20, Figure 10). We wondered whether the mechanism of activation of the K539L mutation could be similar to that of V617F. According to the model (20), K539 forms a salt bridge with residue D620, also located in the loop containing V617 (Figure 10).

[00126] We hypothesized that the K539-D620 salt bridge confers a certain rigidity to the loop containing D620 and V617F, and that mutation of K539 to Leu would eliminate the salt bridge, allowing greater flexibility of the loop containing D620 and V617. If the activation mechanisms of these two mutations were similar, this increased flexibility would permit V617 to reach 'higher up', and make a hydrophobic contact with F595, inducing a similar activation effect as V617F-F595 upon the JH1 αC-helix, but of lesser intensity, since Val is a considerably smaller hydrophic residue compared to Phe and not capable of making pi-stacking interactions.

[00127] We first established the significance of the predicted K539-D620 salt bridge by mutating D620 to Leu. In a luciferase assay in JAK2-deficient γ-2A cells, the JAK2 D620L mutant displayed a STAT5 transcriptional activity similar to that of K539L and almost 50% higher than WT, pointing to the elimination of the salt bridge by disrupting either interaction partner, playing a role in the constitutive activation of JAK2 . A previously described JAK2 D620E mutation in an isolated case of unclassifiable myeloproliferative disease (MPD, 26) supported the notion that not only is the presence of the salt bridge between positions D620 and K539 important, its precise position and 'length' are also carefully regulated. E620 should theoretically be able to support a salt bridge with K539 as well as D620, but Glu is one carbon longer than Asp.

[00128] We then mutated F595 to Ala in the context of K539L, which we expected to decrease the constitutive activity of the K539L single mutation, if the activation mechanism were similar to that of V617F. As can be observed from Figure 11a, the level of STAT5 transcriptional activity of the K539L mutant was markedly decreased when F595A was present, suggesting that the mechanism of activation of the K539L mutation occurs, at least in part, through engaging the hydrophobic interaction between positions F595 and V617.

Pseudokinase domain αC helix residue F595 is also required for constitutive activation of the JAK2 mutants T875N and R683G

[00129] We next asked the question whether the integrity of F595 also plays a role in the constitutive activity of other previously-described JAK2 mutants. We presented in the previous paragraph the situation of mutation JAK2 K539L: In 3% of PV patients where the V617F mutation is not present, four somatic gain-of-function mutations in exon 12 of JAK2 have been reported, out of which JAK2 K539L, located in the linker region between the SH2 and JH2 domains, was the most common (37). These exon 12 mutations are typical for each

patient, and what they have in common is that K539 is either mutated (e.g. K539L) or deleted. Therefore the JAK2 K539L is the prototype for all class of exon 12 JAK2 mutations. Another point mutation first identified in a megakaryoblastic cell line derived from an acute megakaryoblastic leukemia (AMKL) patient, and subsequently detected in a screen of human AMKL cell lines for STAT5 activation, presented a T875N substitution in the kinase domain of JAK2 (38). The JAK2 R683G mutation located in the hinge region between N-terminal and C-terminal lobes of the pseudokinase domain, was identified in a screen of pediatric acute lymphoblastic leukemia (ALL) patient samples (39). JAK2 K539L, T875N and R683G were shown to exhibit constitutive STAT5 activation and to transform Ba/F3 cells expressing the EpoR to cytokine-independence (37, 38, 39).

[00130] We introduced the F595A mutation individually in the context of JAK2 K539L, JAK2 T875N and JAK2 R683G and studied its effect on the activity of each mutant by quantifying the STAT5 levels in a luciferase assay of transiently transfected γ-2A cells. In all three mutants, JAK2 K539L, T875N and R683G, and similar to JAK2 V617F, the STAT5 activity was strongly inhibited by the presence of the F595A mutation (Figure 11A), indicating that F595 is also required for constitutive activation of these JAK2 mutants.

[00131] The signaling effects of the F595A mutation were also examined in sorted Ba/F3-EpoR cells expressing JAK2 K539L/F595A, JAK2 T875N/F595A and JAK2 R683G/F595A (Figure 11B). Cells were washed and maintained in medium free of growth factors for 7 days. We observed that Ba/F3-EpoR cells expressing JAK2 V617F, JAK2 K539L, JAK2 T875N and JAK2 R683G could proliferate in this medium, while the same mutants with the additional F595A mutation lost most of their autonomous growth. In the case of JAK2 K539L/F595A, the presence of the F595A mutation induced a decrease in the initial proliferation rate, but by day 7, these cells had regained the same proliferation ability as JAK2 K539L. These results suggested that residue F595 is also important in the constitutive signaling of JAK2 T875N and JAK2 R683G and initially to JAK2 K539L, implying that activation of catalysis by JAK mutations may be mediated by conformational changes in the JH2 αC helix.

The JAK1 helix C residue F636 is also required for constitutive activity of JAK1 V658F

[00132] We have previously shown that the JAK2 V617F homologous mutation in JAK1, V658F, induces constitutive activation of JAK1 (30). Recently the JAK1 V658F mutation was also identified from a screen of acute leukemia patients (40). We now investigated

whether the F595 homologous residue in JAK1, F636, also plays a role in the constitutive activation of this kinase, in a similar manner to JAK2. We substituted JAK1 residue F636 to Ala in the context of V658F, and quantified the STAT3 activity of this mutant in the JAK1-deficient cell line, U4C. As can be seen in Figure 12, substitution of F636 to Ala induces a large decrease in the constitutive activity of JAK1 V658F, indicating that the necessity of F636 is maintained in JAK1, similar to JAK2. This similar requirement of an intact F636 further points to the possibility that our data might also be relevant for JAK1 mutations recently identified in T-cell adult lymphoblastic leukemia (T-ALL) (40, 41). In these diseases, either the JAK1 V658F mutation was identified (a mutation reported in Staerk J, Kallin A, Royer Y, Diaconu CC, Dusa A, Demoulin JB, Vainchenker W, and Constantinescu SN, *JAK2, the JAK2 V617F mutant and cytokine receptors.* Pathol Biol (Paris). 2007 Mar;55(2):88-91.) or several other mutations in the pseudokinase domain which are likely to behave similarly and depend on F636.

Discussion

[00133] Despite an overabundance of data underlining the role of JAK2 V617F in various myeloproliferative syndromes, the mechanism of its constitutive activation has remained elusive. We propose a mechanism of constitutive activation of JAK2 V617F based on a specific interaction between F617 and F595 located in the αC-helix of the JH2 domain.

[00134] Residue F595, residing in the middle of the pseudokinase domain αC helix, plays a key role in the constitutive activation of JAK2 V617F and of several JAK2 mutants located in different locations of the protein, JAK2 K539L, JAK2 T875N and JAK2 R683G. Residue F595 is not crucial for the mechanism of ligand-induced JAK2 activation, as shown by the signaling abilities of the F595 single and double mutants in the presence of various concentrations of Epo ligand. Also, mutation of F595 to several different residues did not induce constitutive signaling from wild-type JAK2.

[00135] Mutation of F595 to Ala, Val, Ile or Lys in the context of V617F drastically decreased the constitutive activity of the V617F mutant in both the JAK2-deficient γ-2A and the hematopoietic Ba/F3 cell line, in transient and stable expression experiments, respectively. On the contrary, when F595 was replaced by an aromatic residue (Tyr or Trp) in the context of V617F, the constitutive activity was maintained. The substitution of F595 to Ala decreased the constitutive activity of JAK2 K539L, JAK2 T875N, and also of JAK2 R683G. Furthermore, constitutive activity of a JAK1 mutant homologous to JAK2 V617F

(JAK1 V658F) was also inhibited by the homologous JAK1 F595 mutation (F636A), suggesting that our results in JAK2 might be relevant for activation of JAK1 mutants recently identified in T-ALL (40, 41).

[00136] Likewise, F595A was able to decrease the constitutive activity of JAK2 V617W, a mutant which we previously showed is able to induce a myeloproliferative phenotype in mice, similar to the one induced by V617F 22. Both Phe and Trp are large aromatic amino acids, pointing to the mechanism of constitutive activation being based on the ability of these two amino acids to form strong pi-stacking hydrophobic interactions with F595, when substituted for Val at position 617. The strict requirement of hydrophobicity is emphasized by the inability of Tyr, which only differs from Phe by an OH group, to induce constitutive activity of JAK2 (22). The nature of the hydrophobic interaction being pi-stacking, is suggested by the strength of the JAK2 V617F and V617W mutations in comparison to the other hydrophobic residues such as Ile, Met, and Leu which can also induce constitutive activity (22).

[00137] A theoretical study was recently published (27), where molecular dynamics simulations indicated that F595 might be responsible for maintaining wild-type JAK2 inactive and, as a consequence, its possible interaction with F617 could alleviate this inhibition and contribute to activation. One the one hand, we agree with Lee *et al* that F595 is crucial for JAK2 V617F activity, but our results support the contrary notion for the wild-type JAK2, that is, F595 does not play a major role in the physiologic ligand-activation of JAK2, or in its inactivity in the basal state (Figures 3B and 7C). On the other hand, we do support a key role for F595 not only in the constitutive activity of JAK2 V617F, but also of several JAK2 oncogenic mutants.

[00138] Our mechanism, the first to suggest how a specific molecular interaction could induce constitutive activation of JAK2, entails that in the absence of ligand, V617F is able to affect the position of the αC-helix of JH1 by affecting the position of αC-helix of JH2. This repositioning of the helices presumably allows formation of the critical K882-E898 salt bridge, ultimately driving kinase activation. Given this scenario, we would expect that, when appended to a dimeric cytokine receptor, JAK2 V617F would signal irrespective of the particular dimeric orientation of the receptor. We investigated this hypothesis by using coiled-coil constructs that imposed all possible relative conformations on a dimeric Epo receptor (Seubert, Royer et al., 2003, 12:1239-1250). When coexpressed with each different dimeric EpoR, JAK2 V617F was able to induce a constitutive signal regardless of the relative

