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Janus kinase 2 activation mechanisms revealed by analysis of suppressing mutations

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Abstract

Background: Janus kinases (JAK1–3, TYK2) mediate cytokine signals in the regulation of hematopoiesis and immunity. JAK2 clinical mutations cause myeloproliferative neoplasms and leukemia and the mutations strongly concentrate in the regulatory pseudokinase domain, JAK homology 2, JH2. Current clinical JAK inhibitors target the tyrosine kinase domain and lack mutation- and pathway-selectivity.

Objective: To characterize mechanisms and differences for pathogenic and cytokineinduced JAK2 activation to enable design of novel selective JAK inhibitors.

Methods: Systematic analysis of JAK2 activation requirements using structure-guided mutagenesis, cell signaling assays, microscopy, and biochemical analysis.

Results: Distinct structural requirements identified for activation of different pathogenic mutations. Specifically, the predominant JAK2 mutation V617F is the most sensitive to structural perturbations in multiple JH2 elements (C helix (αC), SH2-JH2 linker and ATPbinding site). In contrast, activation of K539L is resistant to most perturbations. Normal cytokine signaling shows distinct differences in activation requirements: JH2 ATP-binding site mutations have only a minor effect on signaling, while JH2 αC mutations reduce homomeric (JAK2-JAK2) EPO signaling, and almost completely abrogate heteromeric (JAK2-JAK1) IFNγ signaling, potentially by disrupting a dimerization interface on JH2.

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Conclusions: These results suggest that therapeutic approaches targeting the JH2 ATPbinding site and αC could be effective in inhibiting most pathogenic mutations. JH2 ATPsite targeting have potential for reduced side-effects by retaining EPO and IFNγ functions. Simultaneously, however, we identify the JH2 αC interface as a potential target for pathway-selective JAK inhibitors in diseases with unmutated JAK2, thus providing new insights for the development of novel pharmacological interventions.

Keywords

Janus kinase; JAK2 V617F; cytokine signaling; myeloproliferative neoplasm; kinase activation; drug design

Introduction

Janus kinases (JAKs) are non-receptor tyrosine kinases critically involved in cellular signaling, regulating the immune system, development, differentiation, and growth [1]. Signaling through JAKs are numerous proinflammatory cytokines, including interleukins (IL-)2, IL-3, IL-4, IL-6, IL-9, IL-12, IL-13, IL-15, IL-23, and granulocyte-macrophage colony stimulating factor (GM-CSF), making JAK inhibition a tempting drug target for the treatment of inflammatory diseases [2]. Similarly, aberrant signaling caused by activating gain-of-function (GOF) mutations in JAKs underlie multiple neoplastic diseases, including myeloproliferative neoplasms [3]. Indeed, the recent advent of JAK inhibitors for the treatment of both of these disease groups has made understanding the mechanisms of JAK-STAT signaling highly relevant to the clinical immunologist [4].

JAKs associate with type I and type II cytokine receptors and mediate cytokine signals from activated receptors to signal transducers and activators of transcription (STATs), which upon phosphorylation by JAKs, move to the nucleus to activate transcription. The four JAKs in mammals (JAKs 1–3, TYK2) signal at homodimeric (JAK2) or heterodimeric/oligomeric (all JAKs) receptors and consist of four domains (N-to-C): a 4.1-band, ezrin, radexin, moiesin (FERM) domain, a Src homology 2 (SH2)-like domain, a pseudokinase domain (JAK homology 2, JH2), and a protein tyrosine kinase domain (JH1). FERM-SH2 mediate association to cytokine receptors [5]. JH2 serves a dual role; it inhibits the tyrosine kinase activity of JH1 in the basal state, and is required for full activation upon cytokine stimulation [6–9].

JH2 is a mutational hotspot for clinical JAK mutations. The somatic JAK2 V617F mutation in exon 14, for example, causes ligand-independent JAK2 activation and underlies >95% of polycythemia vera and >50% of essential thrombocythemia and primary myelofibrosis cases [10]. Other JAK2 GOF mutations are located in JH2 in exon 12 (residues 506–547, including K539L), exon 16 (including R683S/G), and some in JH1 [3]. JH2 GOF mutations in other JAKs cause leukemias and loss-of-function mutations cause immune deficiencies [3], highlighting the dual regulatory role of JH2 [6,7,11]. Recent structural information of the JH2JH1 interaction explains the inhibitory function of JH2 [9,12]. In this interaction, the C helix (αC) side of JH2 binds to JH1 in a front-to-back orientation (Figure 1 A) leading to conformational restriction of JH1 and inhibition of kinase activity [12].

While multiple GOF mutations lie in the JH2-JH1 interface, disruption of the JH2-JH1 interaction alone does not fully explain the high activation potential of all GOF mutations—including JAK2 V617F or JAK2 K539L. We speculate that these mutations utilize the known, but molecularly incompletely characterized, stimulatory function of JH2 to activate JAK2.

Current clinical JAK inhibitors used to treat diseases caused by JAK2 GOF mutations target JH1 and thus do not distinguish between mutated and wild-type (WT) JAK2 and are unable to eradicate the disease. Furthermore, they frequently lead to anemia caused by suppression of normal erythropoietin (EPO) signaling due to inhibition of JAK2 WT functions [13]. In contrast, in inflammatory diseases, in which usually no JAK mutations are present (with rare exceptions, see e.g., ref [14]) current inhibitors are effective in approximately half of the patients, but also affect unwanted cytokine functions and show side-effects such as reactivation of viral infections and anemia [4]. Thus, there is a clinical need for more effective and selective JAK inhibitors able to discriminate between pathogenic and cytokine-induced signaling and/or discriminate between different types of JAK-mediated signaling pathways.

However, a potential paradigm shift in JAK inhibition is emerging, as molecular characterization of JH2 is suggesting an alternative approach and implies JH2 to be a valid target for novel modulators of JAK activity [15]. JH2 harbors the majority of human pathogenic JAK mutations, and we recently identified the JAK JH2 ATP-binding site as a potential drug target by demonstrating that activation by the pathogenic JAK2 JH2 GOF mutations K539L, V617F, and R683S is reliant on the stabilizing effect of ATP binding on JH2 [16]. Furthermore an ATP-competitive compound targeting TYK2 JH2 has been demonstrated to efficiently and specifically inhibit cytokine signaling [17].

