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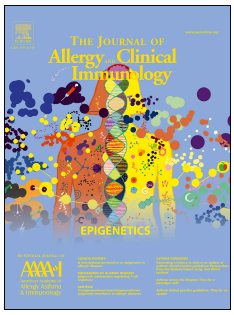

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Differential effect of inhibitory strategies of the V617 mutant of JAK2 on cytokine receptor signaling

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ABSTRACT

Background: Janus Kinase 2 (JAK2) plays pivotal roles in signaling by several cytokine receptors. The mutant JAK2 V617F is the most common molecular event associated with myeloproliferative neoplasms. Selective targeting of the mutant would be ideal for treating these pathologies by sparing essential JAK2 functions.

Objective: We characterize inhibitory strategies for JAK2 V617F and assess their impact on physiological signaling by distinct cytokine receptors.

Methods: Via structure-guided mutagenesis, we assessed the role of key residues around F617 and used a combination of cellular and biochemical assays to measure the activity of JAKs in reconstituted cells. We also assessed the effect of several specific JAK2 V617F inhibitory mutations on receptor dimerization using the NanoBiT protein complementation approach.

Results: We identified a novel JH2 αC mutation, A598F, suggested to inhibit the aromatic stacking between F617 with F594 and F595. Like other JAK2 V617F inhibitory mutations, A598F decreased oncogenic activation and spared cytokine activation, while preventing JAK2 V617F-promoted EpoR dimerization.

Surprisingly, A598F and other V617F inhibiting mutations (F595A, E596R, F537A) significantly impaired IFNγ signaling. This was specific for IFNγ since the inhibitory mutations preserved responses to ligand of a series of receptor complexes. Similarly, homologous mutations in JAK1 prevented signaling by IFNγ.

Conclusions: The region of the JH2 αC, which is required for JAK2 V617F hyperactivation, is crucial for relaying cytokine-induced signaling of the interferon gamma receptor. We discuss how strategies aiming to inhibit JAK2 V617F could be used for identifying inhibitors of IFNγ signaling.

KEY MESSAGES

* Inhibitory strategies for active JAK1 and JAK2 forms differently impact homodimeric and heteromeric cytokine receptors

* There is a common structural requirement between JAK2 V617F oncogenic activation and IFNγ cytokine-triggered activation of IFNGR.

* Potential future pharmacological blockers of JAK2 V617F are predicted to inhibit IFNγ signaling.

CAPSULE SUMMARY

Inhibitory strategies for JAK2 V617F severely impair IFNγ-dependent signaling. Specific inhibitors of JAK2 V617F are predicted to be immunosuppressive via IFNGR inhibition, but they could be useful in pathologies involving excessive IFNγ signaling.

KEY WORDS

JAK2 V617F, Janus Kinases, Myeloproliferative disorders, Cytokine receptor signaling, IFNGR, JAK1, kinases

ABBREVIATIONS

JAK, Janus Kinase; JH, JAK homology; MPN, myeloproliferative neoplasms, PV, polycythemia vera; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; STAT, signal transducers and activators of transcription; EpoR, erythropoietin receptor; TpoR, thrombopoietin receptor; IFNGR, Interferon gamma receptor; IL-2R, Interleukin receptor 2; IRES, internal ribosomal entry site; GFP, green fluorescent protein

INTRODUCTION

V617F is the most prevalent JAK2 mutation in myeloproliferative diseases (MPN) (1-4). The mechanism by which V617F, located on the pseudokinase domain (JH2) of JAK2 promotes the kinase activity of the adjacent kinase domain (JH1) is incompletely understood and is yet to be targeted for specific inhibition. Recent studies on TYK2 (5) and JAK2 (6) provided details of the interface between the two domains and the inhibitory role of JH2. JAKs (JAK1-JAK3 and TYK2) consist of four different domains (FERM, SH2-like, pseudokinase/JH2 and the kinase/JH1) (7). The comparison of the crystal structures of JAK2-JH2 WT and JAK2-JH2 V617F indicates that the αC changes conformation upon the valine to phenylalanine substitution and displays a more rigid configuration with an additional N-terminal turn (8), likely due to an aromatic interaction between phenylalanines residues. In fact, F594 and F595 from the αC and F617, are involved in a double T-shaped π-π stacking interaction where F595 plays a pivotal role in maintaining these interactions. Mutagenesis data showed that the two phenylalanines from JH2 αC are necessary for JAK2 V617F, as their mutation suppressed its hyperactivity (9, 10). In addition, the aromatic interaction between F617 and F595 liberates F537, which along with a re-configuration of the SH2-JH2, might lead to activation of JH1 (11, 12).

Current JAK2 inhibitors are exclusively ATP mimetics targeting the active site of JH1 (13) with relative but not absolute specificity for JAK2, as illustrated by ruxolitinib (14). To date, no compound can distinguish between WT JAK2 and V617F. Given the position of this mutation in a domain remote from the active site, we suggest that identifying allosteric regions to be targeted by small molecules could provide specificity and/or potency towards the mutant (15). Allosteric sites have been identified in various enzymes, which paved the way for the development of a new type of pharmaceuticals (16). These compounds overcome the effect of resistant mutations (17), and exhibit enhanced potency in combination with ATP competitive inhibitors (18).

JH2 negatively regulates JH1 to maintain JAK2 inactive through an inhibitory interface that contains the JH2 αC (6). The V617F activating mutation destabilizes the *in cis* inhibitory interface, but also induces a positive action on JH1. While the intra-molecular interaction is beginning to be uncovered, the molecular basis of the inter-domain communication in the receptor/JAK2 complex has yet to be elucidated.

Receptors such as the erythropoietin receptor (EpoR) and the thrombopoietin receptor (TpoR) are composed of two alpha chains leading to homo-dimerization of JAK2 molecules. JAK2-utilizing receptors like EpoR or TpoR can exist as full or partial preformed dimers, which, upon ligand binding, undergo conformational changes leading to their activation (19-21). However, approaches that can follow the trajectory of receptor molecules pointed to EpoR monomers at the surface, which can be dimerized by Epo (22). The influence of JAK2 V617F on receptor dimerization is still unclear. In contrast, the other cytokine receptors such as type-I interferon (IFN) receptor, type-II IFN receptor (IFNGR) or IL2-R/IL9-R are made of different receptor chains enabling hetero complexes of JAKs to be formed. EpoR represent model receptors for performing mechanistic studies on JAK2 in a homodimeric configuration. Still, JAK2 might function differently when paired with another JAKs member such as JAK1 in receptor complex

(IFNGR). Especially, IFNγ binds to IFNGR1 and IFNGR2 to form a 1:2:2 signaling complex (23) thereby juxtaposing two JAK1 to two JAK2 molecules.

We hypothesized that the altered conformation of the JH2 αC driven by V617F is the first molecular event activating JAK2. Therefore, we sought to find ways to selectively disrupt the αC in the mutant JAK2 to counteract the activation process. We explored the hydrophobic environment around the αC residues through structure-guided mutagenesis. We identified a novel mutation A598F, which acts as a second site suppressor of V617F by blocking constitutive activity and maintaining cytokine-induced signaling. We show that this mutant behaves like other previously reported secondary inhibitory mutations for JAK2 V617F, namely (F595A, E596R, F537A). We tested the role of such mutations and others in the region around A598 in signaling driven by a distinct cytokine receptors from type I and type II subfamilies that utilize JAK2 (24). We also investigated the effect of similar inhibitory mutations on JAK1 V658F (synonymous with JAK2 V617F), which has been described in AML and ALL patients (25, 26). These studies led us to propose a novel avenue for specific JAK2 V617F or JAK1 V658F inhibition and allowed us to observe an unexpected overlap between the function of the JH2 αC in JAK2 V617F oncogenic activation and in IFNGR physiologic activation.

MATERIAL AND METHODS

cDNA

JAK1, JAK2, JAK3 and TYK2 were subcloned in the pMX-IRES-GFP and EpoR, EpoR:gp130, IFNAR1/2, IFNGR1/2, IL-2R and IL-9R in the pMX-IRES-CD4 (27). JAK2 JH2 domain (amino acids 535-812) was subcloned in the pFast-Bac1 vector (Life Technologies). Site-directed mutagenesis was performed as described (11). *Jak2* cDNA was cloned into the pHT-C and pNL-C vectors from the NanoBRET PPI starter system (N1821) from Promega (Leiden, Netherlands) using the NheI and SacI restriction sites. EpoR was cloned into both pBiT-C -LgBiT 1.1 and -SmBiT 2.1 vectors from the NanoBiT PPI starter system (Promega, N2014) using NheI and EcoRI restriction sites. Sequences were verified by Sanger sequencing.

Cell cultures

γ2A are JAK2 deficient (28), U4C are JAK1 deficient (29) and 11.1 are TYK2 deficient (30) fibrosarcoma cells maintained in DMEM and 10% FBS. Pro-B IL-3 (interleukin 3) dependent hematopoietic Ba/F3 cells were maintained in RPMI 1640 and 10% FBS supplemented with IL-3 (31). Autonomous cells expressing JAK2 V617F were cultured without IL-3.

Retroviral transduction and generation of cell lines

High-titer ectopic retroviruses were generated by transient transfection of BOSC packaging cells with the bicistronic pMX-IRES-GFP encoding the JAK2 variants and pMX-IRES-CD4 encoding EpoR (32). Transduction was performed as previously described in (11).

Proliferation assay

Ba/F3-EpoR cells expressing the different JAK2 variants were washed three times in PBS and resuspended in RPMI +10% FBS free of cytokine. Cell numbers were recorded over a period of five days.

Dual luciferase assay

Transcriptional activity of STATs was assessed by measuring luciferase activity using Spi-Luc (33), Gas-Luc (34) and pGRR5-Luc (35) reporters were for STAT5, STAT1 and STAT1/3/5 activities, respectively, as described (11).

Western blots

Western blots were performed as described (11). Antibodies information can be found in the supplemental materials.

Protein production and Thermal shift assays.

JAK2 JH2 recombinant proteins were expressed as previously described (11). Thermal shift assays were performed as described in (36).

Protein complementation assays.

For detailed protocols related to these experiments see supplemental materials in the article's Online Repository.

Structural modeling

The coordinates for the JAK2 JH2 V617F were obtained from the PDB database (accession code: 4FVR). PyMOL Molecular Graphics System (Version 1.8 Schrödinger, LLC) was used to model the position of the phenylalanine substitutions.