dimeric conformation. JAK2 WT, on the other hand, was activated best in the presence of the particular conformations corresponding to an activated EpoR dimer, emphasizing the requirement of a ligand induced conformation. The double mutant F595A/V617F lost its ability to signal constitutively from all conformations of the EpoR dimer, and maintained an activity level similar to WT, pointing to the specific interaction between F595 and F617 being responsible for conferring the constitutive activity. Our results are in line with those of previous publications (28, 29) that showed that the mere presence of a dimeric cytokine receptor promotes JAK2 V617F signaling. Furthermore, at higher expression levels, JAK2 V617F can induce signaling in the absence of co-expressed EpoR (30, 31), probably via endogenous receptors, such as IL3 receptor beta (29), also pointing towards an intrinsic driving mechanism for activation of the kinase domain. Our data suggest that a specific interaction might occur between F595 in the JH2 αC helix and F617, predicted to be located in the loop between β-strands 4 and 5 (Figure 1C) (20). In the case of JAK2 V617F, a Phe, Trp or Tyr at position 595 are able to maintain constitutive activity, suggesting an aromatic requirement at this position, and pointing to a possible specific stacking interaction between residues F595 and F617 as the most productive manner to activate the kinase domain. In the absence of ligand, such an aromatic stacking interaction could induce a conformational change in the JH2 αC helix and trigger catalytic activity via repositioning of key catalytic residues. While this might represent the most efficient path to autoactivation, other, possibly less efficient paths must exist, that similarly rely on F595, since other JAK2 V617 mutations are also constitutively active (*i.e.*, V617M, V617L, V617I (22), and since the F595A mutation inhibits their activation. Given this scenario, we expected that when appended to a dimeric cytokine receptor, JAK2 V617F would signal irrespective of the particular dimeric orientation of the receptor, and that other less active mutants (V617M, V617L, V617I) might still depend on a conformational change of a dimeric receptor. This was indeed the case. When co-expressed with both active and inactive EpoR dimers, JAK2 V617F was able to induce a constitutive signal, regardless of the relative dimeric conformation (Figure 7B). JAK2 mutants V617I, V617L or V617M could signal from both inactive and active cc-EpoR dimer conformations, but they signaled stronger from active cc-EpoR dimers. JAK2 wild-type, on the other hand, was activated only in the presence of the particular conformations corresponding to an activated EpoR dimer. Taken together, these data indicate that the F617: F595 pair is optimal for constitutive activation, irrespective of receptor conformation, but that other bulky aliphatic residues at 617 can induce constitutive activation of JAK2 via F595,

with the caveat that such mutants might additionally require a conformational change of the scaffold receptor, in this case EpoR. In any case, F595 is pivotal for initiating autoactivation of JAK2, while it is not crucial for cytokine-induced JAK2 activation. In the wild-type scenario, a large conformational change of the receptor, that involves rotation (23) and a scissors-like movement (43), brings about kinase domain activation (model, Figure 7C).

[00139] Our data also provide evidence that pseudokinase domains might be pivotal to triggering kinase domain activation, even when mutations are not located in the pseudokinase domain itself, as is the case with JAK2 K539L, JAK2 T875N and JAK2 R683G (Figure 11c). That pseudokinase domains appear in certain proteins to assume "active" conformations (44, 45) suggests that they evolved roles in transmission of conformational changes, and thus could be targets for inhibitors. In support of this, we show that while certain mutations upstream of F595 (R588A, E592A) do not abolish constitutive activity of JAK2 V617F, others downstream, such as M600A, M601A or S602A, Q603A prevent constitutive activation of JAK2 V617F, but do not impair ligand-induced activation. Thus, the middle region of the JH2 helix C seems crucially involved in mediating constitutive activation of mutated JAK2.

[00140] In conclusion, constitutive activation of JAK2 V617F requires F595 of the αC-helix of the JH2 domain with which F617 forms a hydrophobic pi-stacking interaction. This mechanism is specific to V617F, as the activity of JAK2 K539L is not affected by mutating position 595. Inspection of several structures of tyrosine kinase domains further supports the notion that F617 and F595 homologous residues can be in close contact. The F617-F595 interaction might be a unique hydrophobic pocket in the pseudokinase domain of JAK2 V617F that could be targeted by small molecules and facilitate isolation of those that would specifically inhibit JAK2 V617F and spare wild type JAK2. Several JAK2 kinase domain inhibitors, that are ATP-binding competitors are in clinical trials for primary or secondary myelofibrosis (46, 47). These inhibitors do not discriminate between wild-type and mutant JAK2, and can induce unwanted effects, such as anemia and thrombocytopenia. An ideal inhibitor for patients harboring JAK mutants would have to preferentially target the mutant JAK and spare signaling by the wild-type JAK. We suggest that the region in the middle of the pseudokinase domain helix C around residue F595 could be the target of such a specific inhibitor.

Methods

JAK2 mutant plasmid constructs

[00141] All murine and human JAK2 (and JAK1) mutants were obtained by PCR reactions with mutagenic complementary forward and reverse primers by the QuickChange Site-Directed Mutagenesis method (Stratagene). All constructs were cloned into the bicistronic retroviral vector pMX-IRES-GFP and verified by sequencing. Human JAK2 WTpMEGIX-GFP was a kind gift of Dr. William Vainchenker, Institut Gustave Roussy, Villejuif, France. Human JAK2 V617F, K539L, D620L, and K539L/F595A mutants were also created via QuickChange Site-Directed Mutagenesis using the same approach, and verified by sequencing. In most cases, 2 independent clones were tested per each mutant.

Cell lines and retroviral transductions

[00142] Gamma-2A and U4C cells are JAK2-deficient and JAK1-deficient human fibrosarcoma cells (36, 48). Ba/F3-EpoR cells are murine IL3-dependent cells that are expressing the murine erythropoietin receptor. Wild-type and mutant JAKs were transfected into BOSC packaging cells to produce retroviruses which were subsequently used to infect Ba/F3-EpoR cells as described (23). GFP positive cells were sorted by FACS 72 hours after infection, washed, and cultured in the absence of cytokines in RPMI medium + 10% FBS. Cell numbers were recorded over a period of seven days with a Coulter cell counter. The V617F mutant which acquired the ability to proliferate in the absence of cytokines was further cultured in RPMI medium + 10% FBS. The sorted Ba/F3-EpoR cells expressing the JAK2 mutants or wild-type (WT) were maintained in RPMI medium + 10% fetal bovine serum (FBS) and WeHI cell supernatant, as a source of IL-3.

Dual luciferase assays

[00143] The STAT5 transcriptional activity of the various mutants was measured in γ-2A cells (fibrosarcoma cells deficient in JAK2) by dual luciferase assays with the STAT reporter, pGRR5-Luc (31). Cells were seeded in 24-well plates overnight and transfected using lipofectamine, with pGRR5, STAT5, EpoR, the cDNA coding for each individual JAK mutant and pRLTK-Luc as an internal control. The STAT3 transcriptional activity of JAK1 mutants was measured in JAK1-deficient U4C cells (48) by transiently transfecting each JAK1 construct, the pGRR5-Luc and pRLTK-Luc reporters and the interleukin-9 receptor (IL-9R). Medium was changed 4 hours after transfection and stimulation with Epo was

performed when stated.. The cells were lysed 24 hours after transfection, and luminescence was recorded on a TD-20/20 or Glomax 96-well plate luminometer. When performing the assay on stably transduced sorted Ba/F3-EpoR cells (expressing each JAK2 mutant), cells were starved overnight in RPMI medium with 1mg/ml BSA and electroporated with the pGRR5-Luc and pRLTK-Luc luciferase reporters. The cells were subsequently cultured for 2 hours, lysed in 100µl 1X passive lysis buffer, and their luminescence was recorded.

Immunoblotting

[00144] $2.5x10^7$ Ba/F3-EpoR cells expressing each murine or human JAK2 mutant were starved overnight in RPMI medium with 1mg/ml BSA, and stimulated or not with 50U/ml Epo for 15 minutes. Cells were then washed twice in cold PBS, resuspended in cold lysis buffer (1%NP40 + 1X Protease Inhibitor Cocktail (Roche), 1mM vanadate, 1mM PMSF). Upon incubation on ice for 30 minutes and spinning for 20 minutes at 20,000 g and 4°C, the supernatant was mixed with an appropriate volume of 2X Laemmli, boiled, and 40µg total protein was loaded on NuPage 4-12% Bis-Tris gels (Invitrogen). Transfer on nitrocellulose membranes was carried out with the iBlot™ Dry Blotting System (Invitrogen). Membranes were blocked in 5% milk/TBS-Tween, immunoblotting was performed overnight at 4°C with rabbit anti-phospho-JAK2 (Y1007/1008) or rabbit anti phospho-STAT5 A/B (Y694) (both from Cell Signaling Technology) in a solution of 5% BSA in TBS-Tween with a 1:1000 antibody dilution. Secondary anti-rabbit-horseradish peroxidase antibodies (GE Healthcare, UK) were used in a 1:10,000 dilution in 5% milk/TBS-Tween. The membranes were stripped and reprobed with anti-JAK2 and anti- STAT5, respectively (both from Cell Signaling Technology), using the same dilutions and secondary antibodies as for the phospho antibodies. Western blotting antibodies were directed against: phospho-JAK2 (Millipore), JAK2 (Santa Cruz), beta-actin (Sigma), and phospho-STAT5 A/B (Tyr694), phospho-Erk1/2 (Tyr202/Tyr204), Erk1/2 (Cell Signaling Technology).

Structural modeling

[00145] The coordinates for the homology model of JAK2 in an inactive conformation were obtained from Dr. Romano Kroemer. PDB coordinates for JAK2 JH1 active structure were obtained from the PDB database (Accession codes 2B7A, 3FUP). All other PDB coordinates were obtained from the PDB database as described in each case. Molecular graphics images were produced using the UCSF Chimera package from the Resource for

Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (32).

References

[00146] 1. Ziemiecki, A., Harpur, A.G. & Wilks, A.F. JAK protein tyrosine kinases: their role in cytokine signalling. Trends Cell Biol. 4, 207-12 (1994).

[00147] 2. Girault, J.A., Labesse, G., Mornon, J.P. & Callebaut, I. Janus kinases and focal adhesion kinases play in the 4.1 band: a superfamily of band 4.1 domains important for cell structure and signal transduction. Mol. Med. 4, 751-69 (1998).

[00148] 3. Huang, L.J., Constantinescu, S.N. & Lodish, H.F. The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. Mol. Cell 8, 1327-38 (2001).

[00149] 4. Radtke, S. *et al.* Novel role of Janus kinase 1 in the regulation of oncostatin M receptor surface expression. J. Biol. Chem. 277, 11297-305 (2002).

[00150] 5. Royer, Y., Staerk, J., Costuleanu, M., Courtoy, P.J. & Constantinescu, S.N. Janus kinases affect thrombopoietin receptor cell surface localization and stability. J. Biol. Chem. 280, 27251-61 (2005).

[00151] 6. Radtke, S. *et al.* The Jak1 SH2 domain does not fulfill a classical SH2 function in Jak/STAT signaling but plays a structural role for receptor interaction and upregulation of receptor surface expression. J. Biol. Chem. 280, 25760-8 (2005).

[00152] 7. Harpur, A.G., Andres, A.C., Ziemiecki, A., Aston, R.R. & Wilks, A.F. JAK2, a third member of the JAK family of protein tyrosine kinases. Oncogene 7, 1347-53 (1992).

[00153] 8. Lucet, I.S. *et al.* The structural basis of Janus kinase 2 inhibition by a potent and specific pan-Janus kinase inhibitor. Blood 107, 176-83 (2006).

[00154] 9. Boggon, T.J., Li, Y., Manley, P.W. & Eck, M.J. Crystal structure of the Jak3 kinase domain in complex with a staurosporine analog. Blood 106, 996-1002 (2005).

[00155] 10. Williams, N.K. *et al.* Dissecting specificity in the Janus kinases: The structures of JAK-specific inhibitors complexed to the JAK1 and JAK2 Protein Tyrosine Kinase Domain. J. Mol. Biol. (2009).