Here, we provide a systematic analysis of the molecular basis for different JH2-targeting intervention strategies. We identify distinct differences in activation mechanisms between clinical JAK2 GOF mutations in terms of reliance on specific activating JH2 molecular interfaces and JAK2-mediated receptor dimerization. Analysis of cytokine-induced signaling shows differences in JH2 interface requirements between homodimeric (EPO) and heterodimeric (Interferon γ, IFNγ) JAK2 activation. These results provide novel insights into pathogenic and cytokine induced JAK2 activation mechanisms that have implications for development of mutant- and potentially pathway-preferring inhibitors.

Materials and Methods

See Supplementary material for full details of Materials and Methods. Briefly, for immunoblotting and luciferase reporter assays, JAK2-deficient γ2A human fibrosarcoma cells [18] were transfected with the designated combination of human JAK2-HA, human HAEPOR (both in pCIneo), and human STAT5-HA (in pXM) using FuGENE HD (Promega) for 24–48 h. For reporter assays, a Firefly luciferase reporter plasmids for STAT5 (Spi-Luc [1]) or STAT1 (IRF-GAS [19]) were added along with a constitutively expressing *Renilla* luciferase plasmid. Cytokine stimulation was done in starvation medium without FBS for 30 min (for immunoblotting) or 5 h (for reporter assays) unless otherwise specified, with recombinant human EPO (Roche), or IFNγ (Peprotech). For immunoblotting, cells

were washed with PBS, lysed in Triton X-100 lysis buffer, and complete lysates run on lab-made SDS-PAGE gels. Immunoblots were blocked with bovine serum albumin and incubated with primary antibodies: HA Tag (Aviva Systems Biology), phospho-JAK2 (Millipore), phospho-STAT5 (Cell Signaling), phospho-STAT1 (Cell Signaling), STAT1 (BD Biosciences), or actin (Millipore), and a mixture of goat-anti-rabbit and goat-anti-mouse DyLight secondary antibodies (both Thermo Fisher Scientific). Blots were read using an Odyssey CLx (LI-COR), and immunoblot signals quantified using Image Studio software (LI-COR) by manually assigning bands (See Supplementary Material and Figure S1). Reporter assays were detected using the DualGlo reporter assay kit (Promega) according to manufacturer's instructions and normalized to readings from wells of unstimulated cells transfected with JAK2-HA WT.

For qPCR analysis, γ2A cells were transfected for 28 h, starved for 16 h, stimulated for 2 h with 10 U/ml EPO or 10 ng/ml IFNγ, and RNA extracted using TRI Reagent (Molecular Research Center) according to manufacturer's instructions. IRF1 gene expression was measured from reverse-transcribed total RNA using specific primers (5'GCATGAGACCCTGGCTAGAG-3' and 5'-CTCCGGAACAAACAGGCATC-3') and normalized to the expression of TATA-box binding protein (TBP).

For *in vitro* kinase assays, recombinant JAK2 JH2-JH1 (residues 513–1132–6×His) WT, I559F, and E592R proteins were expressed in High Five insect cells (Thermo Fisher Scientific) using the Bac-to-Bac expression system (Invitrogen) according to manufacturer's instructions. Cells were lysed by freeze-thawing, clarified by centrifugation, and recombinant proteins purified using Ni-NTA agarose (Qiagen) followed by sizeexclusion chromatography in a HiLoad 16/600 Superdex 75 pg column (GE Healthcare). Protein concentrations were measured by Bradford assay (Bio-Rad) and enzymatic activity determined with Lance Ultra kinase assay (PerkinElmer) under conditions recommended by the manufacturer. Kinase reactions were performed in triplicate and results shown are representative from 2–3 individual experiments.

For microscopy, cells were seeded on 35 mm glass bottom dish (MatTek), transfected with JAK2-YFP fusion constructs (in pEGFP) or EPOR-YFP/EPOR-CFP (in pBOF [20]) overnight and starved for 8 h. Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 15 minutes at room temperature, washed, and kept in PBS at 4 °C before imaging on a Zeiss LSM 780 laser scanning confocal microscope using a Plan Apochromat 63×/1.4 oil immersion objective. FRET was monitored by acceptor photobleaching [21] and FRET efficiency was calculated from manually segmented cell membrane areas.

Results

Suppressing mutations reveal differences in activation mechanisms of GOF mutations

Studies on JAK2 activation mechanisms have identified several mutations capable of suppressing activation by pathogenic JAK2 GOF mutations (see Table 1, Figure 1, Figure 2). These mutations, termed here 'suppressing mutations', localize in JH2 αC (F595A), in the C-terminus of the SH2-JH2 linker (F537A) and in the JH2 ATP-binding site (see Table 1). Recently Leroy et al. identified an additional residue in the outer face of JH2 αC (JAK2

E596) as an important link in the activation mechanism of V617F, but not of K539L, R683G, or of T875N [22]. Notably, these suppressing mutations are functionally distinct from mutations that completely destabilize JH2 structure (e.g. JAK2 F739R refs [16,23] or deletion of JH2 αG [8]), which mimic JH2 deletion resulting in increased basal activation and irresponsiveness to cytokines.

The activation mechanisms and requirements of regulatory interfaces for different GOF mutations and cytokine-induced JAK2 activation have not been systemically analyzed. We thus set out to compare ligand-independent (pathogenic) and normal ligand-dependent JAK2 activation using suppressing mutations in JAK2-deficient γ2A fibroblast cells. We focused on the three previously identified regulatory regions in JH2: the ATP-binding site (JAK2 mutations I559F, G552A+G554A, or K677E) [16], the outer face of JH2 including αC (F595A, E596R) [12,22–25], and the C-terminus of the SH2-JH2 linker (F537A) [26]. Additionally, we tested a novel JH2 αC outer face mutation, E592R, in order to analyze the involvement of the N-terminus of the JH2 αC. We further hypothesized that JH2 functions as a structural linker between FERM-SH2 and JH1 and, when structurally sound, is able to position JH1 for trans-autophosphorylation. To test this, we aimed to break up the putative interaction between FERM-SH2 and JH2 by introducing V511R to disrupt the short β sheet between the SH2-JH2 linker and the FERM F1-F2 loop (Figure 1). We also included JH1 αC outer face mutations (E896A+E900A) analogous to the JH2 mutations E592R/E596R to test the function of JH1 αC as a potential interaction interface.