RESULTS

1. Identification of A598F as a novel inhibitory mutation for JAK2 V617F.

We hypothesized that large bulky substitutions of JH2 αC residues around F594 and F595 might disrupt the aromatic interactions with F617, inhibiting JAK2 V617F. Based on structural analysis of JH2 V617F (PDB: 4FVR), we introduced phenylalanines at position 598 (alanine in the WT JAK2, **Figure 1D**) and at other nearby positions. Stable hematopoietic Ba/F3 cells expressing the JAK2 variants and the EpoR were generated. These cells depend on IL-3 and Epo for survival. While Ba/F3 cells expressing JAK2 V617F grew autonomously, cells expressing JAK2 V617F/A598F did not (**Figure 1A**). This was confirmed by transient expression of JAK2 variants along with EpoR and STAT5 in JAK2-deficient cell line (γ2A) (**Figure 1B-C**). STAT5 transcriptional activity was measured and revealed that A598F prevents JAK2 V617F constitutive activity without altering cytokine inducibility. Besides, A598F does not significantly alter cytokine inducibility of JAK2 WT (**Figure 1B**). JAK2 and STAT5 phosphorylation were then analyzed by immunoblotting (**Figure 1C**). A598F decreased the basal phosphorylation of JAK2 V617F and STAT5, but enabled Epo-induced activation (right panel). A880F mutation, which prevents ATP binging on JH1 without affecting the kinase scaffold, was used as a kinase-dead mutant control (37).

The crystal structure of JAK2 V617F suggests potential avenues by which the A598F mutation could be acting on JAK2 V617F JH2 function. In addition to F594 (αC) and F595 (αC) and nearby residues, the Cβ atom of the side chain of A598 lies within 6 Å of several residues, including F537 (SH2 JH2 linker), S602 (αC), N612 (β4), L624 (β5), D699 and P700 (of the canonical DPG motif in JAK2 JH2) (**Figure 1 F**). Since N612, L624, D699, and P700 are important structural elements of the JH2 fold, it is possible that A598F distorts one or more of these residues, thereby destabilizing the JH2 domain. It has been previously shown that mutation of D699 to alanine prevents the expression of soluble JAK2 JH2 protein (38). To assess the effects of A598F on JH2 folding and function, we produced recombinant JAK2 JH2 proteins (JH2 WT, JH2 V617F and JH2 V617F/A598F) (8) and performed thermal shift assays (TSAs) in the absence or the presence of ATP or a small molecule binder (36). JAK2 JH2 exhibits a non-canonical nucleotide-binding pocket that is known to interact with ATP and Mg^{++}(8). More recently, it was shown that this same JH2 pocket could also bind small molecules such as TPCA-1 or related compounds (39).
We found that A598F does exert a modest destabilizing effect on the structure of JH2 V617F in the absence of nucleotides by decreasing the Tm by 4℃ (**Figure 1E**). However, JH2 V617F/A598F was still able to bind ATP and TPCA-1, as shown by the positive ΔTm (**Figure 1E**). TPCA-1 even increased the thermal stability of JH2 V617F/A598F (ΔTm= 4℃) to a higher extent than JH2 WT and JH2 V617F (ΔTm =2℃). Hence, it is unlikely that inhibition of JAK2 V617F hyperactivity by A598F is the result of a major perturbation of the JAK2 JH2 domain fold. Together with our previous results indicating that mutation of S602 has minimal effects on JAK2 V617F activity (11), it is very likely that the A598F substitution exerts its effects by altering the conformation/positioning of F594, F595 and/or F537.

The position A598 appears to be highly specific, as replacing residues at other positions along the JH2 αC (S591 and S599) and close to F617 (V615) with phenylalanines did not achieve an inhibition of JAK2

V617F (**Figure 2A**). This highlights the relevance of position 598 on the inner face of JH2 αC for the placement of inhibitory bulky hydrophobic mutations (**Figure 2C**). Of importance, introducing a phenylalanine on the opposite helical interface (E592F), pointing toward JH1 (**Figure 2C**) can lead to hyperactivation of JAK2 (**Figure 2B**), presumably by hyperstabilizing the helix.

2. A598F decreases the activity of other clinically relevant JAK2 mutants, while preserving normal Epo response

We assessed the effect of the inhibitory mutation A598F on other clinically relevant JAK2 mutants including R683G; detected in B-cell acute lymphoblastic leukemia (26), T875N, detected in a megakaryoblastic leukemia cell line (40) and K539L, which is representative of the "exon 12" class of mutations associated with polycythemia vera (PV) (41). A598F decreased the hyperactivity of R683G and T875N mutants, but not of K539L (**Figure 3A**). Molecular dynamics simulation indicated that both R683G and T875N are located in the inhibitory JH1/JH2 interface (**Figure 3B**) (6). These data suggest that similar to V617F, R683G and T875N require a specific JH2 αC conformation for their hyperactivity, while K539L directly activates JH1 without requiring a specific JH2 αC conformation. Additionally, we studied the effect of A598F on the wild-type protein (**Figure 3C**), along with other JAK2 V617F secondary inhibitory mutations previously described (F595A (9), E596 (11) and (F537A (12). All the JAK2 variants were still able to confer Epo sensitivity to EpoR with a very marginal decrease, especially for JAK2 F537A. Surprisingly, all the secondary mutations also significantly lowered JAK2 basal signal.

3. Impact of the secondary site inhibitory mutations on EpoR cytosolic tail dimerization.

We assessed the effect of JAK2 V617F suppressive mutations, the novel A598F and the ones previously described by us (F595A, E596 (9, 11) and by others (F537A (12)), on dimerization of cytosolic tails of EpoR using the NanoBiT protein complementation approach (42). EpoR-LgBiT and EpoR-SmBiT fusion proteins were transfected in γ2A with JAK2 variants at a EpoR-JAK2 1:1 DNA ratio (**Figure 4C**). JAK2 V617F highly increased EpoR cytosolic tail dimerization compared to JAK2 WT (**Figure 4A**). Furthermore, JAK2 V617F having either the A598F or other inhibitory mutations did not promote EpoR dimerization (**Figure 4A**). Of importance, these inhibitory mutations did not significantly affect EpoR dimerization in a JAK2 WT background when compared to JAK2 WT alone (**Figure 4A**). Additionally, in the presence of JAK2 WT, EpoR can be further dimerized in the presence of Epo at time points 0 (instant), 5' and 10' after addition of cytokine (**Figure 4B**). The levels of expression of the fusion proteins were monitored by western blot as shown in the **Figure E1 in the Online Repository**. These results indicate that JAK2 V617F acts by promoting close apposition of EpoR cytosolic tails, which could occur both within a preformed dimer (19, 21, 22, 43) or by switching monomeric receptors to dimers (19, 21, 22, 43).

4. The mutant protein JAK1 V658F is inhibited by the homologous A598F mutation (A639F).

To test the functional conservation of A598F inhibitory mutation, we performed similar studies in JAK1 (**Figure 5)**. JAK1 can be activated by a mutation (V658F) analogous to V617F (44), which is associated with T-cell lymphoblastic leukemia (T-ALL), as other JAK1 and JAK3 mutations (25, 45). Homologous A598F substitution in JAK1 (A639F) decreases constitutive signaling of the hyperactive V658F via a series of cytokine receptor complexes belonging to type I (IL-9R, IL-2R) and type II (IFNAR) heteromeric cytokine receptors and via an artificial EpoR-gp130 homodimeric receptor **(Figure 5)**. In all such complexes, the double mutant JAK1 V658F/A639F showed no constitutive activity and allowed cytokine stimulation to similar levels as wild-type JAK1.

5. Inhibitory mutations severely impair IFNγ signaling.

We investigated the effect of the inhibitory Ala to Phe substitution on IFNγ signaling via either JAK1 or JAK2. IFNγ signals via a heteromeric receptor, through JAK1 and JAK2 forming hetero-tetramers (**Figure 6A**). First, U4C (JAK1-deficient) and Y2A (JAK2-deficient) were transfected with JAK1 and JAK2 variants, respectively, along with IFNGR and STAT1 prior to transcriptional activity measurement (**Figure 6B**) and immunoblotting (**Figure 6C**). JAK2 V617F only very weakly induced constitutive activation of IFNGR signaling, in contrast to the strong activation induced by JAK1 V658F (**Figure 6B, C and Figure E2, A in the Online Repository**) and other active forms of JAK2 (**Figure E2, B in the Online Repository**). JAK1 V658F/A639F prevented constitutive activation and, unexpectedly, prevented IFNγ-induced STAT1 transcriptional activity.

Secondly, we extended our analysis to all the suppressive mutations and tested their impact on IFNγ signaling when in the background of the WT JAK1 or JAK2. When co-transfected with IFNGR, the JAK2 WT carrying the single inhibitory mutations strongly affected IFNγ responses, but such mutants could still support a two-fold increase response compared to basal (**Figure 6D**). Strikingly, all the inhibitory mutations, when in the background of JAK1 WT totally blocked IFNγ signaling (**Figure 6D**). Altogether, our data indicate that inhibitory substitutions preserve cytokine inducibility of homodimeric dimeric complexes such as EpoR through JAK2 (**Figure 3**), while they strongly inhibit IFNGR signaling when placed in JAK2, and completely abolish signaling by IFNγ, when placed in JAK1.

6. Inhibitory JAK mutants can inhibit active JAK mutants *in trans*.

We also have addressed the question of whether one JAK carrying the inhibitory Ala to Phe mutation would be able to inhibit in *trans* an active JAK within a heteromeric complex. We chose the IL-2R and IL-9R, as prototype heteromeric complexes. We tested the ability of JAK1 A639F (homologous to JAK2 A598F) to inhibit the active JAK3 A572V mutant, associated with an acute megakaryoblastic leukemia cell

line (46) known to trigger constitutive STAT3 transcriptional activity via the common γ chain in the context of both IL-2R and IL-9R (47). The A572V mutation is located of the JH2 αC of JAK3 and might be activated by similar molecular mechanism than JAK2 V617F. When co-expressed with JAK1 A639F, JAK3 A572V cytokine-independent hyperactivity returned to normal without any alteration of the cytokine-induced activation (**See Figure E3 in the Online Repository).** Altogether, these data show that inhibitory Ala to Phe substitution in the JH2 αC of one JAK can uncouple constitutive from cytokine-induced activation *in cis* within the same molecule, but also of the other JAK *in trans*.