[00156] 11. Sicheri, F., Moarefi, I. & Kuriyan, J. Crystal structure of the Src family tyrosine kinase Hck. Nature 385, 602-9 (1997).

[00157] 12. De Bondt, H.L. *et al.* Crystal structure of cyclin-dependent kinase 2. Nature 363, 595-602 (1993).

[00158] 13. James, C. *et al.* A unique clonal JAK2 mutation leading to constitutive signaling causes polycythaemia vera. Nature 434, 1144-8 (2005).

[00159] 14. Baxter, E.J. *et al.* Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 365, 1054-61 (2005).

[00160] 15. Levine, R.L. *et al.* Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 7, 387-97 (2005).

[00161] 16. Kralovics, R. *et al.* A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 352, 1779-90 (2005).

[00162] 17. Scott, L.M. *et al.* JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med 356, 459-68 (2007).

[00163] 18. Huse, M. & Kuriyan, J. The conformational plasticity of protein kinases. Cell 109, 275-82 (2002).

[00164] 19. Xu, W., Harrison, S.C. & Eck, M.J. Three-dimensional structure of the tyrosine kinase c-Src. Nature 385, 595-602 (1997).

[00165] 20. Lindauer, K., Loerting, T., Liedl, K.R. & Kroemer, R.T. Prediction of the structure of human Janus kinase 2 (JAK2) comprising the two carboxy-terminal domains reveals a mechanism for autoregulation. Protein Eng 14, 27-37 (2001).

[00166] 21. Gonfloni, S., Frischknecht, F., Way, M. & Superti-Furga, G. Leucine 255 of Src couples intramolecular interactions to inhibition of catalysis. Nat Struct Biol 6, 760-4 (1999).

[00167] 22. Dusa, A. *et al.* Substitution of pseudokinase domain residue Val-617 by large non-polar amino acids causes activation of JAK2. J Biol Chem 283, 12941-8 (2008).

[00168] 23. Seubert, N. *et al.* Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer. Mol Cell 12, 1239-50 (2003).

[00169] 24. Johnson, L.N., Noble, M.E. & Owen, D.J. Active and inactive protein kinases: structural basis for regulation. Cell 85, 149-58 (1996).

[00170] 25. Hubbard, S.R., Wei, L., Ellis, L. & Hendrickson, W.A. Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature 372, 746-54 (1994).

[00171] 26. Li, S. *et al.* Clonal heterogeneity in polycythemia vera patients with JAK2 exon12 and JAK2-V617F mutations. Blood (2008).

[00172] 27. Lee, T.S. *et al.* Mechanisms of constitutive activation of Janus kinase 2-V617F revealed at the atomic level through molecular dynamics simulations. Cancer 115, 1692-700 (2009).

[00173] 28. Lu, X. *et al.* Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. Proc Natl Acad Sci U S A 102, 18962-7 (2005).

[00174] 29. Lu, X., Huang, L.J. & Lodish, H.F. Dimerization by a cytokine receptor is necessary for constitutive activation of JAK2V617F. J Biol Chem 283, 5258-66 (2008).

[00175] 30. Staerk, J., Kallin, A., Demoulin, J.B., Vainchenker, W. & Constantinescu, S.N. JAK1 and Tyk2 activation by the homologous polycythemia vera JAK2 V617F mutation: cross-talk with IGF1 receptor. J. Biol. Chem. 280, 41893-9 (2005).

[00176] 31. Dumoutier, L., Van Roost, E., Colau, D. & Renauld, J.C. Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor. Proc Natl Acad Sci U S A 97, 10144-9 (2000).

[00177] 32. Pettersen, E.F. *et al.* UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem 25, 1605-12 (2004).

[00178] 33. Williams JC, Weijland A, Gonfloni S, Thompson A, Courtneidge SA, *et al.* (1997) The 2.35 A crystal structure of the inactivated form of chicken Src: a dynamic molecule with multiple regulatory interactions. J Mol Biol 274: 757-775.

[00179] 34. Filippakopoulos P, Kofler M, Hantschel O, Gish GD, Grebien F, *et al.* (2008) Structural coupling of SH2-kinase domains links Fes and Abl substrate recognition and kinase activation. Cell 134: 793-803.

[00180] 35. Deindl S, Kadlecek TA, Brdicka T, Cao X, Weiss A, *et al.* (2007) Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. Cell 129: 735-746.

[00181] 36. Kohlhuber F, Rogers NC, Watling D, Feng J, Guschin D, *et al.* (1997) A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. Mol Cell Biol 17: 695-706.

[00182] 37. Scott LM, Tong W, Levine RL, Scott MA, Beer PA, *et al.* (2007) JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med 356: 459-468.

[00183] 38. Mercher T, Wernig G, Moore SA, Levine RL, Gu TL, *et al.* (2006) JAK2T875N is a novel activating mutation that results in myeloproliferative disease with features of megakaryoblastic leukemia in a murine bone marrow transplantation model. Blood 108: 2770-2779.

[00184] 39. Mullighan CG, Zhang J, Harvey RC, Collins-Underwood JR, Schulman BA, *et al.* (2009) JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 106: 9414-9418.

[00185] 40. Jeong EG, Kim MS, Nam HK, Min CK, Lee S, *et al.* (2008) Somatic mutations of JAK1 and JAK3 in acute leukemias and solid cancers. Clin Cancer Res 14: 3716-3721.

[00186] 41. Flex E, Petrangeli V, Stella L, Chiaretti S, Hornakova T, *et al.* (2008) Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia. J Exp Med 205: 751-758.

[00187] 42. Kubatzky KF, Liu W, Goldgraben K, Simmerling C, Smith SO, *et al.* (2005) Structural requirements of the extracellular to transmembrane domain junction for erythropoietin receptor function. J Biol Chem 280: 14844-14854.

[00188] 43. Remy I, Wilson IA, Michnick SW (1999) Erythropoietin receptor activation by a ligand-induced conformation change. Science 283: 990-993.

[00189] 44. Mukherjee K, Sharma M, Urlaub H, Bourenkov GP, Jahn R, *et al.* (2008) CASK Functions as a Mg2+-independent neurexin kinase. Cell 133: 328-339.

[00190] 45. Zeqiraj E, Filippi BM, Goldie S, Navratilova I, Boudeau J, *et al.* (2009) ATP and MO25alpha regulate the conformational state of the STRADalpha pseudokinase and activation of the LKB1 tumour suppressor. PLoS Biol 7: e1000126.

[00191] 46. Wernig G, Kharas MG, Okabe R, Moore SA, Leeman DS, *et al.* (2008) Efficacy of TG101348, a selective JAK2 inhibitor, in treatment of a murine model of JAK2V617F-induced polycythemia vera. Cancer Cell 13: 311-320.

[00192] 47. Mesa RA, Tefferi A (2009) Emerging drugs for the therapy of primary and post essential thrombocythemia, post polycythemia vera myelofibrosis. Expert Opin Emerg Drugs 14: 471-479.

[00193] 48. Muller M, Briscoe J, Laxton C, Guschin D, Ziemiecki A, *et al.* (1993) The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. Nature 366: 129-135.

[00194] All references listed herein, including those listed above, are incorporated herein by reference.

Claims

What is claimed is:

1. A method of identifying an agent that modulates the interaction of amino acid F617, L539, N875, or G683 with amino acid F595 in a mutant Janus kinase 2 (JAK2) with a V617F, a K539L, a T875N, or an R683G mutation comprising:

contacting the mutant JAK2 with a test agent under suitable conditions for the test agent to interfere with the interaction of F617, L539, N857, or G683 with F595; and

determining the activity of the mutant JAK2 in the presence of the test agent.

2. The method of claim 1, further comprising

comparing the activity of the mutant JAK2 to a reference or control activity, wherein if the activity in the presence of the test agent is less than the reference or control activity, then the test agent is identified as an agent that inhibits the activity of the mutant JAK2, or if the activity in the presence of the test agent is higher than the reference or control activity, then the test agent is identified as an agent that stimulates the activity of the mutant JAK2.

3. The method of claim 1, further comprising

contacting wild type JAK2 with a test agent identified to inhibit or a test agent identified to stimulate the activity of the mutant JAK2 under suitable conditions for the test agent to interfere with the interaction of the mutated amino acid with amino acid F595, and

determining the activity of the wild type JAK2 in the presence of the test agent, and

comparing the activity of the wild type JAK2 to a reference or control activity, wherein if the wild type JAK2 activity in the presence of the inhibitory test agent is not decreased as compared to the reference or control activity, then the test agent is identified as an agent that inhibits the interaction of the mutated amino acid with amino acid F595 in the mutant JAK2 but does not inhibit wild type JAK2, or

if the wild type JAK2 activity in the presence of the stimulatory test agent is not increased as compared to the reference or control activity, then the test agent is identified as an agent that activates the interaction of the mutated amino acid with amino acid F595 in the mutant JAK2 but does not activate wild type JAK2.

4. A method of identifying an agent that modulates the interaction of amino acid 617, 539, 875, or 683 with amino acid F595 in a mutant Janus kinase 2 (JAK2) comprising:

contacting the mutant JAK2 with a test agent under suitable conditions for the test agent to interfere with the interaction of amino acid 617, 539, 875, or 683 with amino acid F595; and

determining the activity of JAK2 in the presence of the test agent.

5. The method of claim 4, further comprising

comparing the activity of the mutant JAK2 to a reference or control activity, and if the activity in the presence of the test agent is less than the reference or control activity, then the test agent is identified as an agent that inhibits the interaction of amino acid 617, 539, 875, or 683 with amino acid F595 in the mutant JAK2, or
if the activity in the presence of the test agent is higher than the reference or control activity, then the test agent is identified as an agent that stimulates the interaction of amino acid 617, 539, 875, or 683 with amino acid F595 in the mutant JAK2.

6. The method of claim 1 or 4, wherein the test agent is a small molecule.

7. The method of claim 1 or 4, wherein the test agent is a peptide.

8. The method of claim 1 or 4, wherein the test agent is a protein.

9. The method of claim 1 or 4, wherein the test agent is an antibody.

10. The method of claim 1 or 4, wherein the suitable conditions are physiological conditions.

11. The method of claim 1 or 4, wherein the activity of JAK2 is reduced in the presence of the test agent.

12. The method of claim 1 or 4, wherein the JAK2 kinase is constitutively active.

13. The method of claim 4, wherein

amino acid 617 is an aromatic amino acid,

amino acid 539 is Leucine,
amino acid 875 is Asparagine, or
amino acid 683 is Glycine.

14. The method of claim 4, wherein amino acid 617 is phenylalanine.

15. The method of claim 4, wherein amino acid 617 is tryptophan.

16. The method of claim 1, wherein the agent disrupts or promotes the pi-stacking interaction of F595 with F617.

17. A method of screening a plurality of test agents to identify an agent that modulates the interaction of amino acid F617, L539, N875, or G683 with amino acid F595 in a mutant Janus kinase 2 (JAK2) with a V617F, a K539L, a T875N, or an R683G mutation comprising

contacting the mutant JAK2 with an individual test agent under suitable conditions for the test agent to interfere with the interaction of F617, L539, N875, or G683 with amino acid F595; and

determining the activity of the mutant JAK2 in the presence of each of the test agents.

