JAK2^{ex13InDel} DRIVES ONCOGENIC TRANSFORMATION AND IS ASSOCIATED WITH CHRONIC EOSINOPHILIC LEUKEMIA AND POLYCYTHEMIA VERA

Running title: *JAK2*^{ex13InDel} is associated with PV/CEL phenotypes

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Key Points:

- A novel insertion/deletion mutation in *JAK2* is associated with eosinophilia and erythrocytosis, possibly representing a new clinical entity.
- *JAK2*^{ex13InDel} leads to constitutive activation and promotes signaling through β common chain–based receptors in the absence of ligand

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ABSTRACT

The V617F mutation in the JH2 domain of JAK2 is an oncogenic driver in several myeloproliferative neoplasms (MPNs), including essential thrombocythemia, myelofibrosis, and polycythemia vera (PV). Other mutations in *JAK2* have been identified in MPNs, most notably exon 12 mutations in PV. Here, we describe a novel recurrent mutation characterized by a common 4-amino acid deletion and variable 1-amino acid insertion (Leu583-Ala586DelInsSer/Gln/Pro) within the JH2 domain of JAK2. All four affected patients had eosinophilia, and both patients with Leu583-Ala586DelInsSer fulfilled diagnostic criteria of both PV and chronic eosinophilic leukemia (CEL). Computational and functional studies revealed that Leu583-Ala586DelInsSer (herein referred to as *JAK2*^{ex13InDel}) deregulates JAK2 through a mechanism similar to JAK2^{V617F}, activates STAT5 and ERK and transforms parental Ba/F3 cells to growth factor independence. In contrast to JAK2^{V617F}, JAK2^{ex13InDel} does not require an exogenous homodimeric type 1 cytokine receptor to transform Ba/F3 cells, and is capable of activating β common chain family cytokine receptor (IL-3R, IL-5R, granulocyte-macrophage colony stimulating factor receptor) signaling in the absence of ligand, with the maximum effect observed for IL-5R, consistent with the clinical phenotype of eosinophilia. Recognizing this new PV/CEL overlap MPN has significant clinical implications, as both PV and CEL patients are at high risk for thrombosis, and concomitant cytoreduction of red cells, neutrophils and eosinophils may be required for prevention of thromboembolic events. Targeted next-generation sequencing for genes recurrently mutated in myeloid malignancies in patients with unexplained eosinophilia may reveal additional cases of Leu583-Ala586DelInsSer/Gln/Pro, allowing for complete characterization of this unique MPN.

INTRODUCTION

JAK2^{V617F}, a somatic mutation in the autoinhibitory JH2 (pseudokinase) domain of the non-receptor tyrosine kinase Janus kinase 2 (*JAK2*), promotes kinase activation and is a common driver in the classical Philadelphia chromosome-negative (Ph$^-$) myeloproliferative neoplasms (MPN), polycythemia vera (PV), essential thrombocythemia and myelofibrosis[1,2]. Rare PV cases, characterized by isolated erythrocytosis, exhibit mutations in *JAK2* exon 12 in the 5' portion of the JH2 domain[3-5]. Additional non-V617F/non-exon 12 JAK2 somatic variants have been described in MPNs[6]. Germline mutations other than *JAK2*^{V617F} localizing to the four-point-one, ezrin, radixin, moesin (FERM), JH2 or the JH1 (kinase) domain of JAK2 have also been reported[7-11]. JAK2 is recruited upon ligand binding to dimeric receptors, including erythropoietin receptor (EPOR), thrombopoietin receptor (MPL), and granulocyte colony stimulating factor receptor (G-CSFR) as well as the β common (βc) chain of granulocyte macrophage-colony stimulating factor receptor (GM-CSFR), IL-3R and IL-5R, resulting in kinase activation and signal transduction[12,13]. In JAK2^{V617F} the substitution of phenylalanine for valine disrupts the autoinhibitory function of the JH2 domain by blocking JH2-mediated phosphorylation of S523 and Y570 and preventing critical conformational changes that depend on F595 and E596 in helix αC of JH2[14,15]. The result is ligand independent constitutive activation of receptors and downstream signaling via signal transduction and activation of transcription (STAT) family transcription factors. One phenotypic consequence is erythropoietin (EPO) independent erythropoiesis, detected *in vitro* by formation of EPO-independent erythroid burst forming units (BFU-E), known as endogenous erythroid colonies (EEC)[16].