DISCUSSION

Our main findings are that targeting the aromatic interaction between F617 and F594/F595/F537 using A598F specifically inhibits the JAK2 V617F activation, and that the entire class of such inhibitory V617F mutations specifically inhibits IFNγ receptor signaling, while they do not generally affect cytokine-induced signaling via several tested cytokine receptors (**See Table E1 in the Online Repository**). Furthermore, homologous mutants in JAK1 completely block IFNγ signaling. Thus, a conformation of the JH2 αC and surrounding residues required for JAK2 V617F activation is redundant with a requirement for IFNγ-induced signaling via its receptor complex.

JH2 has a dual role in the function of JAKs. First, it is essential for maintaining a low basal level of activity through its auto-inhibitory function that likely occurs *in cis.* We show that A598F and other JH2 inhibitory mutations are able to reduce the basal activity even further when co-expressed homodimeric receptors such as EpoR (**Figure 3C**), therefore reinforcing the role of JH2 in maintaining basal function of JAK2 (48). Secondly, JH2 also positively contributes to the V617F- and cytokine-driven activation of JAK2, which likely occurs *in trans*. A recent model for JAK2 activation mechanism described a JH2-mediated JAK2 dimerization where JH2 might act *in trans* as a steric scaffold bringing the two downstream JH1 domains together in a position suitable for trans-phosphorylation (49). Our results suggest that V617F-inhibitory mutations clustering around the JH2 αC weaken this JH2-mediated activation without affecting cytokine-driven signaling.

While A598F is suggested to locally disrupt the F-F-F triad preventing activation, we show that it also inhibits R683G and T875N. We propose that A598F destabilize the JH2 αC conformation, which is critical for the JH2:JH1 interplay in these two mutants unlike for K539L, which probably activate JAK2 kinase domain through a different and more direct mechanism.

Models of the inactive JH1-JH2 complex (5, 6) indicate that the JH2 αC is located in the JH1-JH2 interface, which possibly hinders its accessibility to small molecules. In the active conformation of JAK2 V617F, it is likely that the JH1-JH2 interface might differ. Indeed, our TSA data show that the presence of the A598F mutation does not prevent binding of small molecules to the JH2 ATP-binding pocket. This might allow the design of bivalent inhibitors in which an ATP-mimetic is tethered to a ligand that targets the F594/F595/F617 interaction, mimicking the effect of A598F.

JAK2 V617F requires binding to a cytokine receptor for its hyperactivity (50). EpoR was shown to be a preformed dimer stabilized by transmembrane interactions using cell-surface co-patching (19), energy resonance transfer (21) and structural studies (43). Most assays explored extracellular domain dimerization and could reflect stabilization of transient or coincident complexes. On the other hand, recent studies employing single molecule tracking of EGFP-tagged EpoR indicated that the fate of individual receptors was mainly monomeric with Epo inducing dimerization (22). Here, we show using NanoBiT technology that close proximity of the EpoR cytosolic tails can be further and significantly driven by JAK2 V617F (**Figure 4**). This could be compatible with both preformed dimeric receptor complexes and with monomeric receptor states, as the C-terminus regions are predicted to be flexible. Importantly, while it has been suggested that the preformed receptor dimer drives JAK2 V617F activity (50), our data indicate

that JAK2 V617F acts by closely dimerizing EpoR cytosolic tails. This is in agreement with the finding that for triggering signaling in the absence of ligand, the two EpoR-bound JAK2 V617F proteins must be physically closer to each other, when compared to EpoR-bound JAK2 (22). Our NanoBiT data show that the JAK2 V617F suppressive mutations are able to decrease the JAK2 V617F-driven EpoR dimerization suggesting that the JH2 αC region is important in this dimerization process. We propose that the JH2 inhibitory mutations slightly destabilize the JH2 αC dimer conformation in JAK2 V617F, which is crucial for JAK2-mediated EpoR dimerization. Such JAK2-driven receptor dimerization controlled by the JH2 αC seems key for activation of signaling by IFNγ.

JAK2 is required for the signaling of numerous cytokine receptors. Here we show that, in contrast to JAK1 V658F, JAK2 V617F does not hyperactivate IFNGR. This is in line with a study that demonstrated the dominant role of JAK1 over JAK2 in the IFNGR complex (51). Indeed, JAK1 was shown to directly mediate STAT1 phosphorylation while JAK2 rather functions as a scaffold (51). We found that inhibitory mutations specific to JAK2 V617F, when in the background of wild-type JAK2, strongly affect IFNγ signaling, while in the background of wild-type JAK1, they abrogate their ability to signal upon cytokine stimulation. Again, the more drastic inhibition by JAK1 mutations is in agreement with a dominant role for JAK1 in IFNGR signaling (51). Our data suggest that the interplay between heteromeric receptors and JAKs is more intricate than for homodimers. In the IFNGR tetrameric complex, it is possible that a JAK2:JAK2 or even a JAK2:JAK1 contact is lost in the presence of inhibitory mutations localized around the JH2 αC. More structural studies of JAKs and receptor tails would be required to understand the structural bases of these differences.

In conclusion, while JH2 αC plays a major role into the mechanism of activation of JAK2 V617F, here we show that the same residues are also critical to mediate IFNγ signaling. Targeting the αC with bulky hydrophobic molecules mimicking A598F might disrupt the JH2 V617F mediated activation, but would be expected to inhibit IFNγ. On the other hand such inhibitory strategy could be used not only for MPNs carrying JAK2 V617F, but also for treatment of autoimmune diseases where IFNγ is hyperactive.

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Figure legends.

Figure 1. Effect of phenylalanines introductions in the JH2 αC on the JAK2 V617F hyperactivity.

a. Ba/F3-EpoR-JAK2 cells were set for proliferation assay in cytokine-free medium during 5 days. Shown are average +/- SD. **b.** STAT5 transcriptional activity in JAK2-deficient cells (γ2A). Shown are average units + S.E.M of three independent experiments done in triplicate (* P < 0.05, Mann Whitney test). **c.** Immunoblot analysis of JAK2 deficient cells (γ2A) co-transfected with EpoR and JAK2 variants in the absence of cytokine or after 20' of Epo stimulation at 10 U/ml. **d.** Representation of the aromatic interaction (F617-F595-F594) and position of A598 on the αC of JH2-V617F (PDB: 4FVR). **e.** Thermal stability of recombinant JAK2 JH2 monitored by TSA. Shown are histograms depicting the Tm values in the absence of ligand (WT = 38℃, V617F = 35,8 ℃, V617F/A598F = 31,5℃) (left). Ther mal stabilization of JH2 WT, V617F and A598F in the presence of ATP or TPCA-1 are shown as Δ Tm (right). **f.** Residues neighboring A598 (PDB: 4FVR). The side chain of A598 lies near those of F537 (SH2 JH2 linker), F594 (αC), F595 (αC), S602 (αC), N612 (β4), L624 (β5), D699 and P700 (of the canonical DFG motif which is DPG in JAK2 JH2) (side chain atoms are shown in stick and transparent spheres and P700 is not displayed for clarity). The aromatic triad (F594, F595 and V617F) is colored purple and A598 is colored orange.

Figure 2. Position A598 on the JH2 αC is highly specific.

a. Left, STAT5 transcriptional activity of JAK2 V617F variants was assessed in γ2A by dual luciferase assay. Only substitution of residue A598 to phenylalanine could suppress the constitutive activity of JAK2 V617F. Right, modeling of the other putative sites where substitution to phenylalanine could possibly contact F617 side chain and affect the activity of JAK2 V617F. **b.** STAT5 transcriptional analysis in γ2A in the absence or the presence of Epo. Shown are average units +/- S.E.M of three independent experiments done in triplicate (** P < 0.01, NS, not significant, Kruskal-Wallis with Dunn's post test). **b**. STAT5 transcriptional activity measurement of JAK2 WT and JAK2 V617F in the presence of the secondary site mutations E592F and E596F in comparison with JAK2 WT and V617F in γ2A co-transfected with EpoR. **c.** Modelling the position of the inhibitory A598F mutation on an opposite interface than the activating mutation E592F. A598F points toward JH2 (in green), while E592F is facing JH1 (in blue). Shown are average units + S.E.M of three independent experiments done in triplicate (** P < 0.01, NS, not significant, Kruskal-Wallis with Dunn's post test).

Figure 3. A598F has an inhibitory effect on the hyperactivity of some, but not all, disease associated JAK2 mutants but no effect on Epo responsiveness.

a. STAT5 transcriptional activity measurement in γ2A. JAK2 mutants K539L, R683G and T875N all induce constitutive STAT5 transcriptional activity similar to JAK2 V617F. A598F decreased the activity of R683G and T875N but not K539L. Shown are means +/ − S.E.M. of three independent experiments done in triplicate. (***P< 0.001, * P< 0.05, NS, not significant; Mann Whitney test). **b.** Spatial position of three oncogenic JAK2 mutants. K539L lies in the SH2-JH2 linker and is distant from the JH2 αC. T875N is located in the kinase domain (JH1) but close to the JH2 αC in the model of the JH1/JH2 complex (6). R683G localizes in JH2, close to the αC, and forms electrostatic interactions with D873 from JH1 across the JH1/JH2 interface. **c.** STAT5 transcriptional activity measurement in γ2A. After transfection, medium

containing several concentrations of Epo was added, as indicated. A598F affected the Epo responsiveness of JAK2 WT at submolecular concentrations (0,1 U/ml) but not at higher concentrations (1 – 100 U/ml). Shown are means +/ − S.E.M. of three independent experiments done in triplicate

Figure 4. In-cell EpoR dimerization measurement

a. JAK2 deficient cells were transfected with the EpoR C-terminal linked to either LgBit or SmBit along with the JAK2 variants. NanoLuc activity was measured 30 hours after transfection. The cytosolic tail dimerization of EpoR is only promoted in the presence of JAK2 V617F. **b.** In the presence of JAK2 WT, EpoR intracellular tail dimerization could be triggered by Epo and that directly after cytokine addition (t 0) and after 5' and 10' of incubation. Shown are means +/- S.E.M of five experiments done in 6 replicates. Data were normalized in each experiment with control condition (JAK2 WT). Kruskall-Wallis test with Steels post-test at 5% significance level. (*** P<0.001; ** P<0.01; * P<0.05). **c.** Cartoon depicting the EpoR-fusion proteins in the presence of JAK2 at the cell membrane.