18. The method of claim 17, further comprising

comparing the activity of the mutant JAK2 to a reference or control activity, wherein if the activity in the presence of the test agent is less than the reference or control activity, then the test agent is identified as an agent that inhibits the activity of the mutant JAK2, or

if the activity in the presence of the test agent is higher than the reference or control activity, then the test agent is identified as an agent that stimulates the activity of the mutant JAK2.

19. The method of claim 17, wherein at least 50 test agents are screened in parallel.

20. The method of claim 17, wherein at least 100 test agents are screened in parallel.

21. The method of claim 17, wherein at least 500 test agents are screened in parallel.

22. The method of claim 17, wherein at least 1000 test agents are screened in parallel.

23. An agent identified by the method of any one of claims 1-22.

24. An agent that interferes with the interaction of F617, L539, N875, or G683 with amino acid F595 in a mutant JAK2.

25. The agent of claim 24, wherein the agent is a small molecule.

26. The agent of claim 24, wherein the agent reduces the activity of the mutant JAK2.

27. The agent of claim 24, wherein the agent has a molecular weight of less than 2,000 g/mol.

28. The agent of claim 24, wherein the agent has a molecular weight of less than 1,500 g/mol.

29. The agent of claim 24, wherein the agent inhibits the mutant JAK2 and does not inhibit wild type JAK2.

30. A method of reducing or increasing the activity of a mutant JAK2 with a V617F, a K539L, a T875N, or an R683G mutation in a cell comprising contacting the cell with an agent identified by the method of any one of claims 1-17.

31. A method of identifying an agent that modulates the interaction of amino acid F658, with amino acid F636 in a mutant Janus kinase 1 (JAK1) with a V658F mutation comprising:

contacting the mutant JAK1 with a test agent under suitable conditions for the test agent to interfere with the interaction of F658 with F595; and

determining the activity of the mutant JAK1 in the presence of the test agent.

32. The method of claim 31, further comprising

comparing the activity of the mutant JAK1 to a reference or control activity, wherein if the activity in the presence of the test agent is less than the reference or control activity, then the test agent is identified as an agent that inhibits the activity of the mutant JAK1, or

if the activity in the presence of the test agent is higher than the reference or control activity, then the test agent is identified as an agent that stimulates the activity of the mutant JAK1.

33. The method of claim 32, further comprising

contacting wild type JAK1 with a test agent identified to inhibit or an agent identified to stimulate the activity of the mutant JAK1 under suitable conditions for the test agent to interfere with the interaction of the mutated amino acid with amino acid F636, and

determining the activity of the wild type JAK1 in the presence of the test agent, and

comparing the activity of the wild type JAK1 to a reference or control activity,

wherein if the wild type JAK1 activity in the presence of the inhibitory test agent is not decreased as compared to the reference or control activity, then the test agent is identified as an agent that inhibits the interaction of the mutated amino acid with amino acid F636 in the mutant JAK1 but does not inhibit wild type JAK1, or

if the wild type JAK1 activity in the presence of the stimulatory test agent is not increased as compared to the reference or control activity, then the test agent is identified as an agent that stimulates the interaction of the mutated amino acid with amino acid F636 in the mutant JAK1 but does not stimulate wild type JAK1.

34. A method of identifying an agent that modulates the interaction of amino acid 658 with amino acid F636 in a mutant Janus kinase 1 (JAK1) comprising:

contacting the mutant JAK1 with a test agent under suitable conditions for the test agent to interfere with the interaction of amino acid 658 with amino acid F636; and

determining the activity of the mutant JAK1 in the presence of the test agent.

35. The method of claim 34, further comprising

comparing the activity of the mutant JAK1 to a reference or control activity, and

if the activity in the presence of the test agent is less than the reference or control activity, then the test agent is identified as an agent that inhibits the interaction of amino acid 658 with amino acid F636 in the mutant JAK1,

if the activity in the presence of the test agent is higher than the reference or control activity, then the test agent is identified as an agent that stimulates the interaction of amino acid 658 with amino acid F636 in the mutant JAK1.

36. The method of claim 31 or 34, wherein the test agent is a small molecule.

37. The method of claim 31 or 34, wherein the test agent is a peptide.

38. The method of claim 31 or 34, wherein the test agent is a protein.

39. The method of claim 31 or 34, wherein the test agent is an antibody.

40. The method of claim 31 or 34, wherein the suitable conditions are physiological conditions.

41. The method of claim 31 or 34, wherein the activity of the mutant JAK1 is reduced in the presence of the test agent.

42. The method of claim 31 or 34, wherein the mutant JAK1 is constitutively active.

43. The method of claim 34, wherein amino acid 658 is Phenylalanine.

44. The method of claim 31, wherein the agent disrupts the pi-stacking interaction of F658 with F636.

45. A method of screening a plurality of test agents to identify an agent that modulates the interaction of amino acid F658 with amino acid F636 in a mutant Janus kinase 1 (JAK1) with a V658F mutation comprising

contacting the mutant JAK1 with an individual test agent under suitable conditions for the test agent to interfere with the interaction of F658 with amino acid F636; and

determining the activity of the mutant JAK1 in the presence of each of the test agents.

46. The method of claim 45, further comprising

comparing the activity of the mutant JAK1 to a reference or control activity, wherein if the activity in the presence of the test agent is less than the reference or control activity, then the test agent is identified as an agent that inhibits the activity of the mutant JAK1, or

if the activity in the presence of the test agent is higher than the reference or control activity, then the test agent is identified as an agent that stimulates the activity of the mutant JAK1.

47. The method of claim 45, wherein at least 50 test agents are screened in parallel.

48. The method of claim 45, wherein at least 100 test agents are screened in parallel.

49. The method of claim 45, wherein at least 500 test agents are screened in parallel.

50. The method of claim 45, wherein at least 1000 test agents are screened in parallel.

51. An agent identified by the method of any one of claims 31-50.

52. An agent that interferes with the interaction of F658 with amino acid F636 in a mutant JAK1.

53. The agent of claim 52, wherein the agent is a small molecule.

54. The agent of claim 52, wherein the agent reduces the activity of the mutant JAK1.

55. The agent of claim 52, wherein the agent has a molecular weight of less than 2,000 g/mol.

56. The agent of claim 52, wherein the agent has a molecular weight of less than 1,500 g/mol.

57. The agent of claim 52, wherein the agent inhibits the mutant JAK1 and does not inhibit wild type JAK1.

58. A method for the design and identification of a potential binding compound for V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1, comprising the steps of:

(a) generating, on a computer, a three-dimensional structure of the mutant JAK2 or the mutant JAK1;

(b) designing and/or selecting a compound that potentially binds to the mutant amino acid residue in the V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1, using the three-dimensional model of the HsPDF active site; and

(c) synthesizing and/or choosing the potential binding compound.

59. The method according to claim 58, the method further comprising the steps of:

(d) contacting the potential binding compound with the mutant JAK2 or the mutant JAK1; and

(e) determining the percent inhibition of the mutant JAK2 activity or mutant JAK1 activity inhibition as compared to wild type JAK2, or wild type JAK1.

60. The method according to claim 58, the method further comprising the steps of:

(d) contacting the potential binding compound with a cell expressing the mutant JAK2 or the mutant JAK1, and

(e) determining the percent inhibition of the proliferation rate of the cell in the presence of the compound as compared to a cell of the same cell type in the absence of the compound.

61. A method for the design and identification of a potential binding compound for V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1, comprising the steps of:

(a) generating, on a computer, a three-dimensional structure of V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1,;

(b) designing and/or selecting a compound that potentially binds to V617F, K539L, T875N, or R683G in the mutant JAK2, or V658F in the mutant JAK1, using the three-dimensional model of the mutant JAK1 or JAK2 from step (b); and

(d) synthesizing and/or choosing the potential binding compound.

62. The method according to claim 61, the method further comprising the steps of:

(e) contacting the potential binding compound with V617F, K539L, T875N, or R683G mutant JAK2, or V658F mutant JAK1; and

(f) determining the percent inhibition of V617F, K539L, T875N, or R683G mutant JAK2 activity, or V658F mutant JAK1 activity.

63. The method according to claim 61, the method further comprising the steps of:

(e) contacting the potential binding compound with a cell, and

(f) determining the percent inhibition of the proliferation rate of the cell in the presence of the compound as compared to a cell of the same cell type in the absence of the compound..

64. A method of identifying an agent that masks amino acid 617, 539, 875, or 683 in Janus kinase 2 (JAK2), or amino acid 658 in Janus Kinase 1 (JAK1), comprising

contacting the JAK2 or the JAK1 with a test agent under suitable conditions for the test agent to bind to the amino acid; and

determining the activity of the JAK2 or the JAK1 in the presence of the test agent.

65. The method of claim 64, further comprising

comparing the activity of the JAK2 or the JAK1 to a reference or control activity, wherein if the activity in the presence of the test agent is less than the reference or control activity, then the test agent is identified as an agent that masks the amino acid 617, 539, 875, or 683 in JAK2, or amino acid 658 in JAK1.

66. The method of claim 64 or claim 65, wherein the JAK2 is wild type JAK2 or a mutant JAK2 having a mutation from the group consisting of V617F, L539, N875, or G683, or the JAK1 is wild type JAK1 or a mutant JAK1 having a V658F mutation.

```
Csk       AQAFLAEASVMTQLRHSN-LVQLLGVIVEEKGGLYIVTEYMAKGSLVDYLRS--------
c-ABL     VEEFLKEAAVMKEIKHPN-LVQLLGVCTREPP-FYIITEFMTYGNLLDYLRE--------
c-KIT     REALMSELKVLSYLGNHMNIVNLLGACTIGGP-TLVITEYCCYGDLLNFLRRKRDSF---
Flt3      REALMSELKMMTQLGSHENIVNLLGACTLSGP-IYLIFEYCCYGDLLNYLRSKREKFSED
IGF1R     RIEFLNEASVMKEFNCHH-VVRLLGVVSQGQP-TLVIMELMTRGDLKSYLRS--------
IRK       RIEFLNEASVMKGFTCHH-VVRLLGVVSKGQP-TLVVMELMAHGDLKSYLRS--------
JAK2_JH2  SESFEAASMMSKLSHKH-LVLNYGVCVCGDE-NILVQEFVKFGSLDTYLKK--------
```

FIGURE 1A

Kinase	Distance between homologous V617 and F595 positions	PDB code
Csk	3.5 Å	1BYG
c-ABL	4.0 Å	1OPJ
c-KIT	3.6 Å	1T45
Flt3	4.2 Å	1RJB
IGF1R	3.9 Å	1M7N
IRK	3.9 Å	1IRK