We analyzed activation by three different pathogenic GOF JAK2 mutations predicted to have differing activation mechanisms (Figure 2): V617F (exon 14), which has been suggested to alter the conformation of the SH2-JH2 linker and thus indirectly affect the inhibitory JH2-JH1 interaction [12,26]; R683S (exon 16), which is predicted to activate primarily by breaking the inhibitory JH2-JH1 interaction [9,12]; and K539L (exon 12), which lies at the N terminus of JH2, and thus might also affect the SH2-JH2 linker, but whose activation mechanism has not been studied in detail.

In accordance with previous reports [16,22,24,25] we found that ligand-independent JAK2 JH1 activation loop (Y1007-Y1008, pJAK2) hyperphosphorylation caused by V617F is suppressed by JH2 ATP pocket and αC mutations (Figure 2 A and B, first panel). V617Finduced pJAK2 is also suppressed by V511R, suggesting that the activation mechanism of V617F requires correct linking of JH2 to SH2. However, JAK2V617F activation is not sensitive to perturbation of JH1 αC (Figure 2 A and B, first panel). Downstream pSTAT1 analysis correlated with pJAK2 levels. Effects of suppressing mutations on STAT5 activation were analyzed in reporter assays with EPOR-HA coexpression (Figure 2 D), where the inhibition profile correlated with pJAK2 and pSTAT1 analysis with strongest inhibition with αC mutations and F537A.

Activation by R683S was sensitive to all suppressing mutations in pJAK2 and pSTAT1 analysis as well as in STAT5 transcriptional activation, and the ATP-binding site mutants showed slightly more suppression than mutations in αC. Interestingly, K539L was clearly the most resistant to suppression, and only the αC mutation F595A strongly suppressed K539L in pJAK2, pSTAT1, and STAT5 activation. JH2 ATP-binding site mutations affected

mainly JAK2 phosphorylation. These data suggest a distinct activation mechanism for K539L over V617F and R683S.

Taken together, these results indicate that interactions involving JH2 are critical for hyperactivation of all JAK2 GOF mutants, but that the specific JH2-mediated interactions differ between the GOF mutations.

The effect of suppressing mutations on cytokine activation

Cytokine stimulation titrations with JAK2-HA V617F+suppressor double-mutant constructs showed that even strong suppression of basal V617F-induced activity did not inhibit cytokine-induced STAT5 transcriptional activation for EPO or STAT1 activation for IFNγ (Figure 3 A and B, respectively). Rather, the most potent suppressor mutations (F537A and all αC mutations) restored EPO sensitivity to be indistinguishable from JAK2 WT (Figure 3 A). For IFNγ, cytokine sensitivity was also restored, which was further corroborated with qPCR of induction of expression of the IFNγ-responsive gene *Interferon regulatory factor 1* (IRF1). Interestingly, however, IFNγ-induced STAT1 activation with αC and F537A mutations with V617F were lower than with JAK2 WT (Figure 3 B and C) suggesting potential specific involvement of these regions in IFNγ signaling.

Previous work has suggested that suppressing mutations do not inhibit cytokine-induced signaling in a JAK2 WT background [16,22,24], but detailed analysis of sensitivity to different modes of JAK2-mediated signaling (homo- vs. heterodimeric) has been lacking. We thus analyzed cytokine-induced JAK2 activation using the mutation panel in a JAK2 WT background. Immunoblot analysis of JAK2-mediated STAT5 phosphorylation on homodimeric EPO receptor showed that, despite lower basal signaling activity, EPOinduced signaling was preserved in suppressing mutations (Figure 4 A), and JH2 ATPbinding site mutations were virtually identical to JAK2 WT in their response to EPO. F595A and E592R in αC and F537A, however, showed diminished EPO-induced STAT5 phosphorylation (Figure 4 A). Reporter assays showed similar results, albeit with differences even more pronounced (Figure 4 C).

Strikingly, the same JH2 αC and SH2-JH2 linker mutations practically abolished heteromeric JAK2-JAK1-mediated STAT1 phosphorylation upon IFNγ stimulation (Figure 4 B), while JH2 ATP-site mutations were again indistinguishable from JAK2 WT. In accordance with pSTAT1-immunoblot data, IFNγ-induced STAT1 transcription activity was almost completely abrogated for all αC mutations (including E596R) and F537A, whereas JH2 ATP-site mutations and V511R showed no significant decrease (Figure 4 D). *IRF1*qPCR further corroborated these results with E592R and I559F (Figure 4 E).

We also measured STAT1 activation with longer IFNγ stimulation times to estimate rule out simply delayed signaling kinetics [27], and found no activation of STAT1 with E592R or F537A mutants even at long time-scales (Figure S2 D).

Characterization of JAK2 GOF activation mechanisms by suppressor mutations

JAK2 V617F hyperactivation relies on the interaction with cytokine receptors but the underlying mechanisms are still elusive [28–30]. Disruption of FERM and receptor binding of

JAKs to receptors is a potential mechanism of suppression [28], and it has been suggested that some JAK3 JH2 mutations (including a JH2 ATP-binding site mutation) could affect subcellular localization of JAK3 [31]. We assessed subcellular localization of our suppressing mutations by imaging JAK2-YFP fusion proteins, but found no effect for either mutation on subcellular localization, either with or without added EPOR-HA (Figure 5 A). In contrast, JAK2-YFP with Y119E, known to cause dissociation from receptors [29,32], showed exclusively cytoplasmic JAK2 (Figure 5 A).

We speculated that mutation-induced JAK2-receptor dimerization is part of the activation mechanism of JAK2 GOF mutations. We thus analyzed whether suppressing mutations directly affect the propensity of JAK2 to dimerize on receptors. Using a FRET-based EPOR-CFP/YFP receptor dimerization assay in the JAK2-deficient γ2A fibroblast cell line (lacking endogenous EPOR expression) along with our JAK2-HA mutant constructs showed that E592R significantly reduces basal JAK2-EPOR dimerization (Figure 5 B). I559F, in contrast, showed a slight increase in dimerization, but this was within experimental noise and not significant.