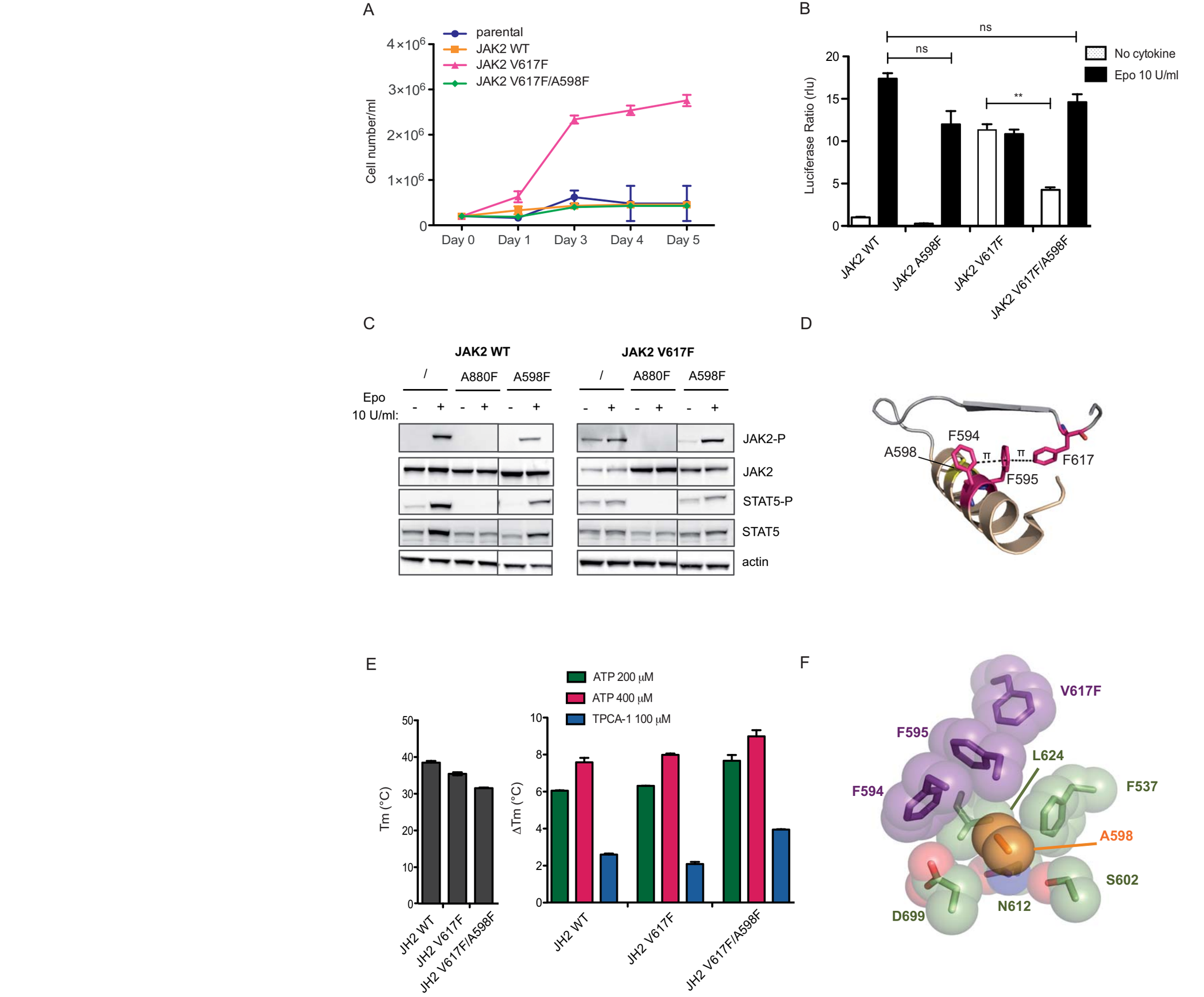
Figure 5. Effect of the homologous A598F mutation (A639F) on JAK1 V658F in different cytokine receptors.

a. Alignment of the JH2 αC sequences containing the alanine at position 598 that is conserved in the other family members **b.** U4C (JAK1-/-) were transfected with the JAK1 variants along with the chimeric receptor, EpoR: gp130. The previously described JAK1 V658F/F636A mutant (synonymous with JAK2 V617F/F595A (9)) was used as a control. STATs transcriptional activity was measured using the pGRR5-Luc in the absence or the presence of the indicated cytokine. **c.** IFNAR signaling was analyzed in U4C for the JAK1 variants. STAT-1 transcriptional activity was measured using Gas-Luc reporter. **d,e.** U4C were transfected with the JAK1 variants and IL-9Rα (d) or IL-2Rα (e) along with the γc. U4C, which are deficient in both JAK1 and JAK3 were also complemented with JAK3 WT where indicated. Besides F636A, E637R (synonymous with JAK2 E596R (11)) was used as a control. STAT5 transcriptional activity was measured using Spi-Luc in the absence or the presence of the indicated cytokines. Shown are average units + S.E.M of three independent experiments done in triplicate. Kruskall-Wallis test with Steels post-test at 5% significance level. (*** P<0.001; ** P<0.01; * P<0.05).

Figure 6. Effect of the inhibitory mutations on IFNγ signaling.

a. Representation of the IFNGR**. b,** IFNGR signaling was assessed in U4C for the JAK1 variants and in γ2A for the JAK2 variants. STAT-1 transcriptional activity was measured without cytokine or after 20 hours of IFNγ stimulation. Shown are average units + S.E.M of four independent experiments done in triplicate. **c.** Immunoblot analysis of U4C and γ2A cells transfected with JAK1 and JAK2 respectively and stimulation with IFNγ for 20 min. **d.** Extended analysis of JAK2 V617F inhibitory mutations in the background of JAK2 WT on IFNγ sensitivity using STAT1 reporter in γ2A, upper panel. Impact of synonymous repressive mutations A639F (=JAK2 A598F), E637R (= JAK2 E596R), F636A (=JAK2 F595A) and F575A (= JAK2