FIGURE 1B

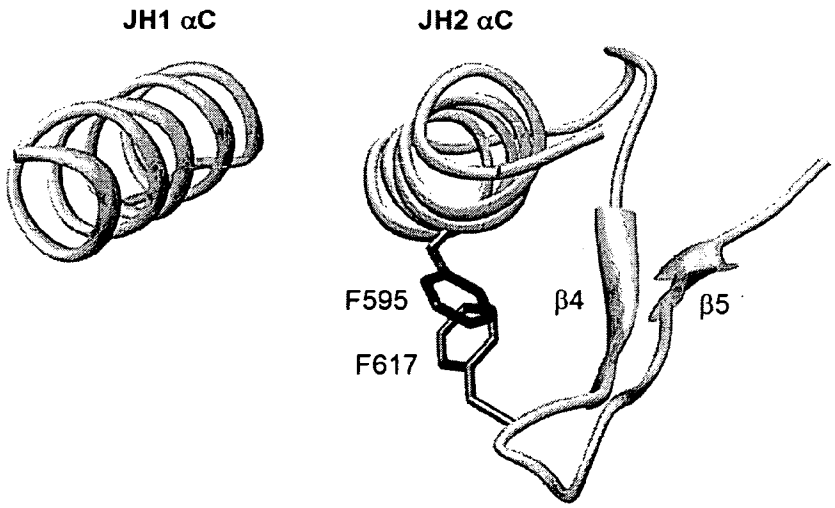

FIGURE 1C

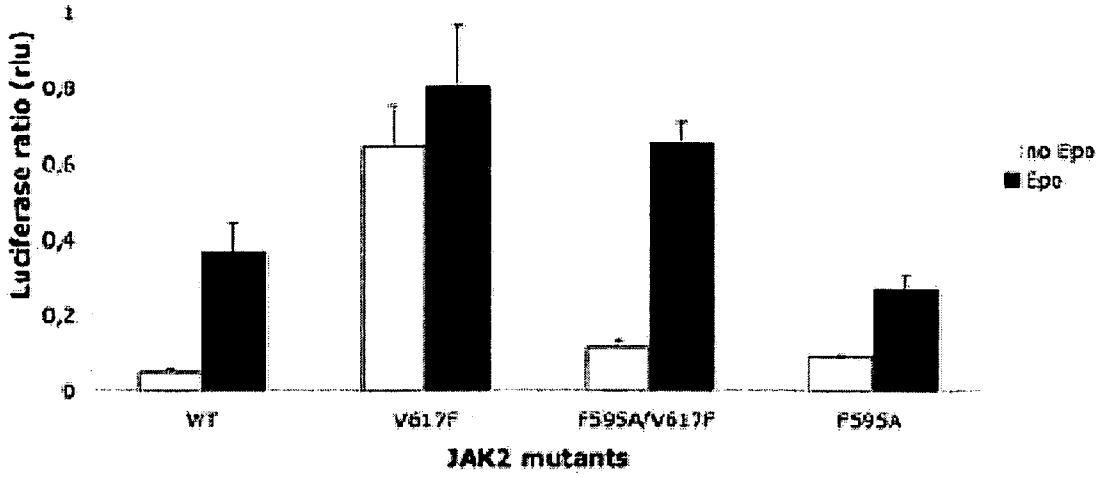
Luciferase ratio (rlu)
1
0,8
0,6
0,4
0,2
0
WT
V617F
F595A/V617F
F595A
JAK2 mutants
no Epo
Epo

FIGURE 2

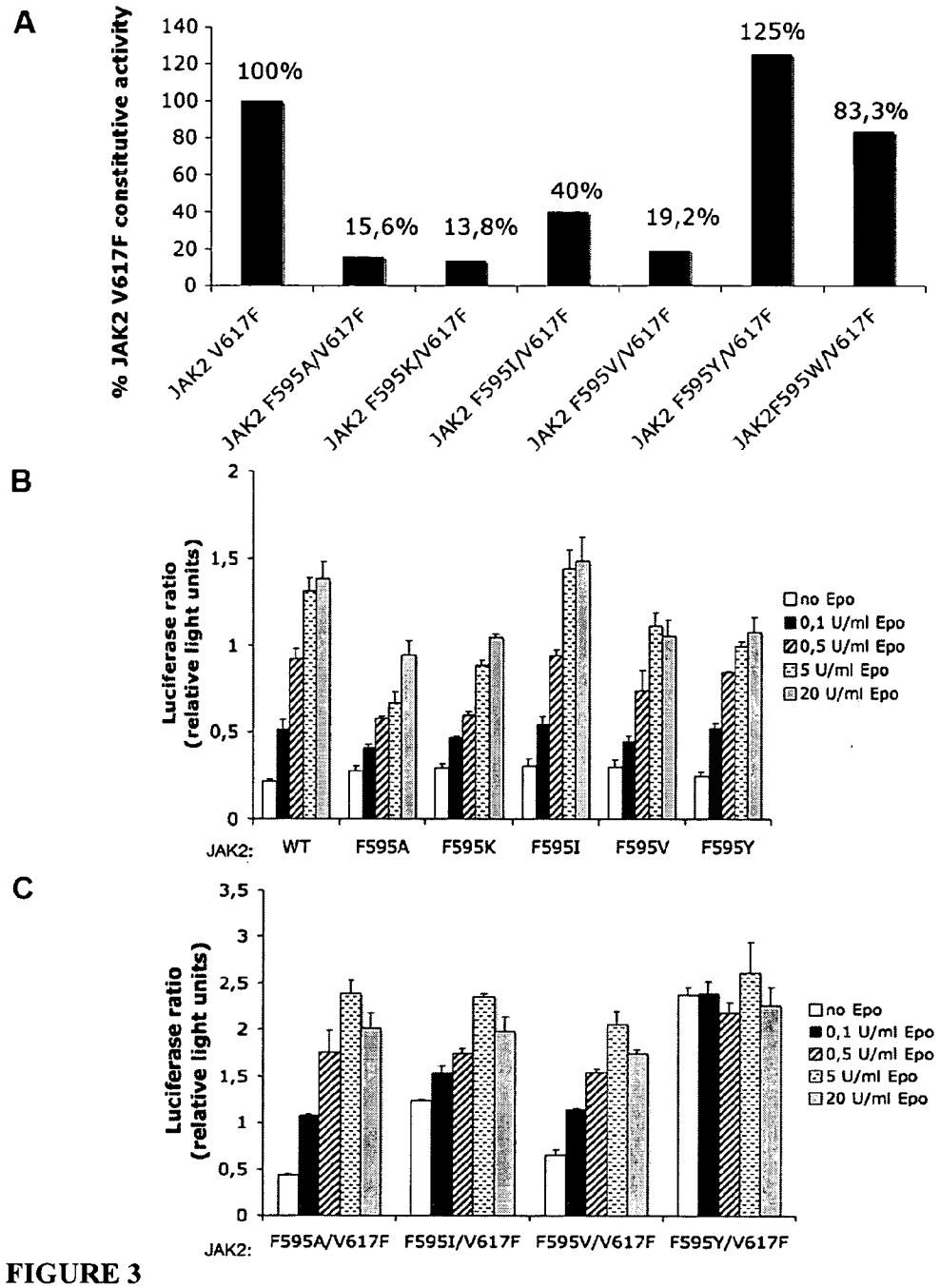
A
% JAK2 V617F constitutive activity
140
120
100
80
60
40
20
0
100%
15,6%
13,8%
40%
19,2%
125%
83,3%
JAK2 V617F
JAK2 F595A/V617F
JAK2 F595K/V617F
JAK2 F595I/V617F
JAK2 F595V/V617F
JAK2 F595Y/V617F
JAK2F595W/V617F
B
Luciferase ratio (relative light units)
2
1,5
1
0,5
0
JAK2: WT F595A F595K F595I F595V F595Y
no Epo
0,1 U/ml Epo
0,5 U/ml Epo
5 U/ml Epo
20 U/ml Epo
C
Luciferase ratio (relative light units)
3,5
3
2,5
2
1,5
1
0,5
0
JAK2: F595A/V617F F595I/V617F F595V/V617F F595Y/V617F
no Epo
0,1 U/ml Epo
0,5 U/ml Epo
5 U/ml Epo
20 U/ml Epo

FIGURE 3

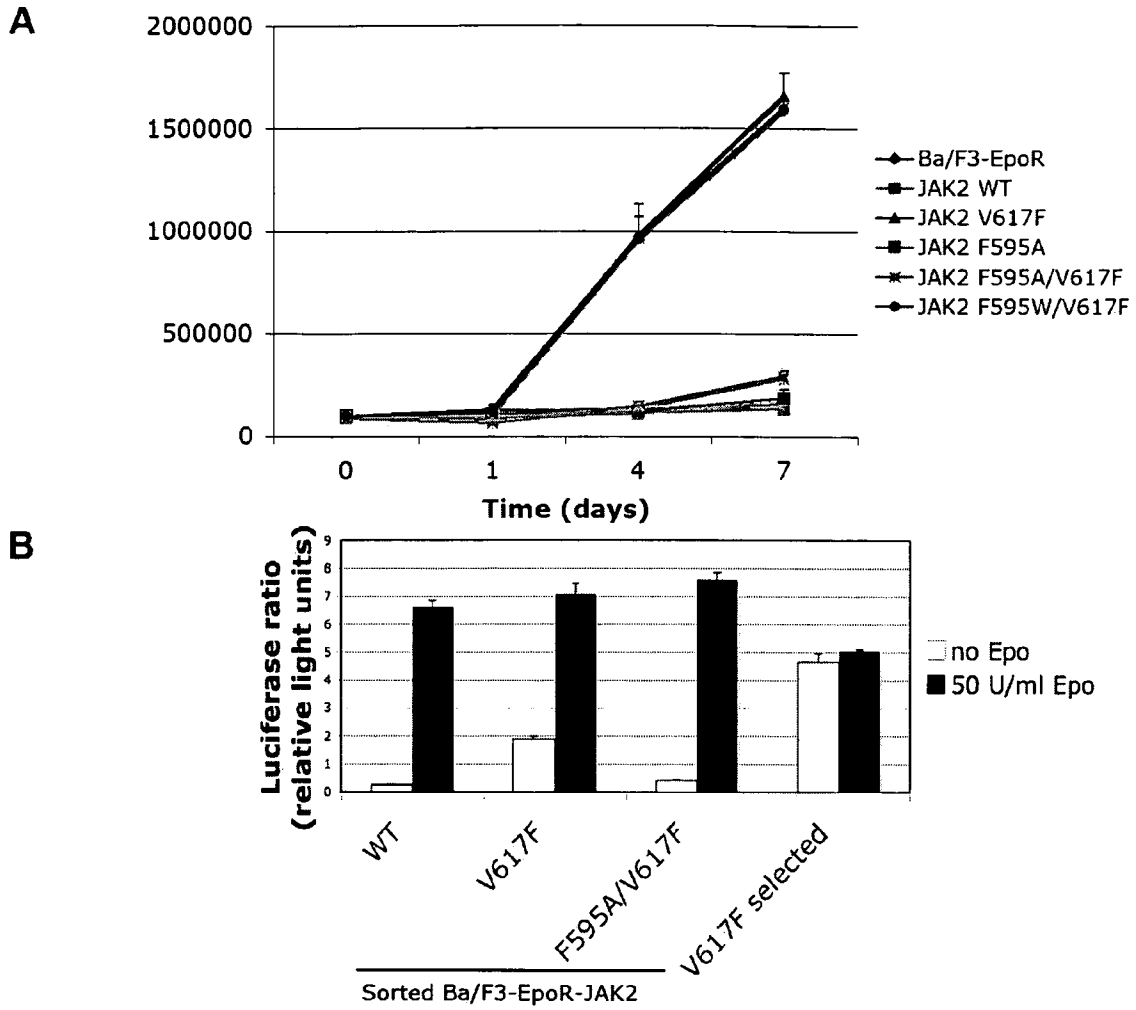
A
2000000
1500000
1000000
500000
0
0
1
4
7
Time (days)
Ba/F3-EpoR
JAK2 WT
JAK2 V617F
JAK2 F595A
JAK2 F595A/V617F
JAK2 F595W/V617F
B
Luciferase ratio (relative light units)
no Epo
50 U/ml Epo
WT
V617F
F595A/V617F
V617F selected
Sorted Ba/F3-EpoR-JAK2