To directly assess whether decreased dimerization propensity also translates to decreased receptor-mediated JAK2 activation, we assessed basal activation of otherwise wild-type JAK2. EPOR-HA overexpression (known to induce ligand-independent activation [16]) alongside expression of JAK2 mutants showed that all suppressing mutations, irrespective of their mode of action, suppress EPOR-induced JAK2 activation (Figure 5 C), suggesting that other mechanisms beyond lowering of dimerization propensity (as shown for E592R (Figure 5 B), and potentially also true for other αC mutations) are likely at play to explain the mode of action of suppressing JH2 ATP-binding site mutations. We thus measured whether mutating the JH2 ATP-binding site or αC directly affects the enzymatic activity of JH1. Indeed, kinase assays with recombinant JAK2 JH2-JH1 fragments *in vitro* showed unchanged $K_{m,\ ATP}$ values for both JH2 mutations I559F and E592R, but lowered kinase reaction catalysis rates (k_{cat}) for I559F (Figure 5 D).

Taken together, these data suggest that the mechanisms of suppression of JH2 ATPbinding site and αC mutations are different. Inhibiting ATP binding to JH2 directly lowers catalytic activity of JH1, potentially through lowering the stability of the JH2 αC [16], and thus strengthening the JH2-JH1 interaction [12]. Altering the outer face charge of αC directly (e.g., with E592R), on the other hand, inhibits the propensity of JAK2 to dimerize and thereby hinders JH1 activity by suppressing trans-autophosphorylation (Figure 5 B, D).

Discussion

The molecular mechanisms of JAK activation by cytokine or mutation have long been elusive and here we have performed a systematic analysis of JAK2 activation mechanisms using structure-guided mutagenesis. Our results shed light on not only the mechanism of cytokine-independent JAK2 activation, but also identify a previously unknown interface on JH2 involved in JAK2-mediated receptor dimerization and needed especially for heteromeric JAK signaling.

Our results enable grouping of activating JAK2 mutations based on their requirements for distinct structural elements and thus activation mechanisms. Both V617F and R683S were sensitive to mutations affecting the JH2 ATP binding site and αC, albeit their suppressing effects showed differences, i.e., αC mutations completely abrogated V617F but not R683S, while JH2 ATP-binding site mutations showed similar suppression of both. The effect of suppressing mutations were more pronounced at pJAK2 and pSTAT1 than on STAT5 reporter assays which may reflect technical differences (e.g., stability of luciferase), or be indicative of signal amplification in the JAK-STAT pathway. Previously, we have shown that a suppressing JH2 ATP-binding site mutation reverts the increased hematocrit in a mouse V617F MPN model [16].

The resistance of K539L compared to V617F and R683S to suppressing mutations is interesting, since K539 and V617 reside near each other in the JH2 structure (Figure 1), and suggests a distinct activation mechanism for K539L. R683S likely activates through breaking the autoinhibitory interface resulting in increased conformational freedom and activation of JH1. This freed JH1 does, however, still rely on correct JH2-mediated positioning for ligand-independent activation, as well as JAK2-mediated receptor dimerization (see V511R and αC mutations, respectively in Figure 2). In contrast, K539L is unlikely to simply interfere with the autoinhibitory interaction, and our inhibitory profile analysis is consistent with a more direct activation mechanism of K539L, potentially involving direct activation of JH1, e.g., through interaction with K857 on JH1 [22]. Consistent with previous reports, K539L is effectively inhibited only by F595A, which is known primarily for participating in stabilizing interactions in JH2 αC in the context of V617F hyperactivation [24,25], but which has been suggested to alter the stability of the JH2 αC also more broadly.

For V617F, our results show complete inhibition by αC mutations including E592R, which our FRET-data indicate to interfere with JAK2-mediated receptor dimerization (Figure 5 B). We thus hypothesize that V617F activates JAK2 mostly by enhancing the propensity of JAK2 to dimerize on a receptor. This is in line with previous reports with recombinant JAK2 and TYK2 JH2-JH1 fragments, which showed only modest activation of kinase activity with the JAK2 V617F or analogous TYK2 V678F mutation in an isolated *in vitro* setting, which does not include JAK-mediated receptor dimerization effects [9,33]. Our mutagenesis data furthermore suggests, that the dimerization interface directly includes the JH2 αC, with E592 (and probably E596) involved.

Our analysis of suppressing mutations in an otherwise wild-type background shows that, contrary to previous reports [16,22,24], suppressing mutations do affect JAK2 WT activity by lowering both basal (Figure 5 C), as well as ligand-dependent activation (Figure 4). Interestingly, quantitative comparison of potency of individual suppressing mutations to inhibit activation by V617F and cytokine reveals that these two correlate clearly (Figure S3). The correlating suppression of ligand-dependent and -independent JAK2 activation suggests that the same JH2 interface (αC and C-terminus of SH2-JH2 linker) is used in both settings. Furthermore, imaging data of JAK2-YFP shows unaltered subcellular distribution of JAK2 carrying suppressing mutations (Figure 5 A) ruling out direct destabilization of JAK2/FERM as an explanation for suppression.

Our results also reveal that JH2 αC suppressing mutations most likely inhibit JAK2 activation by suppressing JAK2 dimerization (Figure 5 B), while JH2 ATP-binding site mutations exert their suppressing effects by directly affecting tyrosine kinase activity of JAK2 JH1 (Figure 5 D), potentially through partial destabilization of JH2 αC [12,16]. We thus conclude, that V617F and R683S most likely activate JAK2 by increasing its propensity for dimerization, and that this is counteracted by suppressing mutations in the JH2 αC (E592R, E596R, F595A) and SH2-JH2 linker (F537A). For the case of R683S, which lies directly in the JH2-JH1 autoinhibitory interface (Figure 1 and refs [9,12]), we speculate that weakening of the autoinhibitory interaction releases JH2, which relieves autoinhibition on JH1, as well as exposing the dimerization interface on JH2.