F537A) when introduced in the background of JAK1 WT in U4C using STAT1 reporter, lower panel. Several concentrations of IFNγ were added and luciferase activity was monitored after 20 hours. Shown are average units + S.E.M of three independent experiments done in triplicate. The expression of exogenous JAK2 in γ2A and exogenous JAK1 in U4C was monitored and revealed no difference in the protein level (right panel). The GFP expression can be correlated to the level of expression of JAK which is cloned upstream of the IRES sequence in the cloning vector.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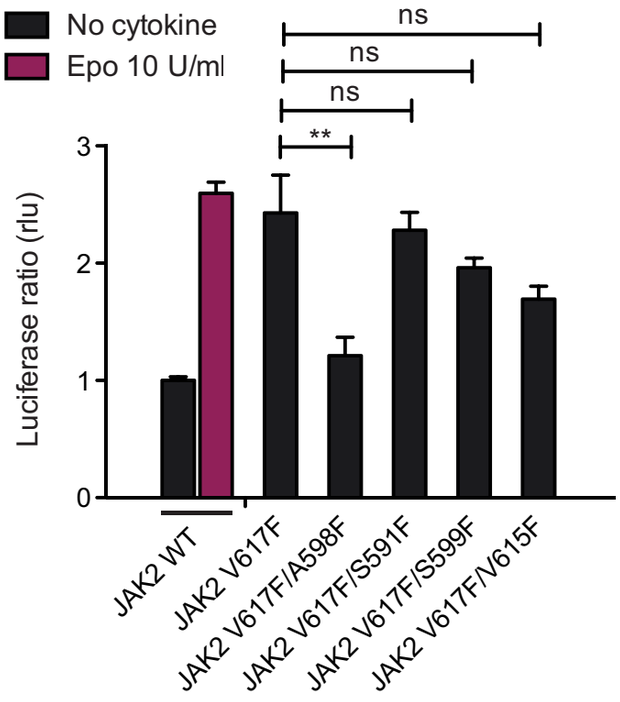
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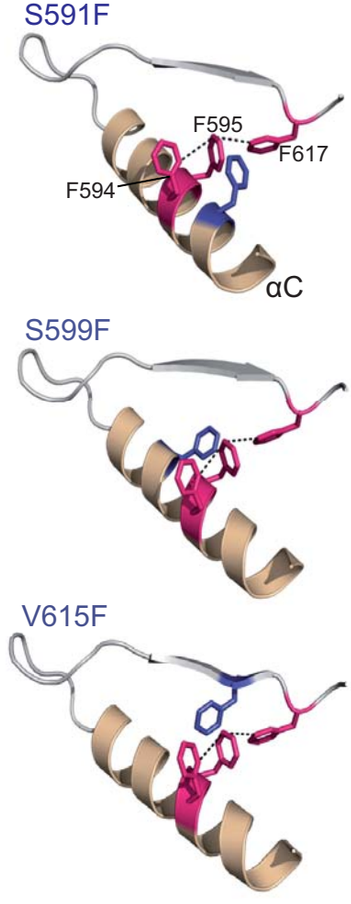
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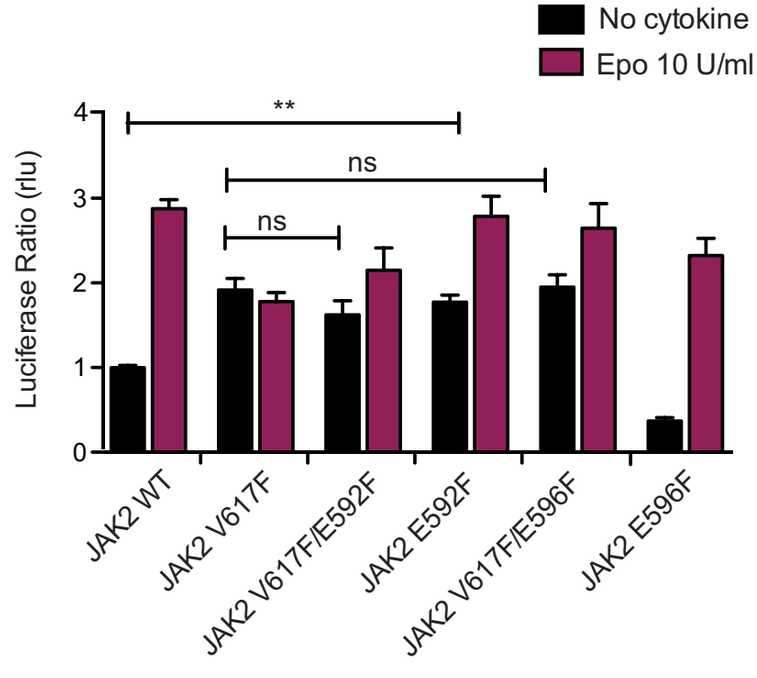
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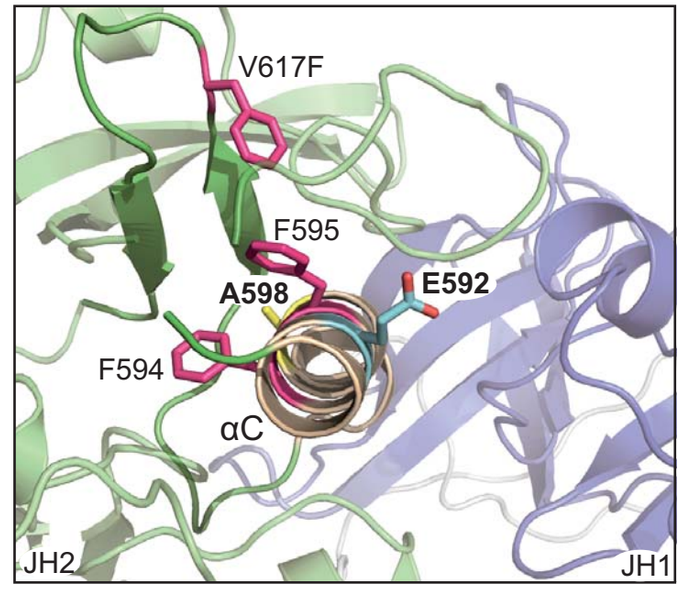
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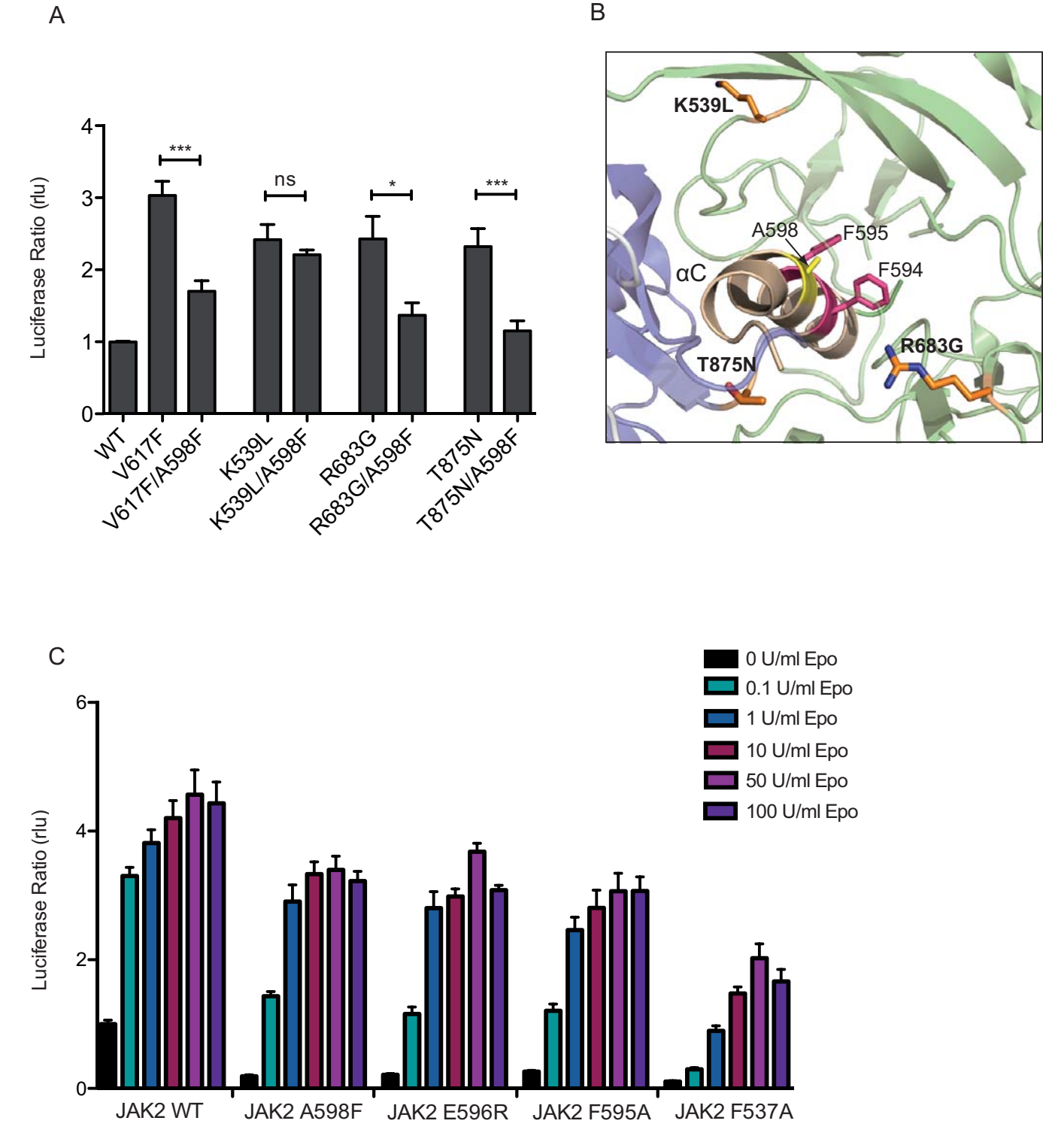
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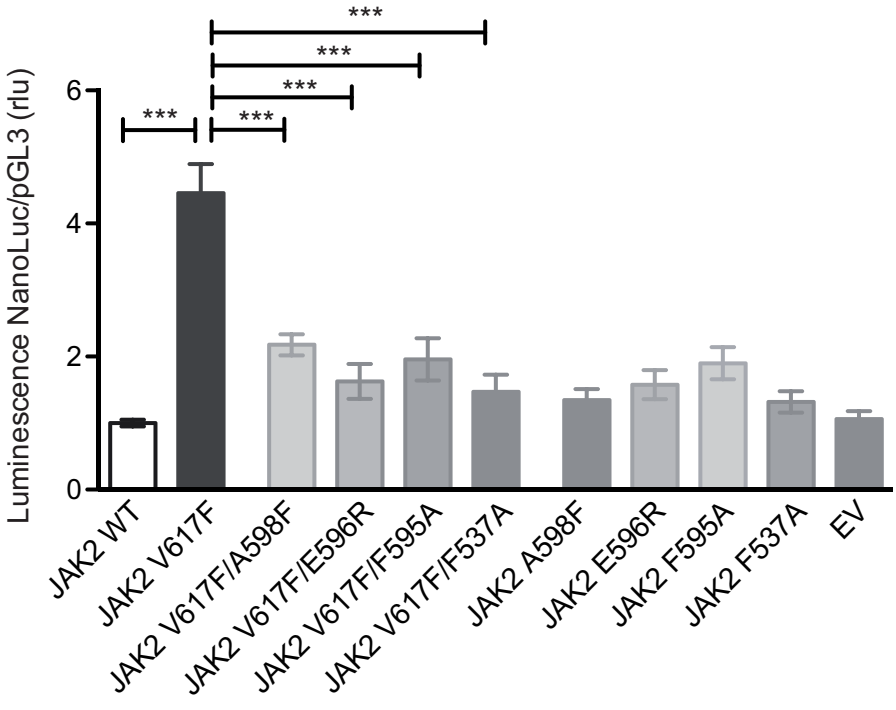
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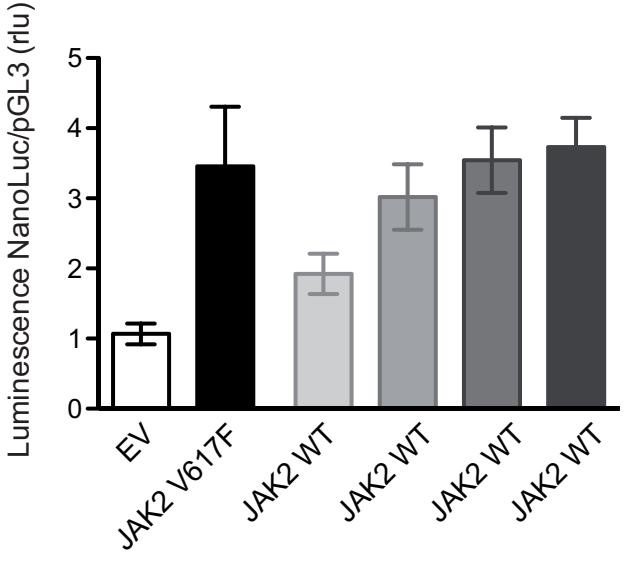
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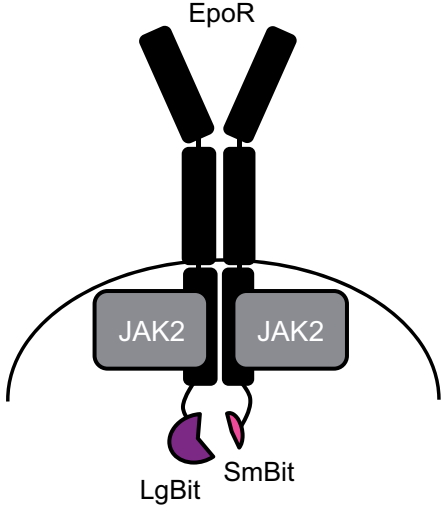
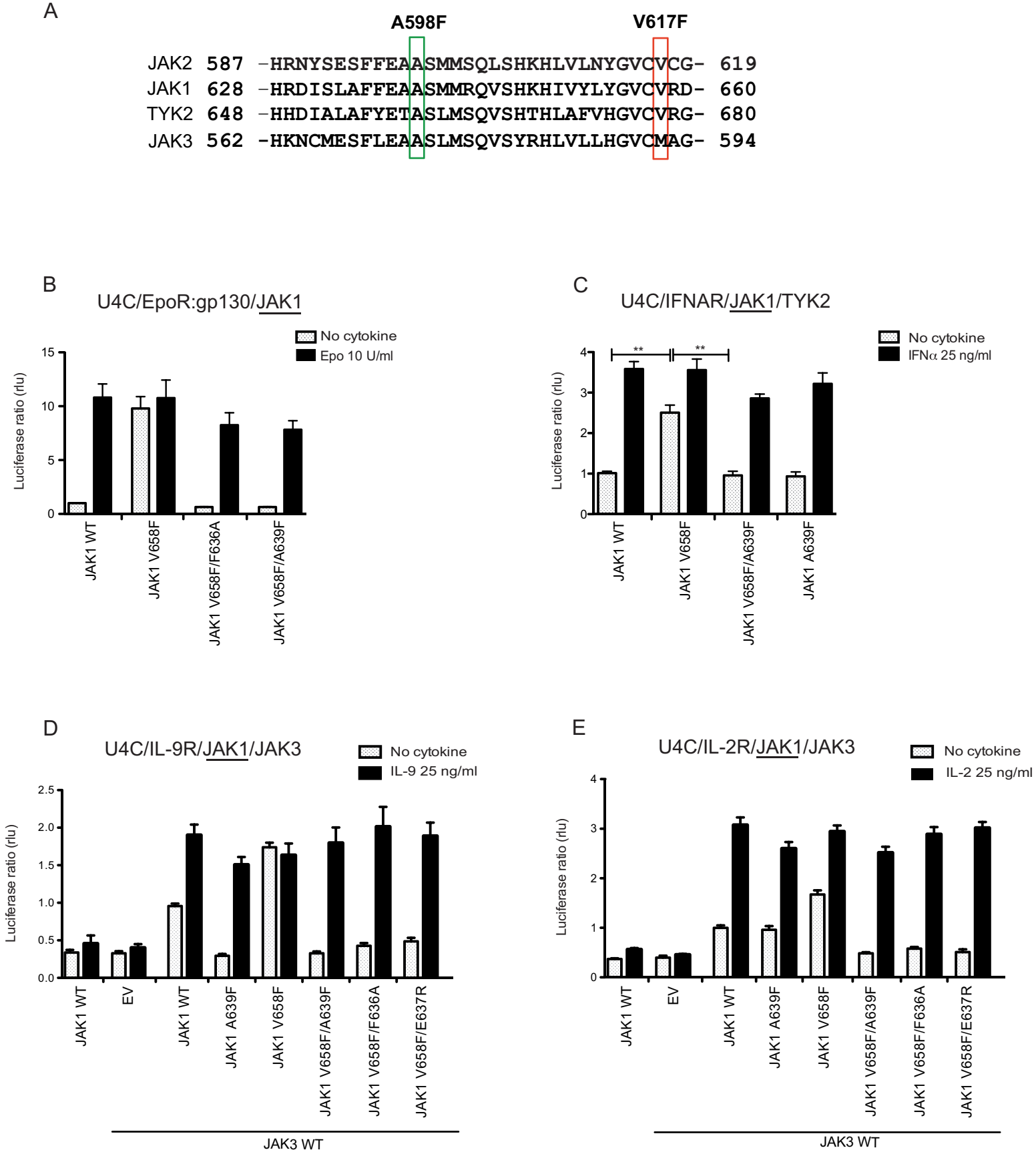
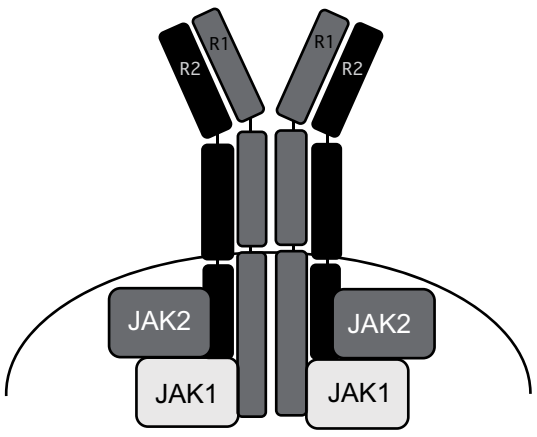
t 5'
t 10'