FIGURE 4

C

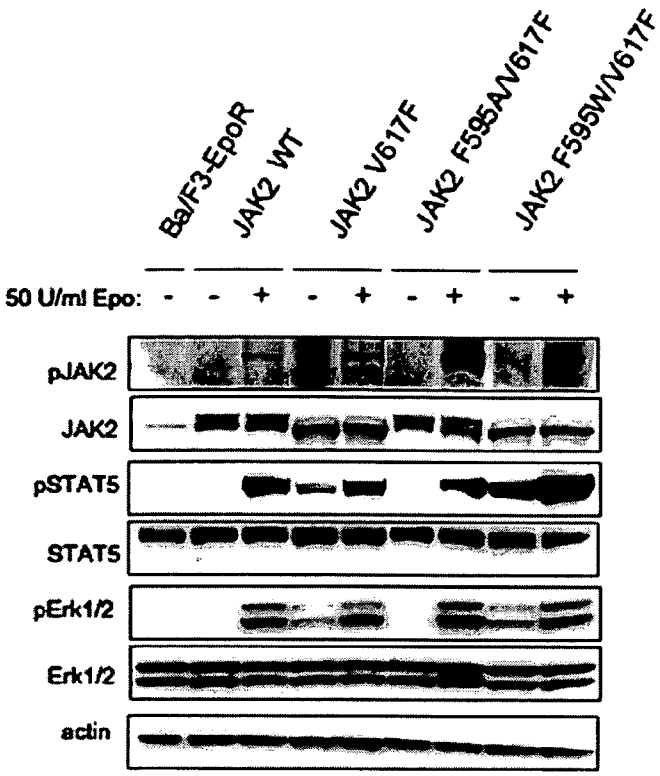

FIGURE 4 (continued)

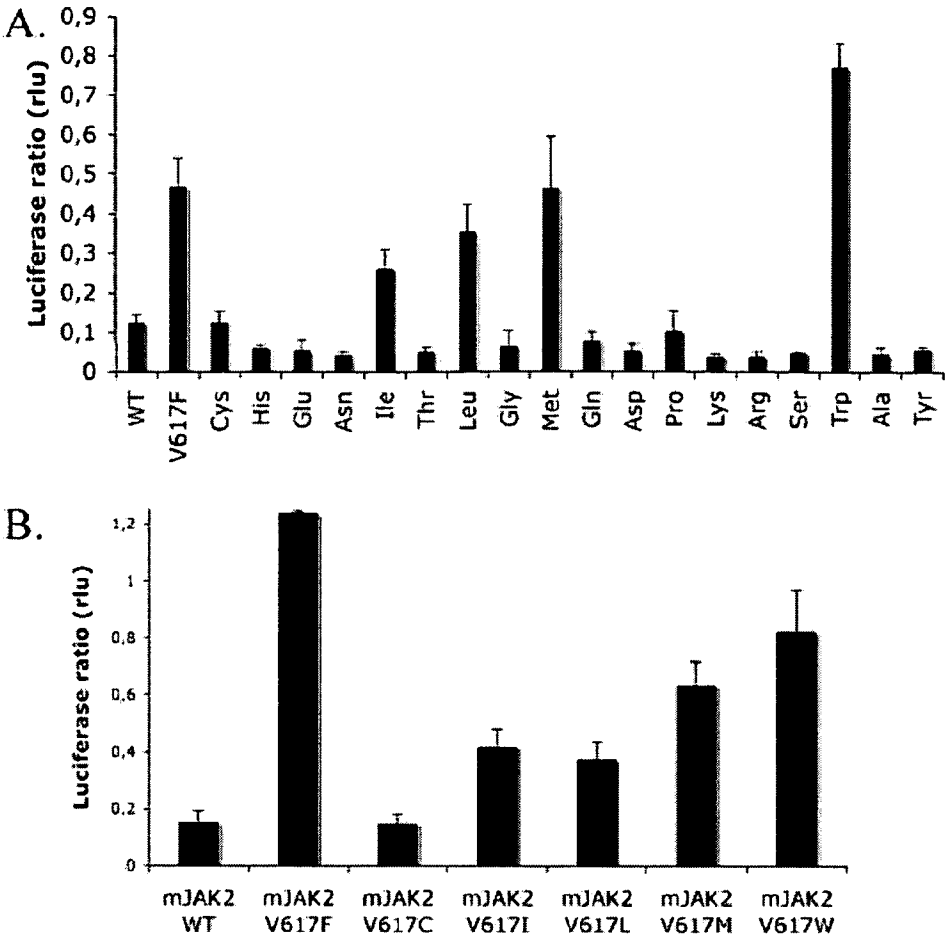
A.
Luciferase ratio (rlu)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
WT
V617F
Cys
His
Glu
Asn
Ile
Thr
Leu
Gly
Met
Gln
Asp
Pro
Lys
Arg
Ser
Trp
Ala
Tyr
B.
Luciferase ratio (rlu)
1,2
1
0,8
0,6
0,4
0,2
0
mJAK2 WT
mJAK2 V617F
mJAK2 V617C
mJAK2 V617I
mJAK2 V617L
mJAK2 V617M
mJAK2 V617W

FIGURE 5

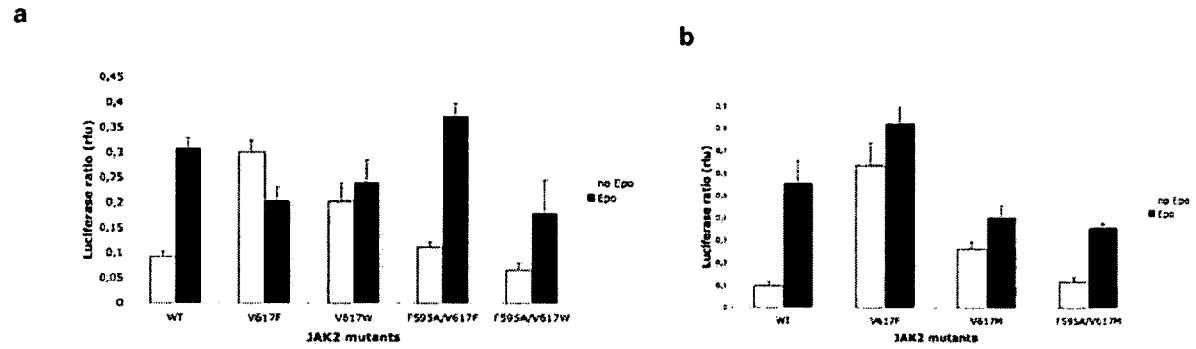
a
b
Luciferase ratio (rlu)
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
WT
V617F
V617W
F595A/V617F
F595A/V617W
JAK2 mutants
no Epo
Epo

FIGURE 6

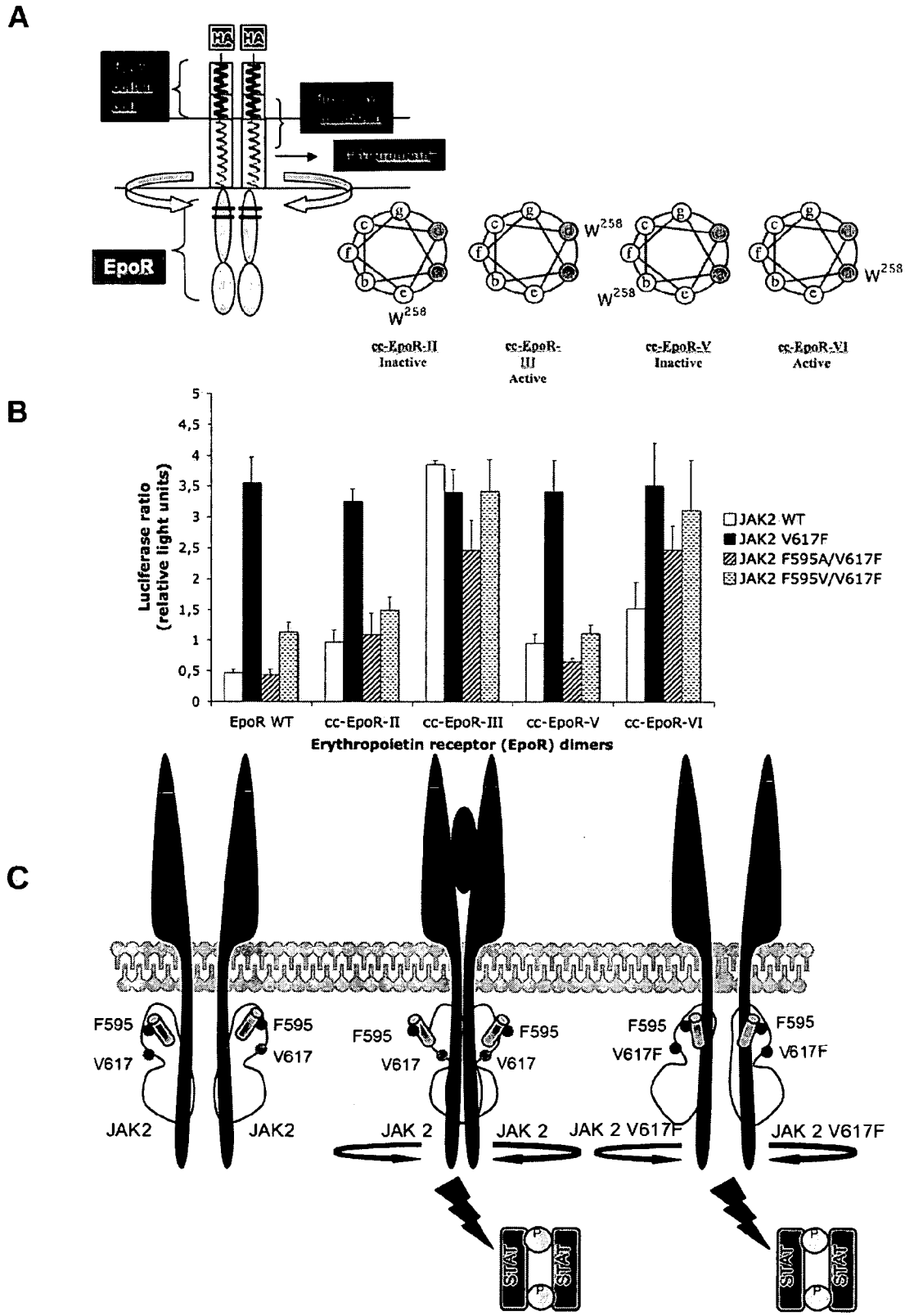
A
HA
HA
EpoR
W258
W258
W258
cc-EpoR-II
Inactive
cc-EpoR-III
Active
cc-EpoR-V
Inactive
cc-EpoR-VI
Active
B
Luciferase ratio (relative light units)
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
JAK2 WT
JAK2 V617F
JAK2 F595A/V617F
JAK2 F595V/V617F
EpoR WT
cc-EpoR-II
cc-EpoR-III
cc-EpoR-V
cc-EpoR-VI
Erythropoietin receptor (EpoR) dimers
C
F595
V617
JAK2
JAK2
F595
V617
JAK 2
JAK 2
F595
V617F
JAK 2 V617F
JAK 2 V617F
STAT
STAT
STAT
STAT