Gain-of-function *JAK2* mutations have been described in myeloid neoplasms other than Ph$^-$ MPNs, including disorders with eosinophilia, most notably those characterized by a pericentriolar material 1 (PCM1)-JAK2 rearrangement[17]. JAK2^{V617F} may occur in up to 4% of patients with hypereosinophilia of unknown significance, and survival of hypereosinophilia patients with *JAK2*^{V617F} is reduced compared to those with *FIP1L1-PDGFRα*[12,18,19]. Here we characterize a novel insertion/deletion JAK2 mutation detected in a patient presenting with an MPN combining features of PV and chronic eosinophilic leukemia (CEL). We identified three additional cases of JAK2 insertion/deletion mutations involving the identical four residues, one of which exhibited a similar phenotype, raising the question of a specific PV/CEL overlap syndrome associated with insertion/deletion mutations in the JAK2 JH2 domain. We demonstrate that JAK2^{ex13InDel} bears mechanistic resemblance to JAK2^{V617F}, but can activate STAT5 in the absence of βc family cytokines IL-3, IL-5 and GM-CSF, conceivably promoting eosinophilic differentiation.

METHODS

Patient samples. Written informed consent was obtained from Patient 1 under University of Utah Institutional Review Board Protocol 45880. Red blood cell lysis was performed using $NH_4Cl/NaHCO_3$. Patient samples from the United Kingdom are described in Supplemental Information.

Cell culture. The IL-3 dependent murine cell line Ba/F3 (DSMZ, Germany) was cultured in RPMI medium supplemented with 10% fetal bovine serum (Sigma-Aldrich. St. Louis, MO), 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin ± 10% WEHI conditioned medium as a source of murine IL-3.

Construction of expression constructs and derivation of Ba/F3 lines. Standard methodology was used. See Supplemental Information.

Immunoblot and immunoprecipitation. Standard methodology was used. See Supplemental Information.

Measurement of drug response by cell proliferation assay. Ruxolitinib and momelotinib were purchased from Selleck Chem (Houston, TX). Ba/F3 cells expressing *JAK2* mutants were seeded at 2000 cells/well in 96-well plates with graded concentrations of inhibitors in medium containing IL-3. At 72 hours, viable cells were quantified using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reagent per manufacturer's instructions (Promega, Madison, WI)[20]. Absorbance at 490 nm was measured with an Epoch Microplate Reader (BioTek Instruments, Winooski, VT).

RNA-based analysis of X-chromosome inactivation. We used a quantitative assay based on transcript analysis of five X-chromosome encoded genes informative in 95% of females[21,22]. The transcription-based clonality assay was performed as previously described[23]. After genotyping exonic single nucleotide polymorphisms (SNP) in five X-chromosome genes (*G6PD, MPP1, FHL1, BTK* and *IDS*), we used quantitative allele-specific PCR to determine the allelic frequency of informative markers (heterozygous at the polymorphic locus being interrogated)[23,24].

EEC colony assays. We followed published protocols[24,25]. See Supplemental Information.

Fragment length analysis and Sanger sequencing. *JAK2*^{ex13InDel} was determined using semi-quantitative fragment length analysis[21] and *JAK3*^{R925S} was tested by Sanger sequencing at the DNA Sequencing Core Facility, University of Utah. PCR was performed using 20 ng of DNA or cDNA, HotStarTaq Master Mix (Qiagen, Germantown, MD), 50 mM $MgCl_2$, 0.2 μM of each primer (JAK2-gDNA: (forward) 5` FAM TTCCTACTTCGTTCTCCATCTTT 3`; (reverse) 5'-TGAGAGCACATCTTTAAACAGCA-3') and (JAK2-cDNA: (forward) 5'-FAM TGAACCAAATGGTGTTTCACA-3'; (reverse) 5'-CAAATTTTACAAACTCCTGAACCA-3') and (JAK3-gDNA: (forward) 5'-GACAGATCCTGCCTTCTCCA-3'; (reverse) 5'-CAAACCACTCCTCAGCCTTC-3')

***Dual-luciferase reporter assay*.** See Supplemental Information.

Computational modeling. The structure of JH2 with the JAK2^{ex13InDel} mutation was predicted using the web portal Phyre2 in intensive mode[28]. The model was compared to the X-ray crystal structures of JH2 JAK2^{WT} (PDB:4FVQ) and JH2 JAK2^{V617F} (PDB: 4FVR)[29]. Images were prepared in PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA).