C

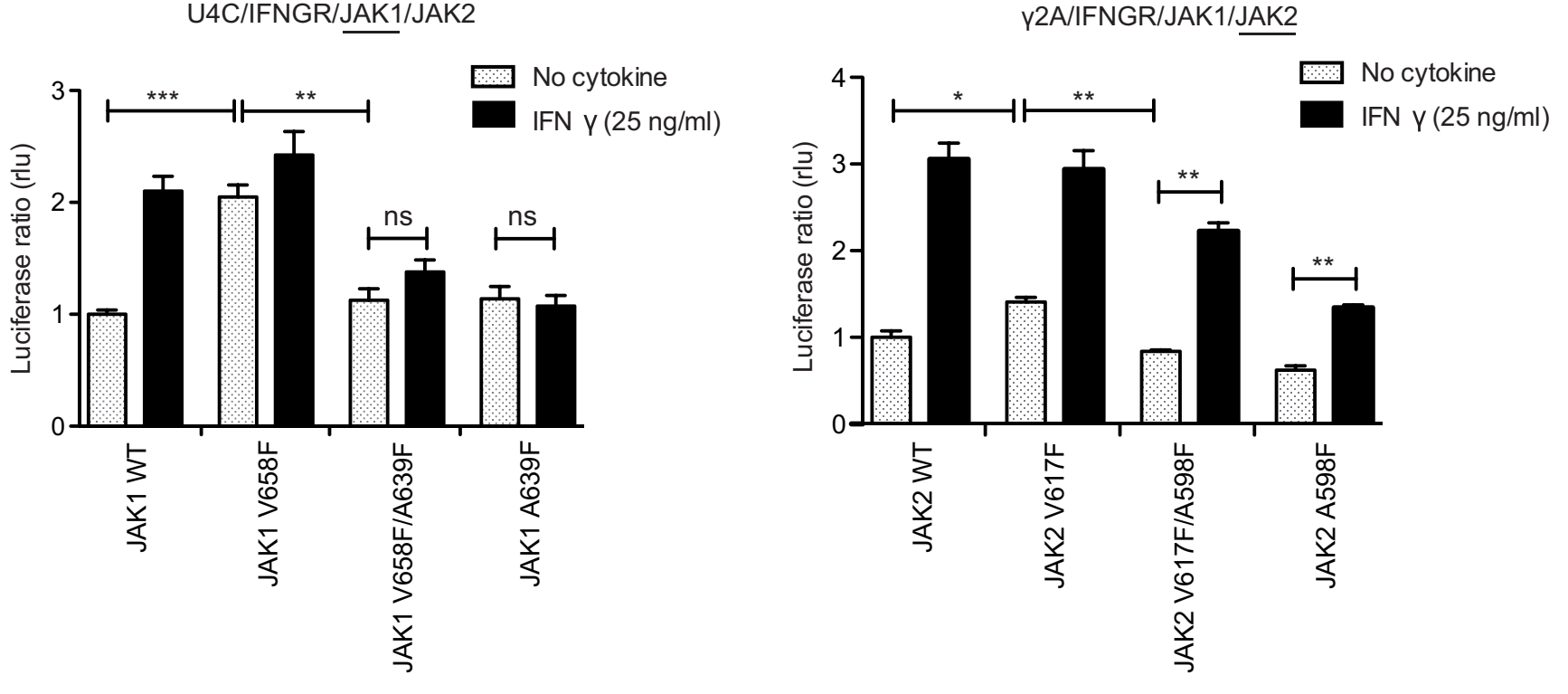

EpoR
JAK2
JAK2
LgBit
SmBit


A
A598F
V617F
JAK2 587 -HRNYSESFFEAASMMSQLSHKHLVLNYGVCVCG- 619
JAK1 628 -HRDISLAFFEAASMMRQVSHKHIVYLYGVCVRD- 660
TYK2 648 -HHDIALAFYETASLMSQVSHTHLAFVHGVCVRG- 680
JAK3 562 -HKNCMESFLEAASLMSQVSYRHLVLLHGVCMAG- 594
B
U4C/EpoR:gp130/JAK1
No cytokine
Epo 10 U/ml
Luciferase ratio (rlu)
JAK1 WT
JAK1 V658F
JAK1 V658F/F636A
JAK1 V658F/A639F
C
U4C/IFNAR/JAK1/TYK2
No cytokine
IFNα 25 ng/ml
**
**
Luciferase ratio (rlu)
JAK1 WT
JAK1 V658F
JAK1 V658F/A639F
JAK1 A639F
D
U4C/IL-9R/JAK1/JAK3
No cytokine
IL-9 25 ng/ml
Luciferase ratio (rlu)
JAK1 WT
EV
JAK1 WT
JAK1 A639F
JAK1 V658F
JAK1 V658F/A639F
JAK1 V658F/F636A
JAK1 V658F/E637R
JAK3 WT
E
U4C/IL-2R/JAK1/JAK3
No cytokine
IL-2 25 ng/ml
Luciferase ratio (rlu)
JAK1 WT
EV
JAK1 WT
JAK1 A639F
JAK1 V658F
JAK1 V658F/A639F
JAK1 V658F/F636A
JAK1 V658F/E637R
JAK3 WT

A


R1
R2
R1
R2
JAK2
JAK2
JAK1
JAK1

B


U4C/IFNGR/JAK1/JAK2
No cytokine
IFN γ (25 ng/ml)
Luciferase ratio (rlu)
***
**
ns
ns
JAK1 WT
JAK1 V658F
JAK1 V658F/A639F
JAK1 A639F
γ2A/IFNGR/JAK1/JAK2
No cytokine
IFN γ (25 ng/ml)
Luciferase ratio (rlu)
*
**
**
**
JAK2 WT
JAK2 V617F
JAK2 V617F/A598F
JAK2 A598F

C

U4C
JAK1 WT
JAK1 A639F
EV
JAK1 V658F
JAK1 V658F/A639F
NT
IFNγ (25 ng/ml): - + - + - + - + - + - +
STAT1-P
STAT1
Actin
Y2A
JAK2 WT
JAK2 A598F
EV
JAK2 V617F
JAK2 V617F/A598F
NT
IFNγ (25 ng/ml): - + - + - + - + - + - +
STAT1-P
STAT1
Actin