The molecular details of heteromeric JAK activation have remained largely unknown. Our analysis of cytokine-stimulation of JAK2 carrying suppressing mutations strikingly suggest that the same interface needed for activation by V617F or R683S by dimerization of (receptor-bound) JAK2, is crucial especially for heteromeric activation of JAK2. This finding refines earlier work that showed a critical role for JH2 in JAK activation [7,11]. Previous studies have shown in several cytokine receptor systems that catalytic activity of both JAKs is not required for heteromeric JAK activation [20,34]. For instance, in IFNγ signaling STAT1 does not require enzymatically functional JAK1 [35], but does require the presence of JAK1 JH2 [36]. Pathway-specific JAK substructures have also been implicated in JAK2 JH1 for EPO signaling [37]. Our results refine these findings by identifying the Cterminus of the SH2-JH2 linker and JH2 αC as critical for heteromeric JAK activation. However, whether the JH2 interface identified here participates in JAK2-JAK2 or JAK2JAK1 dimers/multimers on IFNGR remains a topic for future research.

Currently available JAK inhibitors show beneficial clinical responses, but there is a clear need for more effective, optimally disease-selective drugs with less side-effects. The key question for this goal is to understand the differential mechanisms defining pathogenic and different cytokine-induced activation modes. Our results presented here provide insights into these questions and identify specific regions in JH2 that are differentially required for JAK2 activation in different contexts. These findings pave the way for the design of novel, potentially mutant and/or pathway-selective pharmacological compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

JAK	Janus kinase
GOF	gain-of-function
MPN	myeloproliferative neoplasm
JH	JAK homology

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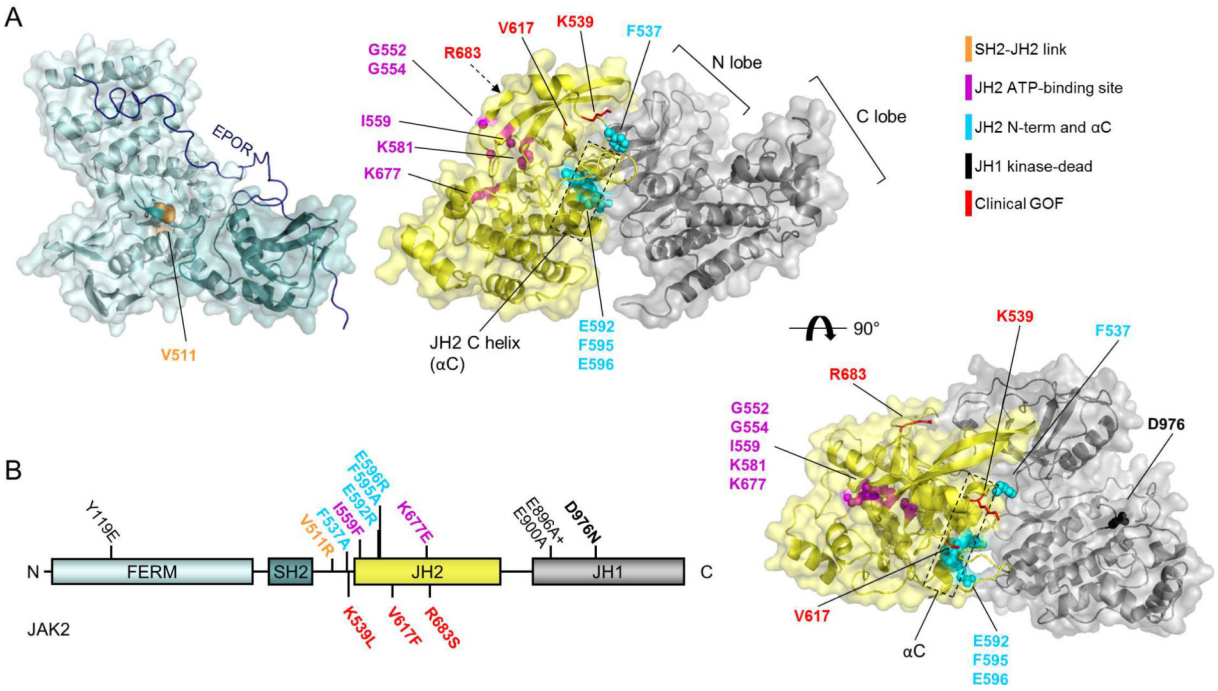

Figure 1: JAK2 domain structure.

A: Structures of JAK2 FERM-SH2 (left, PDB: 4Z32) with model of EPOR JAK2-binding peptide shown in dark blue (modelled based on Interferon λ 1 receptor (IFNLR1) peptide bound to JAK1 FERM-SH2, PDB: 5L04), and JAK2 JH2-JH1 inhibitory interaction [12]. Right: JAK2 JH2-JH1 top view. B: Domain structure of JAK2. See also Table 1.