Statistics. Results are provided as mean ± SEM. Data were analyzed by 2-way ANOVA with Tukey correction for multiple comparisons or a 2-tailed Student *t* test.

RESULTS

Insertion/deletion mutations in the JH2 domain of JAK2 are associated with eosinophilia

A 69 year-old woman (patient 1) with a history of eosinophilic fasciitis and presumed immune thrombocytopenic purpura (ITP) treated with eltrombopag presented for evaluation of steroid-refractory hypereosinophilic syndrome. She had a several year history of peripheral blood eosinophilia with an absolute eosinophil count of up to 17,500/μL. The white blood cell count was 30×10^3/μL (57% eosinophils, 32% neutrophils, 5% lymphocytes, 4% monocytes, 1% immature granulocytes, 1% basophils), Hgb 15.7 g/dL, Hct 48.3% and platelets 193×10^3/μL. Initial EPO concentration was 2.5 mU/mL (normal range, 4-27). Bone marrow biopsy was hypercellular with trilineage hematopoiesis, increased atypical (hyperlobated) megakaryocytes, markedly increased eosinophils with abnormal granulation and nuclear lobation, but no increase in blasts. Cytogenetic examination showed a normal female karyotype and SNP microarray was negative for copy number alterations or copy-neutral loss of heterozygosity. Fluorescence in situ hybridization for *FIP1L1/CHIC2/PDGFRα*, *FGFR1*, *PDGFR*β and *CBF*β rearrangements was negative, as were T-cell clonality studies. Next generation sequencing (NGS) on 52 myeloid malignancy associated genes revealed an insertion/deletion mutation in exon 13 of *JAK2* (*JAK2*^{ex13InDel}: Leu583-Ala586DelInsSer, c.1747_1756DelInsT) with a variant allele frequency of 10%, but no other mutations (Figure 1A). Genotyping of DNA from the patient's fingernails failed to detect *JAK2*^{ex13InDel}, validating *JAK2*^{ex13InDel} as a somatic mutation. Based on the presence of two major criteria (Hct >48%; presence of a *JAK2* mutation) and one minor criterion (reduced EPO), the patient fulfilled diagnostic criteria for PV, while also meeting criteria for CEL[17]. Computed tomography (CT) scan of the chest revealed ground glass opacities consistent with eosinophilic pulmonary involvement and a left ventricular filling defect consistent with a cardiac thrombus. The patient was placed on anticoagulation with warfarin. Ruxolitinib was started, with reduction of eosinophil counts (Figure 1B). Eltrombopag was discontinued, and repeat echocardiogram showed resolution of cardiac thrombus. Hematologic response continued for 18 months, when platelets suddenly dropped to $<6\times10^3$/μL, failed to recover upon discontinuation of ruxolitinib, and were unresponsive to a trial of steroids. Bone marrow biopsy was unchanged, without increase in blasts, and NGS continued to demonstrate JAK2^{ex13InDel} at 9.5% VAF, with a new *TET2* mutation (c.3195_3198del, pThr1066fs) at 1.6% VAF. The patient was started on 5-azacitidine, with recovery of platelet counts, but persistent eosinophilia. Ruxolitinib was added, with reduction of eosinophil counts. Therapy continued with 5-azacitidine combined with ruxolitinib, with acceptable platelet and eosinophil counts.

To understand whether insertion/deletion mutations of JAK2 occur more frequently in eosinophilic conditions, we screened 173 cases from the Wessex Regional Genetics Laboratory, using a custom targeted amplicon NGS covering *JAK2* exon 13 (see Supplemental Information for NGS methods). All 173 cases were negative for *FIP1L1-PDGFRA*, *STAT5* N642H and other eosinophilia-associated abnormalities. We identified two additional patients with JAK2 insertion/deletion variants (VAFs 16.7% and 44%). One patient had the identical mutation as the index case and presented with a dual PV/CEL clinical phenotype. A second patient had *JAK2*:

Leu583_Ala586DelInsGln, c.1748_1756del. The identical deletion with a proline insertion was reported in a single case in an NGS study performed on patients with WHO-defined hypereosinophilic syndrome and idiopathic hypereosinophilia (Table 1)[30]. A survey of the catalogue of somatic mutations in cancer (COSMIC, accessed April 28, 2019) revealed no additional insertion/deletion mutations in this region of the JH2 domain of JAK2.