D

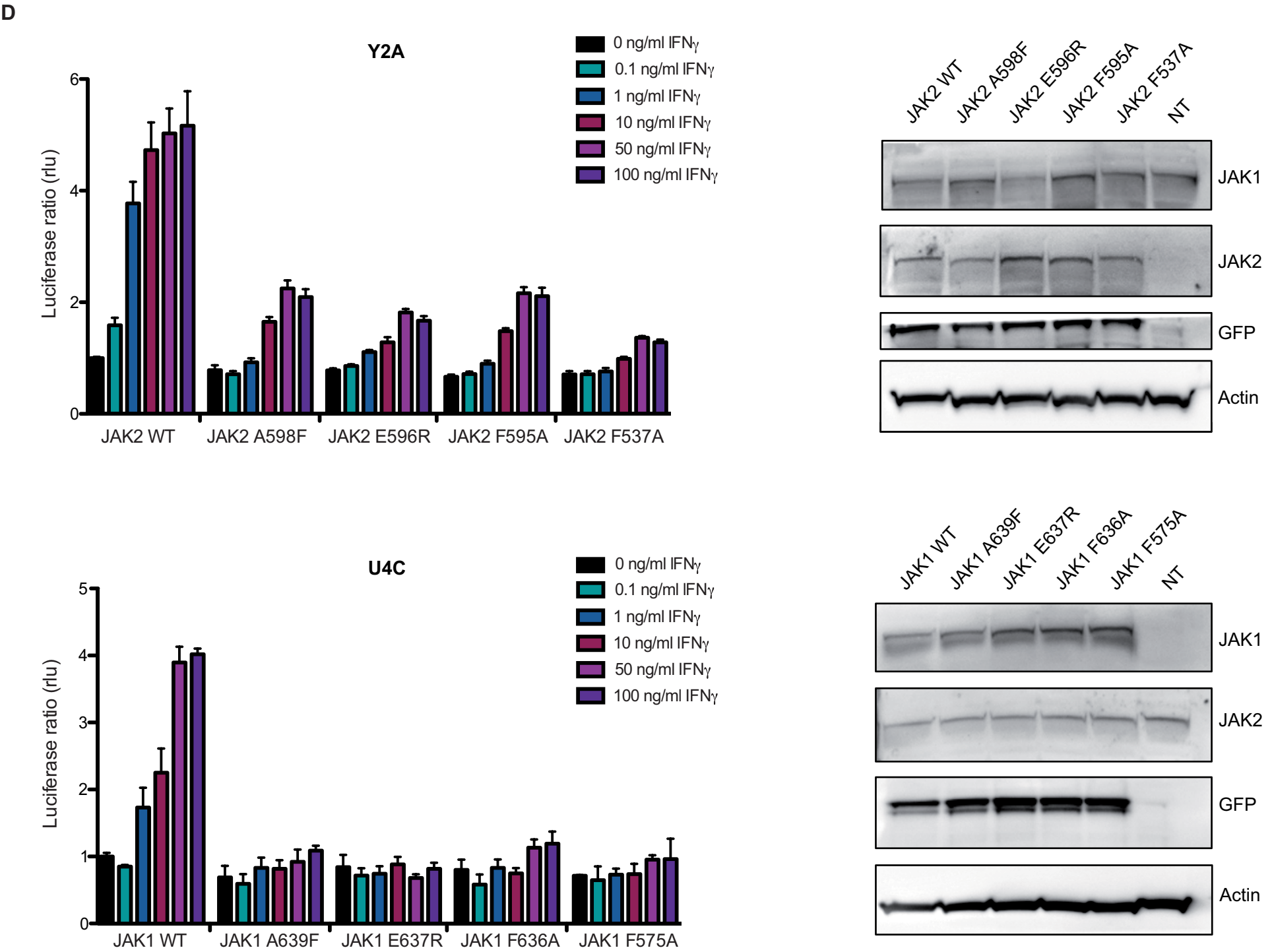

Y2A
0 ng/ml IFNγ
0.1 ng/ml IFNγ
1 ng/ml IFNγ
10 ng/ml IFNγ
50 ng/ml IFNγ
100 ng/ml IFNγ
Luciferase ratio (rlu)
JAK2 WT
JAK2 A598F
JAK2 E596R
JAK2 F595A
JAK2 F537A
JAK2 WT
JAK2 A598F
JAK2 E596R
JAK2 F595A
JAK2 F537A
NT
JAK1
JAK2
GFP
Actin
U4C
0 ng/ml IFNγ
0.1 ng/ml IFNγ
1 ng/ml IFNγ
10 ng/ml IFNγ
50 ng/ml IFNγ
100 ng/ml IFNγ
Luciferase ratio (rlu)
JAK1 WT
JAK1 A639F
JAK1 E637R
JAK1 F636A
JAK1 F575A
JAK1 WT
JAK1 A639F
JAK1 E637R
JAK1 F636A
JAK1 F575A
NT
JAK1
JAK2
GFP
Actin

Table E1

Secondary mutations

↓

JAK Pair		Receptor	Constitutive Basal	Cytokine response
JAK2 VtF	*JAK2 VtF*	EpoR	Decreased	Not affected
JAK2 VtF	JAK1 WT	IFNGR	None	Slightly affected
JAK1 VtF	JAK2 WT	IFNGR	Decreased	Strongly affected
JAK1 VtF	*JAK1 VtF*	Artificial EpoR:gp130	Decreased	Not affected
JAK1 VtF	JAK3 WT	IL2-R, IL-9R	Decreased	Not affected
JAK1 VtF	TYK2 WT	IFNAR	Decreased	Not affected

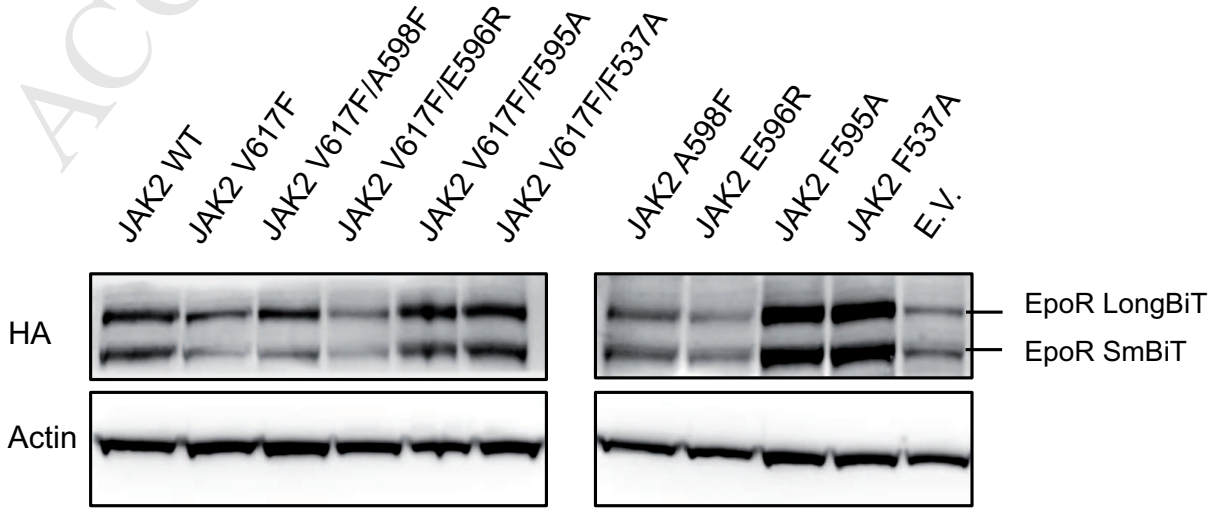
Secondary mutations

↓

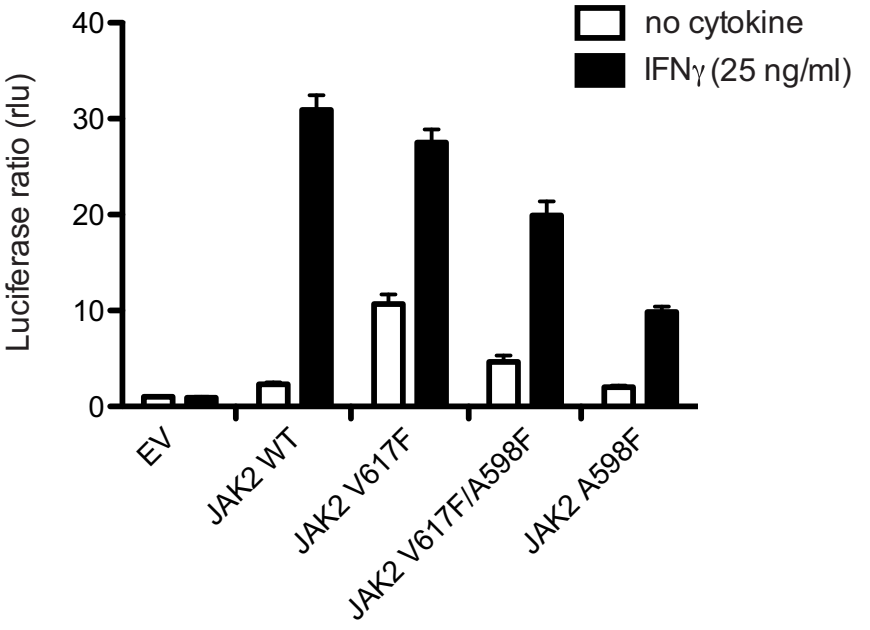
JAK Pair		Receptor	Constitutive Basal	Cytokine response
JAK2 WT	JAK2 WT	EpoR	Decreased	Slightly Affected
JAK2 WT	JAK1 WT	IFNGR	Not affected	Affected
JAK1 WT	JAK2 WT	IFNGR	Not affected	Strongly Affected
JAK1 WT	JAK1 WT	Artificial EpoR:gp130	Not affected	Not affected
JAK1 WT	JAK3 WT	IL2-R, IL-9R	Not affected	Not affected
JAK1 WT	TYK2 WT	IFNAR	Not affected	Not affected

Effects of inhibitory secondary mutations on different JAK combination in respective cytokine receptor complexes.