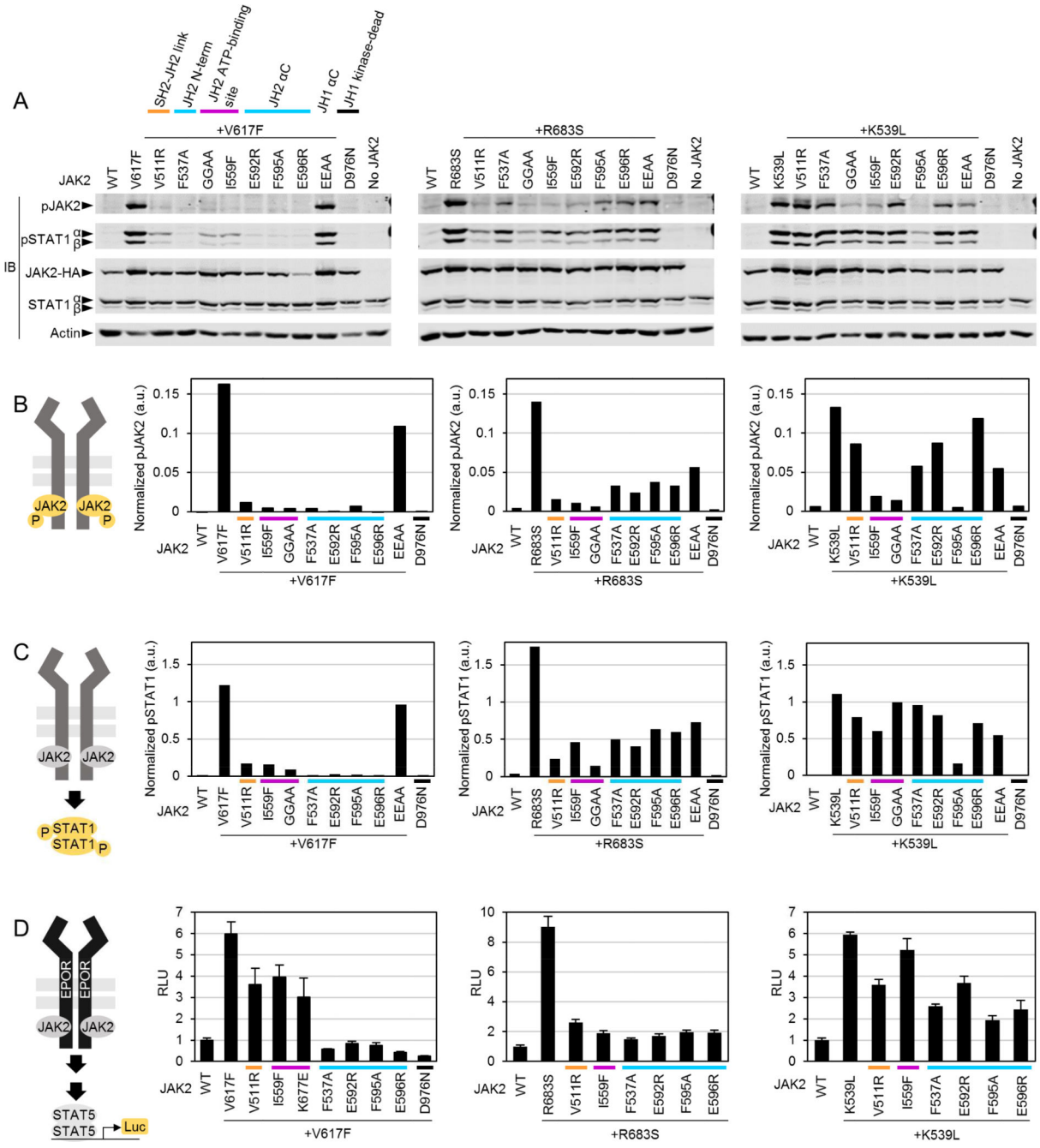

Figure 2: Suppressing JAK2 mutations reveal distinct activation mechanisms for different JAK2 gain-of-function (GOF) mutations.

A: Representative immunoblots of whole-cell lysates from JAK2-deficient γ2A cells transiently transfected with full-length JAK2-HA mutants as indicated. pJAK2, JAK2 activation loop phosphorylation JAK2(Y1007/1008); pSTAT1, STAT1(Y701) phosphorylation. GGAA, G552A+G554A. EEAA, E896A+E900A. Experiment was repeated twice with similar results. B and C: Quantification of immunoblots shown in A. a.u., arbitrary units. D: STAT5 reporter assay in the presence of transfected EPOR-HA. Averages and standard deviations from triplicate wells are shown as fold induction relative

to unstimulated JAK2 WT. RLU, relative luminescence units. All reporter experiments were repeated twice with similar results.

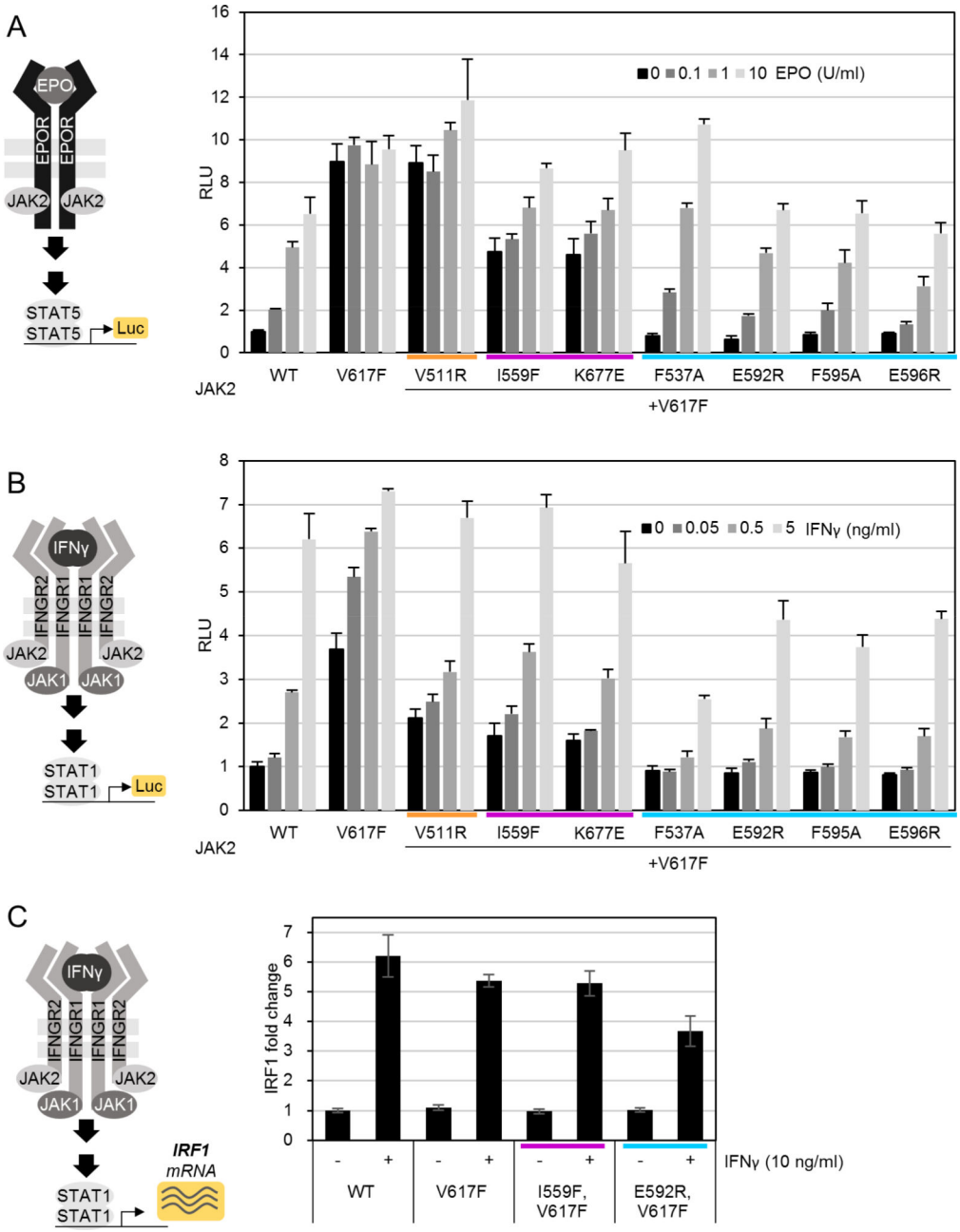

Figure 3: Suppression of V617F activation by secondary mutations restores cytokine sensitivity.