FIGURE 7

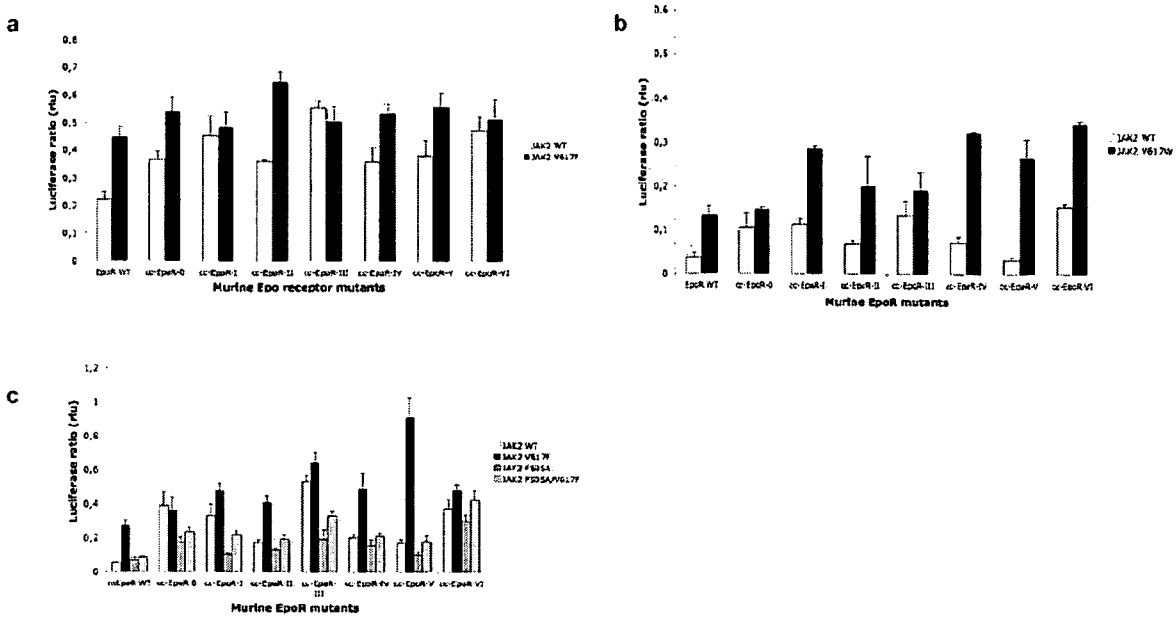

FIGURE 8

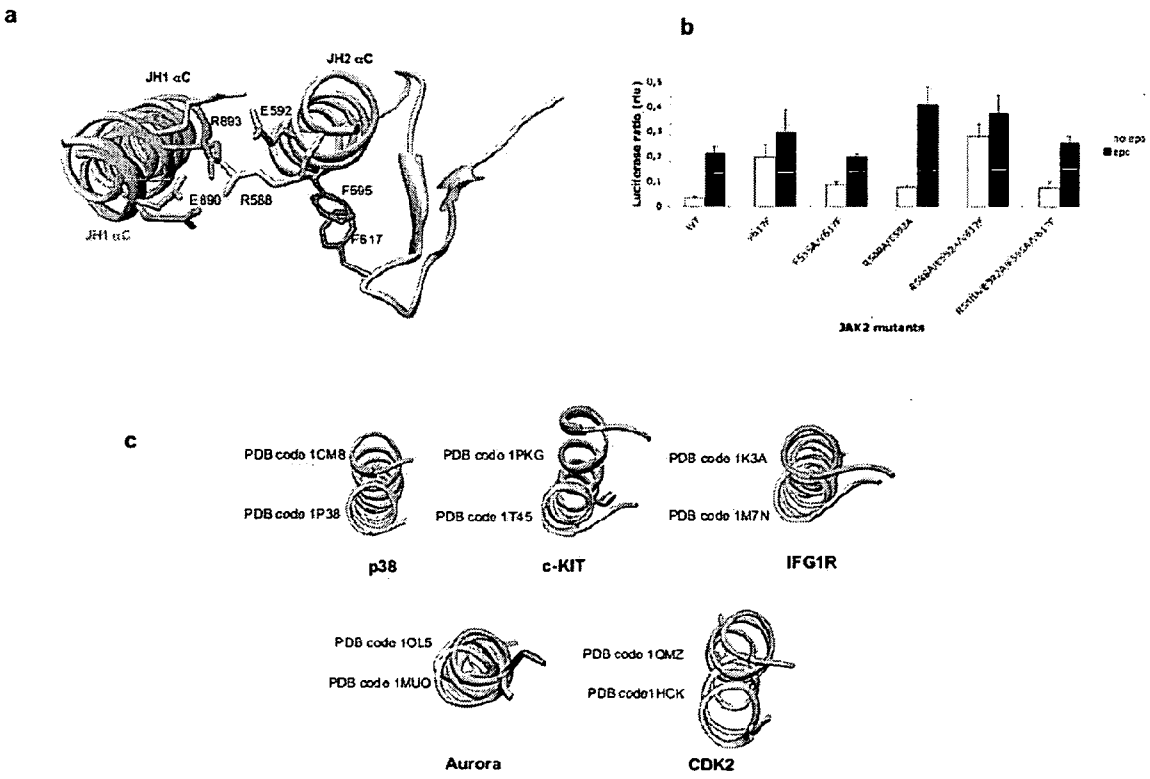

FIGURE 9

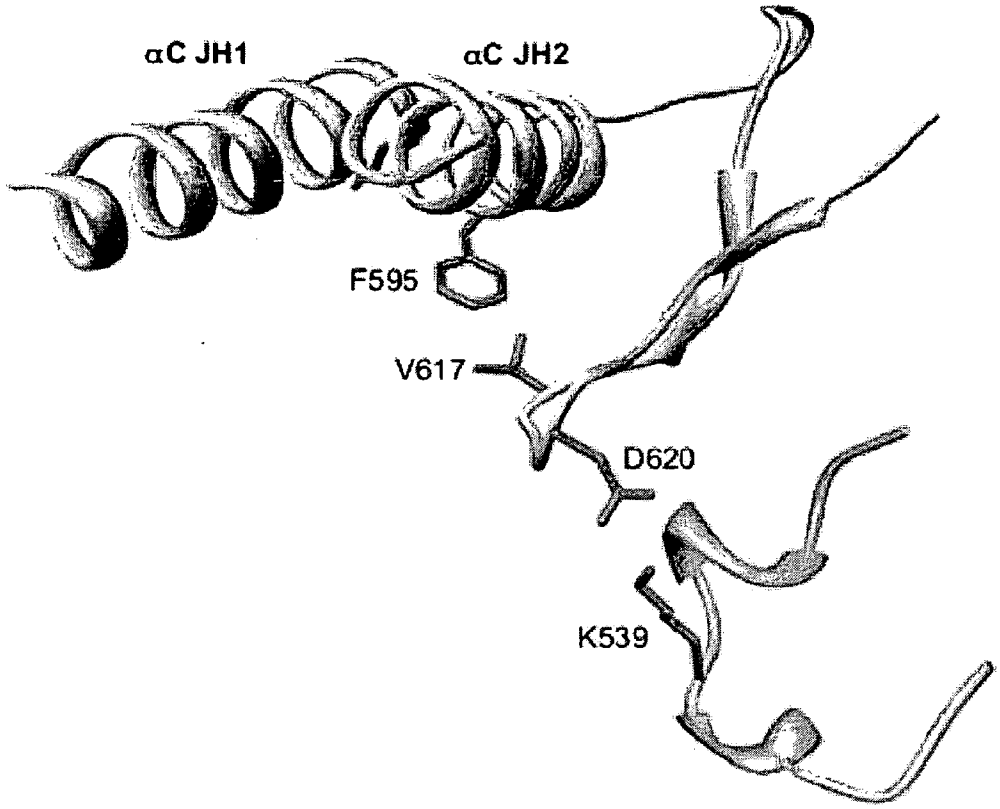

FIGURE 10

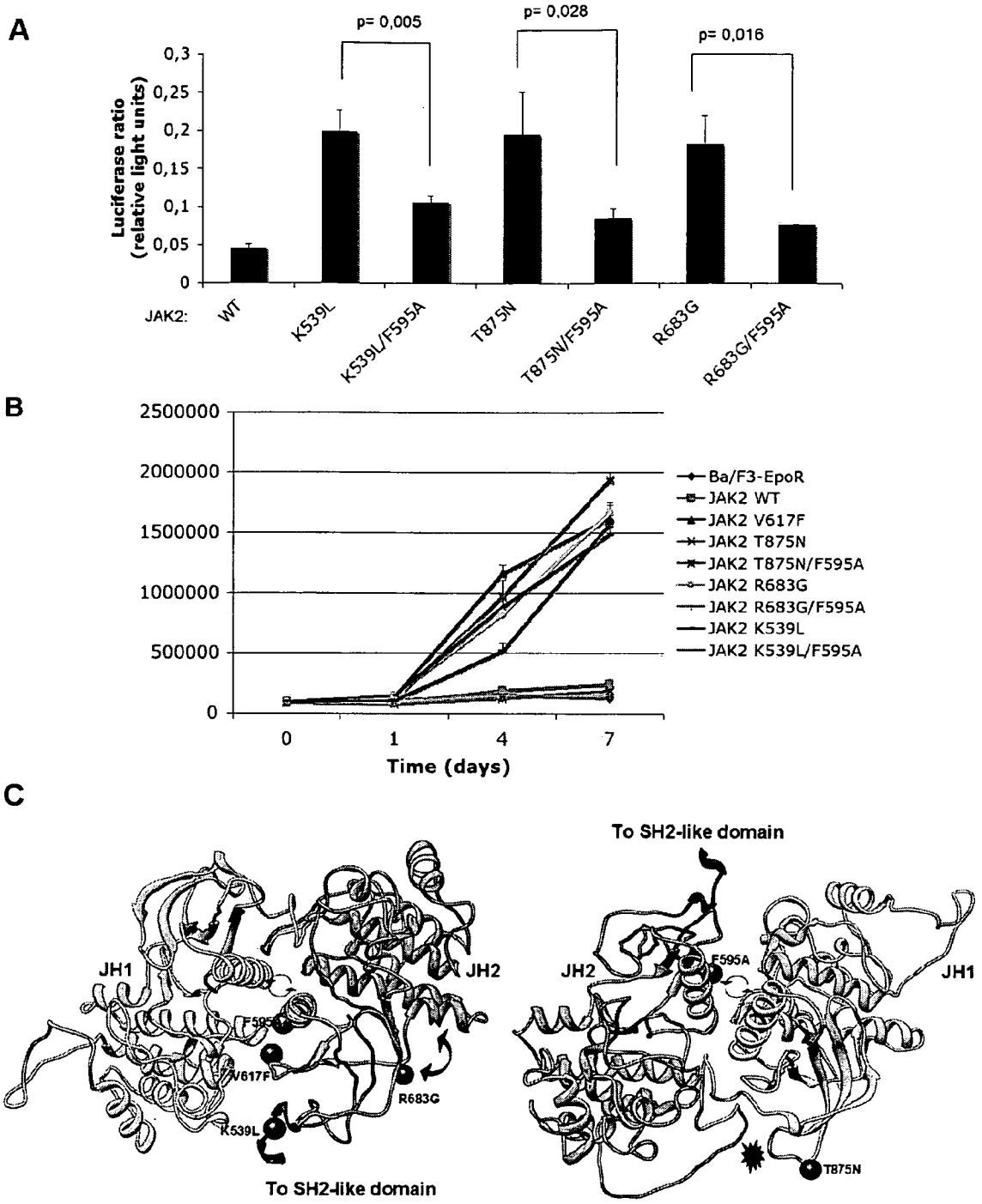
A
p= 0,005
p= 0,028
p= 0,016
Luciferase ratio (relative light units)
0,3
0,25
0,2
0,15
0,1
0,05
0
JAK2:
WT
K539L
K539L/F595A
T875N
T875N/F595A
R683G
R683G/F595A
B
2500000
2000000
1500000
1000000
500000
0
0
1
4
7
Time (days)
Ba/F3-EpoR
JAK2 WT
JAK2 V617F
JAK2 T875N
JAK2 T875N/F595A
JAK2 R683G
JAK2 R683G/F595A
JAK2 K539L
JAK2 K539L/F595A
C
JH1
JH2
V617F
R683G
K539L
To SH2-like domain
To SH2-like domain
JH2
F595A
JH1
T875N

FIGURE 11

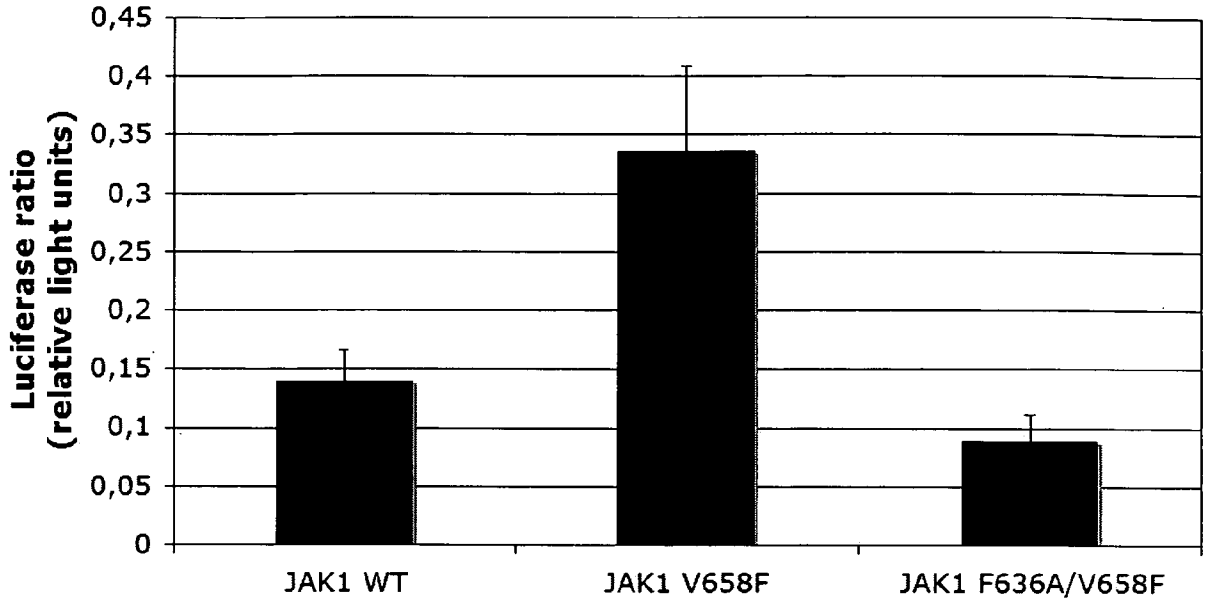

FIGURE 12

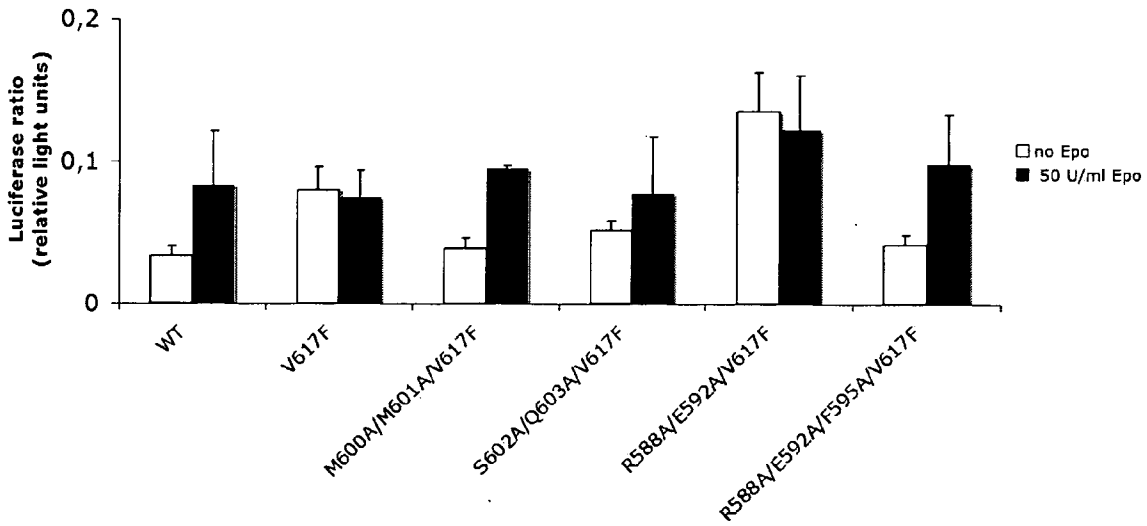

FIGURE 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/001538

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KISS R ET AL: "Identification of a novel inhibitor of JAK2 tyrosine kinase by structure-based virtual screening" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, ELSEVIER SCIENCE, GB LNKD-DOI:10.1016/J.BMCL.2009.04.138, vol. 19, no. 13, 5 May 2009 (2009-05-05), pages 3598-3601, XP026155116 ISSN: 0960-894X [retrieved on 2009-05-05] whole doc., in particular fig. 4, p. 3600, right col.	1-22

-/--

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 September 2010	15/11/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lüdemann, Susanna

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/001538

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUSA ALEXANDRA ET AL: "Substitution of pseudokinase domain residue Val-617 by large non-polar amino acids causes activation of JAK2" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 283, no. 19, May 2008 (2008-05), pages 12941-12948, XP002601734 ISSN: 0021-9258 p. 12947, right col. -12948, left col.	1-22
A	ROBERS MATTHEW B ET AL: "Cellular LanthaScreen and beta-lactamase reporter assays for high-throughput screening of JAK2 inhibitors" ASSAY AND DRUG DEVELOPMENT TECHNOLOGIES, vol. 6, no. 4, August 2008 (2008-08), pages 519-529, XP002601735 ISSN: 1540-658X whole doc, in particular abstract, p. 524, right col. - p. 528, left col.,p.529	1-22