EEC formation and hypersensitivity to EPO are hallmarks of PV[31]. Peripheral blood mononuclear cells from patient 1 were cultured in the presence of increasing concentrations of EPO[32]. The erythroid progenitor cells grew EECs and her BFU-Es were hypersensitive to EPO (Figure 1C). Genotyping of colonies revealed heterozygosity for *JAK2*^{ex13InDel} in seven of eight EECs and JAK2^{WT} in the remaining colony (Figure 1D). In contrast to PV, where most EECs are homozygous for *JAK2*^{V617F} as a result of somatic uniparental disomy, loss of heterozygosity of *JAK2*^{ex13InDel} was not detected, consistent with normal SNP array results[24,33,34].

To test whether the patient's *JAK2*^{WT} cells were also clonal, we used an RNA-based clonality assay[35]. Genomic DNA from the patient's MNCs was genotyped for exonic SNPs in five X-chromosomal genes (*G6PD, MPP1, FHL1, BTK* and *IDS*) and found to be heterozygous for *G6PD (*C/T, cds no. 1311, dbSNP: rs2230037*)* and *MPP1 (*G/T, cds no. 358, dbSNP:rs1126762*),* thus informative for a transcriptionally-based clonality assay[35]. Analysis of hematopoietic lineages revealed clonal platelets, neutrophils and eosinophils (Table 2). Fragment analysis of neutrophils and eosinophils showed *JAK2*^{ex13InDel} allele frequencies of 15% and 45%, respectively, indicating that *JAK2*^{ex13InDel} is present in virtually all eosinophils, but only a subset of neutrophils. This suggests that clonal hematopoiesis in this patient is driven by one or more unknown somatic mutation(s) and that subsequent acquisition of *JAK2*^{ex13InDel} biases myelopoiesis toward eosinophil differentiation. The clonal background upon which JAK2^{V617F} mutations occur has been similarly postulated to influence MPN phenotype[24,36,37]. Colony assays using mononuclear cells from our patient and healthy controls did not reveal a bias toward BFU-U, CFU-GM or CFU-GEMM formation (Supplemental Figure 1), but results should be interpreted with caution given the variation in colony type and number observed amongst controls.

JAK2^{ex13InDel} confers cytokine-independent growth to Ba/F3 cells

Parental Ba/F3 cells lacking exogenous type I homodimeric cytokine receptors were transduced with human *JAK2* retroviral constructs for expression of JAK2^{ex13InDel}, JAK2^{V617F} or JAK2^{WT} and cultured with IL-3 supplementation. At 48 hours following infection, GFP^{+} cells were assessed by FACS and percentages found to be comparable across genotypes in three independent experiments (Supplemental Table 2). Following confirmation of GFP-positivity, IL-3 was withdrawn and the culture monitored for GFP by flow cytometry. JAK2^{ex13InDel} expressing cells rapidly expanded, reaching close to 100% after 5 days (Figure 2A). In contrast, JAK2^{V617F} expressing cells reached only 25% GFP positivity after 10 days. In a separate set of experiments, parental Ba/F3 cells and GFP-sorted Ba/F3 cells expressing JAK2 constructs were plated at equal cell numbers and grown ± IL-3. Parental Ba/F3 cells and those expressing JAK2^{WT} or JAK2^{V617F} failed to proliferate in the absence of IL-3, while JAK2^{ex13InDel} cells demonstrated exponential growth under both medium conditions (Figure