A: STAT5 reporter assay in the presence of transfected EPOR-HA. B: IFNγ/STAT1 reporter. A and B as described for Figure 2. C: quantitative PCR (qPCR) of IFNγ-induced *interferon regulatory factor 1* (*IRF1*). Averages and standard deviations from two biological replicates each done in technical triplicates in qPCR are shown. Mutations are color-coded by type as in Figure 2.

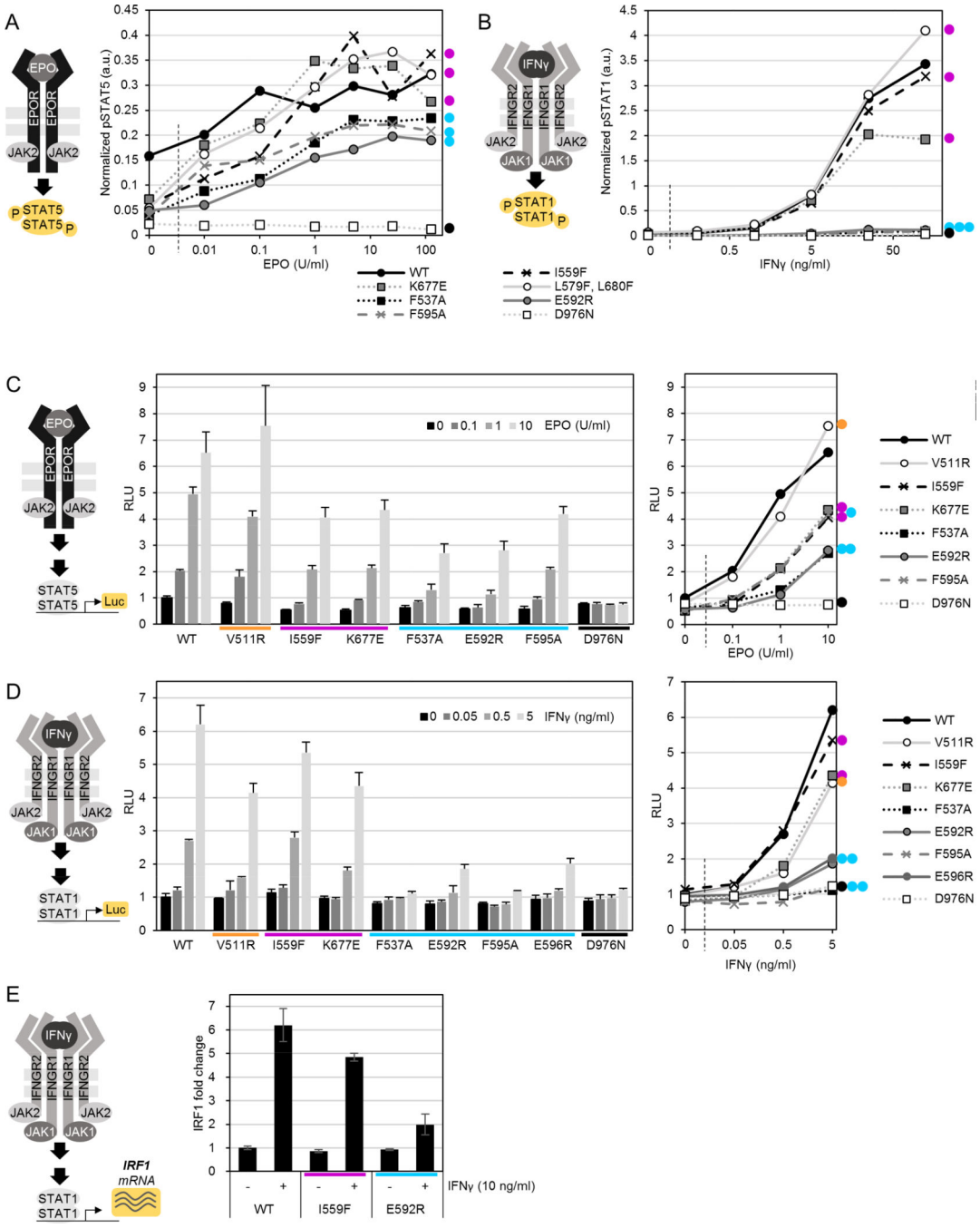

Figure 4: Analysis of suppressing mutations in JAK2 WT background.

A and B: Quantifications from immunoblots, see also Figure S2 A and B. C: STAT5 reporter assay in the presence of transfected EPOR-HA. D: IFNγ/STAT1 reporter assay. C and D as described for Figure 2. E: qPCR of *IRF1* expression as described for Figure 3. Wild-type sample (WT) same as in Figure 3 C. Mutations are color-coded by type as in Figure 2. Experiments were repeated twice with similar results.