A	J. Sayyah et al: "Identification and Characterization of a Novel Jak2 Tyrosine Kinase Inhibitor" ASH Annual meeting abstracts, [Online] vol. 108, 2006, pages ABSTRACT-3604, XP002601736 ASH Annual meeting abstracts Retrieved from the Internet: URL:http://abstracts.hematologylibrary.org/cgi/content/abstract/ashmtg;108/11/3604?maxtoshow=&hits=10&RESULTFORMAT=1&author1=sayyah&andorexacttitle=and&andorexacttitleabs=and&andorexactfulltext=and&searchid=1&FIRSTINDEX=0&sortspec=relevance&volume=108&resourcetype=HWCIT> [retrieved on 2010-09-17] abstract	1-22
T	DUSA ALEXANDRA ET AL: "JAK2 V617F Constitutive Activation Requires JH2 Residue F595: A Pseudokinase Domain Target for Specific Inhibitors" PLOS ONE, vol. 5, no. 6, June 2010 (2010-06), XP002601737 ISSN: 1932-6203 the whole document	1-22

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 23-30, 51-57

Present claims 23-30,51-57 encompass compounds (claim 30: method of treatment for said compounds) defined only by their desired function, contrary to the requirements of clarity of Article 6 PCT, because the result-to-be-achieved type of definition does not allow the scope of the claim to be ascertained. The fact that any compound could be screened does not overcome this objection, as the skilled person would not have knowledge beforehand as to whether it would fall within the scope claimed. Undue experimentation would be required to screen compounds randomly.
The non-compliance with the substantive provisions is to such an extent that no meaningful search of claims 23-30, 51-57 could be carried out at all (Article 17(2) PCT).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2)PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/001538

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 23-30, 51-57
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-22

A method of identifying/screening an agenthat modulates the interaction of amino acid F617, L539, N 875 OR G 684 with amino acid F 595in a mutant JAK 2 with a V617F, a K539L, a T875N OR an R683G mutation.

---

2. claims: 31-50

A method of identifying/screening an agenthat modulates the interaction of amino acid F658 with amino acid F636 in a mutant JAK1 with a V658F mutation.

---

3. claims: 58-66

A method for the design/identification of an agent bind to/masking amino acids 617, 539, 875, 683 in a mutant or wt JAK2 or amino acid 658 in a mutant or wt JAK1.