2B). Only Ba/F3 JAK2^{ex13InDel} cells exhibited colony growth in the absence of IL-3 (Supplemental Figure 2). Parental, *JAK2*^{WT} and *JAK2*^{V617F} infected Ba/F3 cells also displayed markedly different growth rates in response to graded concentrations of IL-3, while the growth of Ba/F3 *JAK2*^{ex13InDel} was relatively insensitive to varying IL-3 dose (Figure 2C). Lastly, we performed cell proliferation experiments with ruxolitinib or momelotinib in the presence of IL-3. The IC_{50} of ruxolitinib and momelotinib was increased ten-fold and two-fold, respectively, in *JAK2*^{ex13InDel} Ba/F3 cells compared to controls cultured in IL-3 containing medium (Figure 2D)The inhibitory effect of ruxolitinib on growth of Ba/F3 JAK2^{ex13InDel} cells was augmented by the absence of IL-3 (Supplemental Figure 3). Altogether, these experiments demonstrate that *JAK2*^{ex13InDel} potently transforms parental Ba/F3 cells to cytokine independence, with superior transforming capacity compared to JAK2^{V617F} and resistance to JAK2 inhibitors.

JAK2^{ex13InDel} and JAK2^{V617F} use similar mechanisms of constitutive kinase activation

JAK2^{ex13InDel} is located in the N-lobe of the JH2 domain that faces the catalytically active JH1 domain, specifically forming the loop between the N-terminal regions of JH2 helix αC and the β3 strand. This loop is structurally close to V617F and JH2 αC[28,38,39] (Figure 3A). To understand how JAK2^{ex13InDel} leads to constitutive activation of JAK2, we modelled the effect of the L583-A586 deletion based on the structure of the JH2 domain of JAK2 (PDB: 4FVQ) using Phyre[2] (intensive mode). Compared to JAK2^{WT}, the JAK2^{V617F} JH2 exhibits a rigid α helix C with an extra N-terminal turn. Strikingly, a similar conformational change is observed in the structural model of the JAK2^{ex13InDel} mutant in which deletion of L583-A586 modifies the conformation of the loop between the JH2 αC and β3 strand, thereby altering the N-terminal part of αC. It has been shown previously that the autoinhibitory function of the JH2 domain can be restored in JAK2^{V617F} via modulation of residues within the JH2 αC, specifically through mutagenesis of residue E596 to positively charged residues such as E596R/K [14]. Based on our structural modeling, we predicted that JAK2^{ex13InDel} uses the same activation mechanism as JAK2^{V617F}. To test this, we generated Ba/F3 cells expressing JAK2^{E596R}, JAK2$^{E596R/V617F}$ and JAK2$^{E596R/ex13InDel}$. In contrast to JAK2^{ex13InDel}, JAK2$^{E596R/ex13InDel}$ did not exhibit IL-3 independent growth (Figure 3B). This indicates that JAK2^{ex13InDel} belongs to the same mechanistic circuit as JAK2^{V617F} and that mutations that eliminate or reverse the charge of E596 disrupt both JAK2^{V617F} and JAK2^{ex13InDel} mediated constitutive kinase activation. It is conceivable that additional mechanisms enhance constitutive kinase activation. For instance, molecular dynamic simulation suggested that phosphorylation of Y570 participates in the maintenance of inhibitory interactions between JH1 and JH2[38]. In JAK2^{ex13InDel}, the altered position of Y570 may disrupt inhibitory interactions to a greater degree than in JAK2^{V617F}. Increased kinase activity could explain the reduced sensitivity of JAK2^{ex13InDel} to JAK2 inhibitors. Unfortunately, repeated attempts to directly compare enzyme kinetics between full length mammalian JAK2^{WT}, JAK2^{ex13InDel} and JAK2^{V617F} were unsuccessful, as we were unable to achieve sufficient concentrations of full-length purified protein, an experimental hurdle known in the field[40].

JAK2^{ex13InDel}, but not JAK2^{V617F}, activates ERK1/2 and STAT5 signaling in Ba/F3 cells in the absence of IL-3

We next assessed canonical signaling pathways activated by JAK2^{V617F} [41]. Ba/F3 cells expressing *JAK2*^{ex13InDel}, *JAK2*^{V617F} or *JAK2*^{WT} maintained in IL-3 were subjected to an extensive washout protocol to remove IL-3, then replated ± IL-3, followed by immunoblot analysis of whole cell extracts. As expected, all cell lines cultured in the presence of IL-3 demonstrated robust activation of ERK1/2, STAT5 and JAK2 (Figure 4A-B). In contrast, activation of ERK1/2, STAT5 and JAK2 persisted in Ba/F3 cells expressing JAK2^{ex13InDel} despite IL-3 withdrawal. No consistent differences were observed for STAT3, SHP2 or p38 mitogen activated protein kinase (Figure 4). JAK2 tyrosine phosphorylation was readily demonstrable in JAK2 immunoprecipitates from all cell lines when cultured with IL-3. In the absence of IL-3, only JAK2^{ex13InDel} showed weak, but reproducible tyrosine phosphorylation. As activation of downstream signaling by JAK2^{V617F} depends on association with a cytokine receptor, such as EPOR, the data are consistent with the lack of EPOR expression in Ba/F3 cells and suggest that JAK2^{ex13InDel} is either capable of a receptor interaction that is not accessible to JAK2^{V617F}, or does not require such interaction.