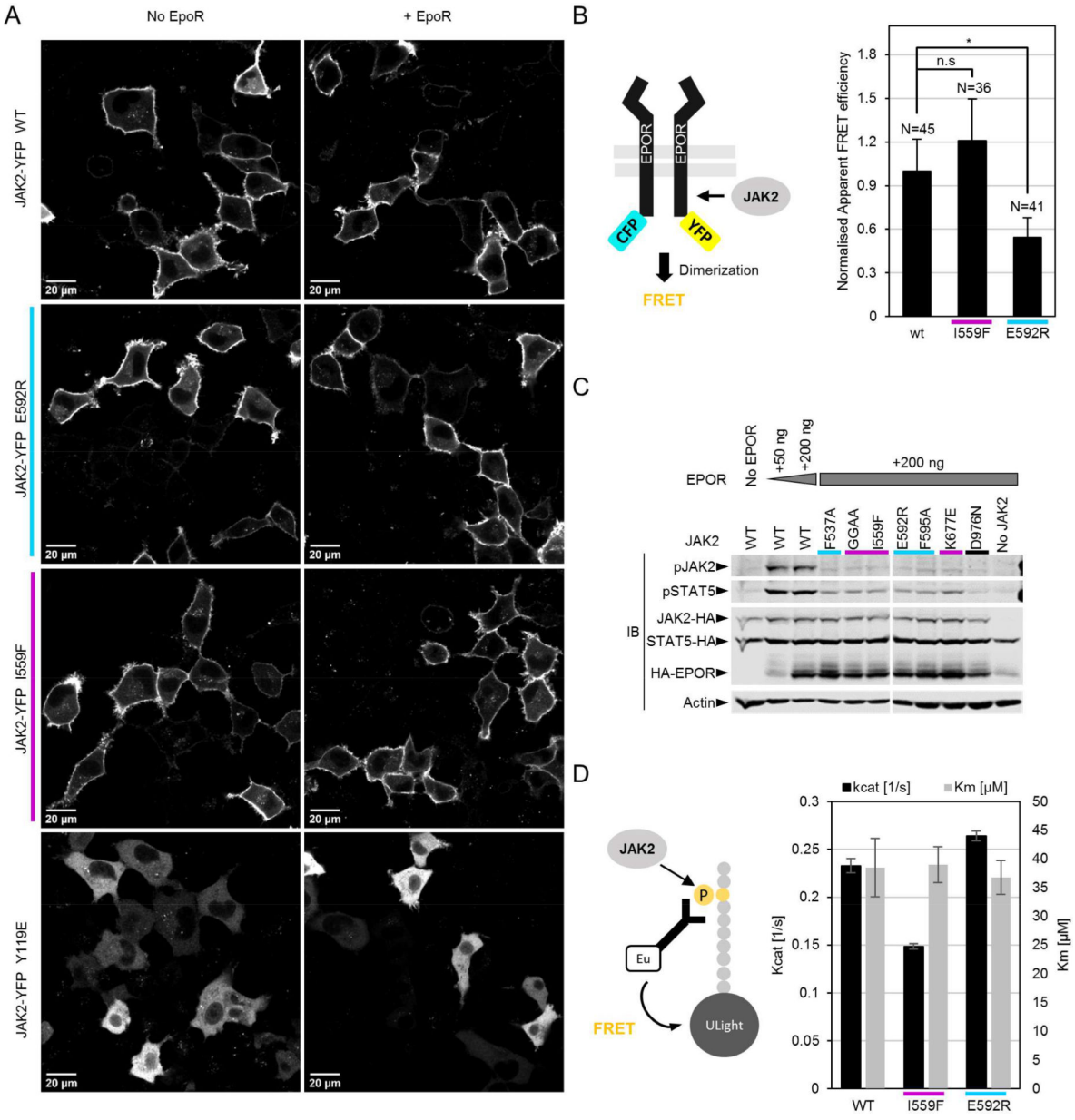

Figure 5: Suppressing mutations localize correctly to the membrane, but differentially affect dimerization of JAK2-EPOR and kinase activity of recombinant JAK2 JH2-JH1.

A: Representative confocal microscopy micrographs of fixed γ2A cells expressing the indicated JAK2-YFP mutations. B: Analysis of basal JAK2-EPOR dimerization. Normalized apparent FRET efficiency calculated from manually segmented cell membranes as detailed in Materials and Methods. Number of individual cells analyzed for each condition is indicated. Significance assessed by Student's t test (unpaired). n.s. = not significant; *$p < 0.05$. C: Immunoblot analysis of whole-cell lysate from γ2A cells transiently transfected with the JAK2-HA constructs and EPOR-HA as shown. D: Kinase assay with purified recombinant JAK2 JH2-JH1. Shown are averages and standard deviation from triplicate measurements.

Table 1:

Used JAK2 mutations and their presumed mode of action or experimental 530 rationale. See also Figure 1.

Mutation	Substructure	Rationale / mode of action	Reference
Y119E	FERM F1	Mimics Y119 phosphorylation. Previously reported to induce dissociation of JAK2 from receptor.	29,32
V511R	SH2	Designed to disrupt SH2-JH2 linker from FERMSH2.	-
F537A	SH2-JH2 link	F537 proposed to stack with F595 in JAK2 JH2 WT. Known to inhibit V617F.	26
K539L	SH2-JH2 link	Activating by unknown mechanism. Causes PV.	38
G552A + G554A	JH2 β1: Glyrich loop	Designed to remove flexible glycines usually needed for ATP binding.	16
I559F	JH2 β2	Designed to sterically inhibit ATP binding. Verified to inhibit ATP binding [16].	16
K581A	JH2 β3	Removes conserved β3 lysine.	16,39
E592R	JH2 αC	Outer face of JH2 αC	12
F595A	JH2 αC	Inner face of JH2 αC. Known to inhibit V617F and others by potentially destabilizing JH2 and making space for F617 (ref [12]).	12,23–25
E596R	JH2 αC	Outer face of JH2 αC. Known to inhibit V617F and others. Mechanism unknown.	22
V617F	JH2 β4-β5 loop	Activating, potentially by disturbing SH2-JH2 linker. Causes MPNs.	40–43
K677E	JH2 β6-β7 loop	Designed to inhibit ATP binding electrostatically. Verified to inhibit ATP binding.	16
R683S	JH2 β7-β8 loop	Activating, probably by breaking R683-D873 interaction over inhibitory JH2-JH1 interface. Causes ALL.	44,45
T875N	JH1 β2-β3 loop	Activating, mechanism probably similar to R683S. Causes AMKL.	46
L884P	JH1 β3-αC loop	Activating by unknown mechanism. Homologous to JAK3 L857P found in ALL.	47
E896A + E900A	JH1 αC	Outer face of JH1 αC.	-
D976N	JH1 β6-β7 loop	D in HRD. Mutation is catalytically inactive (i.e., kinase dead).	-