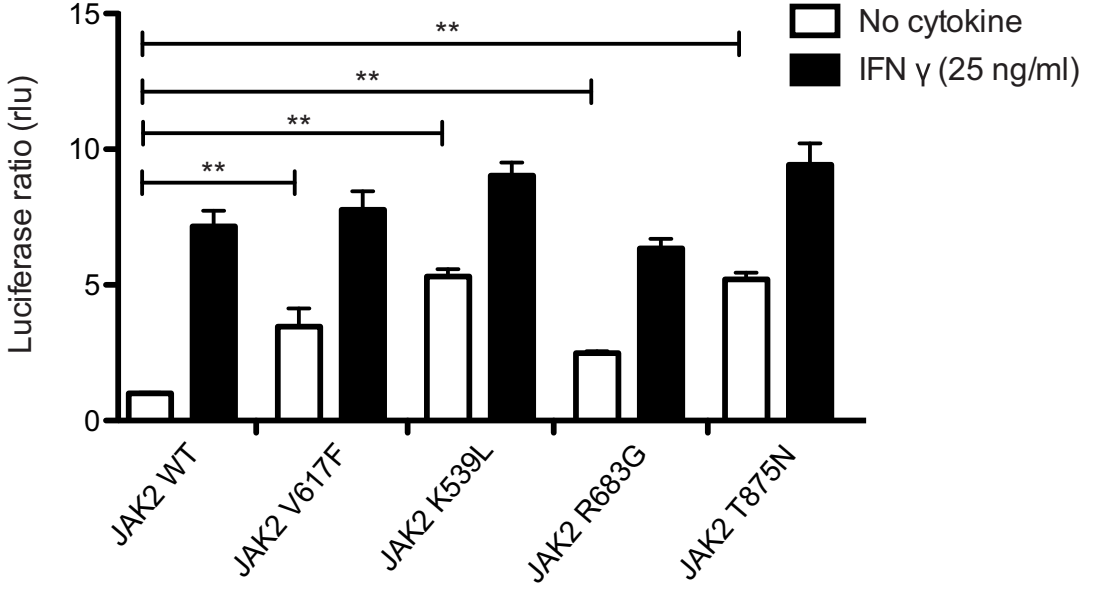
Figure E1

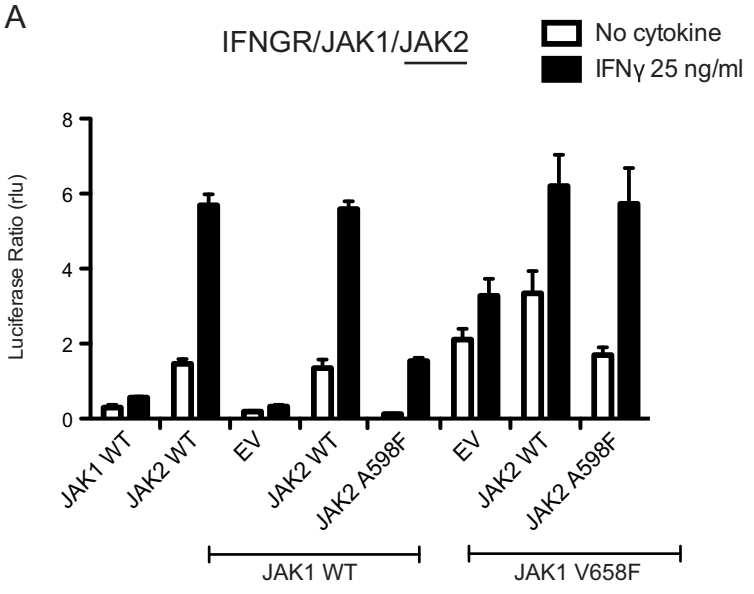


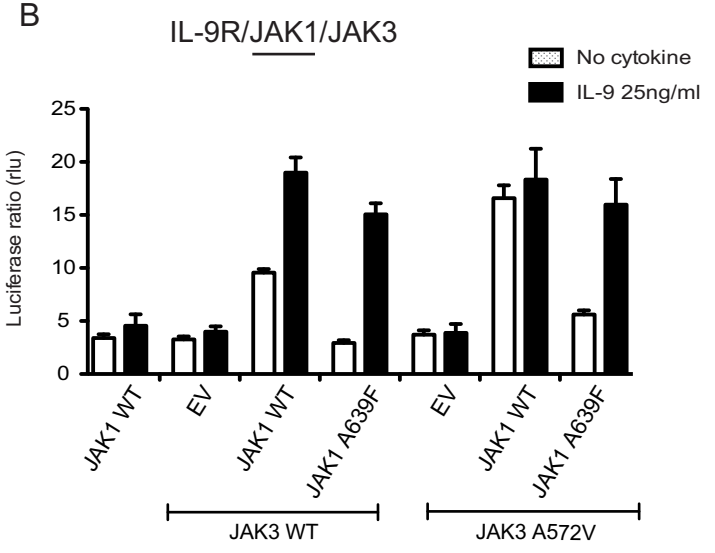
A

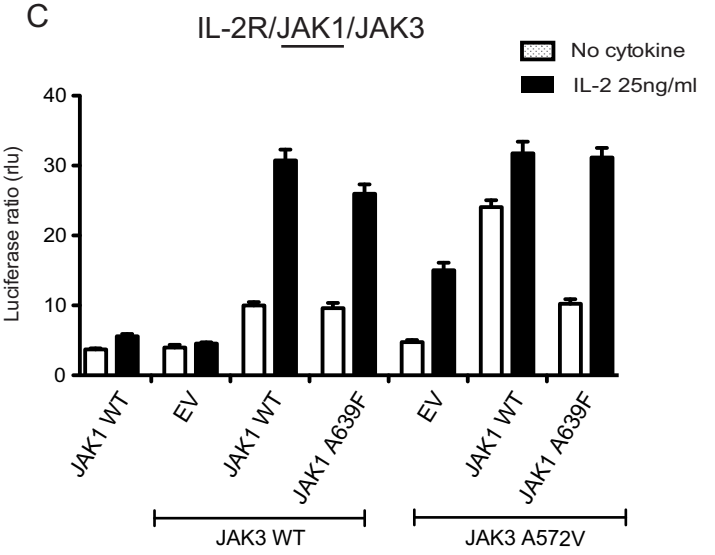

40
30
20
10
0
Luciferase ratio (rlu)
no cytokine
IFNγ (25 ng/ml)
EV
JAK2 WT
JAK2 V617F
JAK2 V617F/A598F
JAK2 A598F

B


15
10
5
0
Luciferase ratio (rlu)
No cytokine
IFN γ (25 ng/ml)
**
**
**
**
JAK2 WT
JAK2 V617F
JAK2 K539L
JAK2 R683G
JAK2 T875N


A
IFNGR/JAK1/JAK2
No cytokine
IFNγ 25 ng/ml
Luciferase Ratio (rlu)
8
6
4
2
0
JAK1 WT
JAK2 WT
EV
JAK2 WT
JAK2 A598F
EV
JAK2 WT
JAK2 A598F
JAK1 WT
JAK1 V658F


B
IL-9R/JAK1/JAK3
No cytokine
IL-9 25ng/ml
Luciferase ratio (rlu)
25
20
15
10
5
0
JAK1 WT
EV
JAK1 WT
JAK1 A639F
EV
JAK1 WT
JAK1 A639F
JAK3 WT
JAK3 A572V


C
IL-2R/JAK1/JAK3
No cytokine
IL-2 25ng/ml
Luciferase ratio (rlu)
40
30
20
10
0
JAK1 WT
EV
JAK1 WT
JAK1 A639F
EV
JAK1 WT
JAK1 A639F
JAK3 WT
JAK3 A572V

Supplementary Figure legends.

FIGURE E1

Measurement of the expression of the fusion proteins used for the NanoBit study. The level of HA tagged-EpoR LongBit and SmBit were monitored using an anti HA antibody.

FIGURE E2

a. STAT1 transcriptional activity was measured in γ2A cells in the absence of exogenous IFNGR complexes with or without cytokine stimulation as indicated. Gas_Luc reporter was used to assess the STAT1 activity. **b.** STAT-1 transcriptional measurement of JAK2 variants in γ2A cells in the absence of exogenous IFNGR. Similarly to JAK2 V617F, JAK2 K539L, JAK2 T875N and JAK2 R683G show constitutive basal activity via endogenous IFNGR. Gas_Luc reporter was used to assess the transcriptional activity in the absence and in the presence of IFNγ.

FIGURE E3

a. Transcriptional activity measurement of STAT1 in the presence of IFNGR in JAK2 deficient cells. **b.** STAT5 transcriptional activity measurement in JAK1 deficient cells in the presence of IL-9R. **c.** STAT5 transcriptional activity measurement in JAK1 deficient cells in the presence of IL-9R.

Supplementary Table legend.

TABLE E1

Summary of the different effects of the secondary site inhibitory mutation A598F (and homologous A639F) in JAK2 V617F and JAK2 WT (and homologous JAK1 V658F and JAK1 WT) in the distinct cytokine receptor complexes.

Supplementary experimental procedures

Western blots

β-Actin was revealed using a murine monoclonal anti β-actin antibody (AC-74, Sigma). Phospho-JAK2 (Y1007/1008) and total-JAK2 were detected using Rabbit mAb C80C3 (Cell Signaling Technologies #3776) and Rabbit mAb D2E12 XP® (Cell Signaling Technologies #3230), respectively. Phospho-Stat5 (Tyr694) Antibody (#9351, Cell Signaling) and STAT5 (D3N2B) Rabbit mAb (#25656, Cell Signaling) were used for STAT5-P and STAT5. Phosopho-STAT1 (Tyr701) (D4A7) Rabbit mAb (#7649, Cell Signaling), STAT1 Rabbit polyclonal antiboby (#9172, Cell Signaling) were used for STAT1-P and STAT1. HA-tagged proteins were detected using a monoclonal anti-HA high affinity IgG1 antibody (3F10, Sigma). HaloTag fusions proteins were detected using HaloTag monoclonal antibody (Promega #G9211). HRP (Horseradish peroxidase)-linked secondary anti-rabbit IgG, anti-mouse IgG and anti-rat IgG antibodies (Cell Signaling Technologies #7074, #7076, #7077) were used to detect the signal.

Protein complementation assay - NanoBiT

γ2a cells plated in DMEM + 10% FBS in a 48-well plate were transiently transfected with vectors coding for the EpoR-LgBiT and EpoR-SmBiT fusion proteins (NanoBiT system: Promega, Leiden, Netherlands) along with a vector coding for JAK2 (pMX-IRES-GFP backbone) at a EpoR-JAK2 1:1 DNA ratio and the pGL3-control vector (Promega), using FuGene (Promega). Cells were kept 24 h post transfection at 37℃, 8% CO_2 before being replated into two 96-well plates in pre-warm DMEM/F12 (no phenol red). 25µL of 5X NanoGlo® Live Cell Reagent (Promega) was added to each well before reading the plate for the NanoBiT luminescence using a GloMax Discover System (Promega). For transfection control, 50µL of LAR II reagent (Promega) was added on top to each well and firefly luciferase luminescence from the pGL3-control vector was read using a 530LP filter using the GloMax Discover System (Promega). The level of HA tagged-EpoR LongBit and SmBit were monitored by western blot using an anti HA High affinity antibody (Roche). NanoBit system is a split Nano-luciferase assay, where two EpoR monomers are C-terminal linked to either the LgBiT (18k Da) or the SmBiT (11 amino-acids). If two EpoR cytosolic domain monomers dimerize, the SmBiT and LgBiT fusion proteins recombine in a fully functional Nano-luciferase creating a quantifiable luminescent signal in the presence of substrate.