JAK2^{ex13InDel}, but not JAK2^{V617F}, activates signaling through βc-associated cytokine receptors

All four patients in our series showed eosinophilia, raising the question whether JAK2^{ex13InDel} causes eosinophil lineage bias. As the βc family cytokines IL-3, IL-5 and GM-CSF have been shown to promote eosinophilic differentiation, we transfected HEK293 cells with the βc chain and the respective α chains of the IL-3, IL-5 and GM-CSF receptors, luciferase-based STAT5 reporters, and *JAK2*^{WT}, *JAK2*^{V617F}, *JAK2*^{ex13InDel} or empty vector (EV) [42]. We chose HEK293 cells as they are human-derived and do not express endogenous βc receptors, allowing us to isolate signaling events[43]. We assessed STAT5 transcriptional activity in the presence and absence of IL-3, IL-5, or GM-CSF (Figure 5A-C). All three cytokines activated STAT5 in EV, JAK2^{WT}, JAK2^{V617F} and JAK2^{ex13InDel} expressing cells, while only JAK2^{ex13InDel} expressing cells displayed STAT5 activation in the absence of IL-3, IL-5 or GM-CSF, with the strongest effect observed in cells expressing IL-5R. Interestingly, the βc chain alone activated the reporter in *JAK2*^{ex13InDel} expressing cells, while IL-5R α-chain alone did not, with the strongest effect observed when both were co-expressed (Supplemental Figure 4). Thus, unlike JAK2^{WT} and JAK2^{V617F}, JAK2^{ex13InDel} is uniquely capable of activating βc family cytokine signaling in HEK293 cells. Additional luciferase-based STAT5 assays in HEK293 cells demonstrate that both JAK2^{V617F} and JAK2^{ex13InDel} are capable of activating EPOR and MPL in the absence of cytokine. While EPOR activation is comparable, cytokine-independent activation of MPL by JAK2^{V617F} is enhanced compared to JAK2^{ex13InDel} (Supplemental Figure 5).

The ability of JAK2^{V617F} to transform hematopoietic cells requires interaction with a cytokine receptor[12], while certain JAK3 mutants have been shown to activate signal transduction without the need for interaction with a cytokine receptor[44]. As tyrosine 114 in the N-terminal FERM domain of JAK2 is essential for cytokine receptor interactions[44-46], we introduced the Y114A mutation into JAK2^{ex13InDel}, and expressed the double mutant in Ba/F3 cells. Sorted Ba/F3 *JAK2*$^{Y114A/ex13InDel}$ cells were cultured ± IL-3 for three days. Ba/F3 cells expressing JAK2$^{Y114A/ex13InDel}$ did not proliferate in the absence of IL-3, while exponential growth was observed with IL-3, suggesting that cellular transformation and oncogenic signaling by JAK2^{ex13InDel} is dependent upon association

with a cytokine receptor (Figure 5D). While JAK2^{V617F} typically associates with a homodimeric type I cytokine receptor, components of certain heterodimeric receptors, including the βc of the GM-CSF, IL-3 and IL-5 receptors, can cooperate with JAK2^{V617F} to activate downstream signaling and induce cellular transformation[47]. Given the differential ability of JAK2^{ex13InDel} compared to JAK2^{V617F} to activate heterodimeric receptor signaling in HEK293 cells (Figure 5A-C), we hypothesized that JAK2^{ex13InDel} may differentially bind to and phosphorylate βc in Ba/F3 cells. To test this, we assessed tyrosine phosphorylation of βc immunoprecipitates from Ba/F3 cells expressing JAK2^{WT}, JAK2^{V617F} or JAK2^{ex13InDel}. We consistently detected JAK2 in βc immunoprecipitates from cells growing in IL-3, while only minimal amounts of JAK2 were detectable in the absence of IL-3, irrespective of genotype (Figure 5E, lower panel; Supplemental Figure 6). In accord with this, we saw no consistent βc phosphorylation in the absence of IL-3, and phosphorylation in the presence of IL-3 was comparable across genotypes (Figure 5E, upper panel). This data suggests that transformation of Ba/F3 cells by JAK2^{ex13InDel} may use another, as yet unidentified cytokine receptor.

DISCUSSION

We describe for the first time the structure and function of a novel *JAK2* exon 13 insertion/deletion mutant identified in a patient fulfilling diagnostic criteria for both PV and CEL. Consistent with the clinical PV phenotype, mononuclear cells from the patient demonstrate EPO hypersensitivity and EEC formation, phenocopying JAK2^{V617F}. Unlike PV subjects with *JAK2*^{V617F} mutation wherein uniparental disomy is detected in most EEC clones[24], we did not detect homozygosity of *JAK2*^{ex13InDel} in our patient's EECs, but heterozygosity. This finding may account for the relatively low proportion of EECs in our patient compared to those observed in classic PV. Further, *JAK2*^{ex13InDel} imparts IL-3-independent growth to Ba/F3 cells in the absence of an exogenous cytokine receptor, resulting in constitutive STAT5 and ERK1/2 activation. Compared to JAK2^{V617F}, the transforming potency of JAK2^{ex13InDel} toward Ba/F3 cells is significantly increased in multiple assays (Figure 2A-D). There are two possible explanations for this striking difference. First, JAK2^{ex13InDel} may have increased intrinsic kinase activity compared to JAK2^{V617F} due to conformational differences. While JAK2^{ex13InDel} operates within the same activation circuit as JAK2^{V617F}, our modelling suggests that unique conformational attributes may mitigate residual autoinhibitory Y570 interactions present in JAK2^{V617F}, thereby potentiating *JAK2*^{ex13InDel} transformation capacity. The reduced sensitivity of JAK2^{ex13InDel} to JAK kinase inhibitors would also be consistent in principle with this notion (Figure 2D), but could also be explained by differences in inhibitor binding affinity to the catalytic site. Unfortunately, we were unable to directly compare kinase activity across genotypes, as multiple attempts to generate sufficient quantities of recombinant full-length JAK2 proteins were unsuccessful.

An alternative explanation is that JAK2^{ex13InDel} may be able to interact with a cytokine receptor or other signaling molecule inaccessible to JAK2^{V617F}. Ba/F3 cells expressing human JAK2^{ex13InDel} were transformed to cytokine-independence in the absence of an exogenous receptor, while Ba/F3 cells expressing JAK2^{V617F} were not, consistent with previous reports that co-expression of a homodimeric cytokine receptor is required for JAK2^{V617}-

mediated Ba/F3 transformation[12]. This is surprising in view of our computational modelling that supports analogous mechanisms of kinase activation in JAK2^{ex13InDel} and JAK2^{V617F} (Figure 3A), and suggests that JAK2^{ex13InDel} has acquired additional functional capabilities. In accord with this idea, JAK2^{ex13InDel} increases STAT5 transcriptional activity in HEK293 cells expressing IL-3R, IL5-R or GM-CSFR, while JAK2^{WT} and JAK2^{V617F} have no effect (Figure 5A-C). This indicates that JAK2^{ex13InDel} can activate the βc family of receptors to induce cytokine-independent STAT5 signaling. Remarkably, effects were most pronounced for IL-5R, the cytokine with the strongest association with eosinophil differentiation, providing a potential mechanistic link to the eosinophilia observed in patients with JAK2 Leu583-Ala586DelInsSer/Gln/Pro. Altogether, our data suggest that alterations within the JH2 domain can affect JAK2 functionalities, in addition to autoinhibition of kinase activity, and that JAK2^{ex13InDel} may skew differentiation toward the eosinophil lineage through ligand-independent activation of IL-5/STAT5 signaling. As JAK2^{ex13InDel} also activates IL-3 and GM-CSF signaling, and synergism between the three βc cytokines is crucial to optimal eosinophilic growth and differentiation, JAK2^{ex13InDel} may be uniquely capable of inducing eosinophil differentiation and growth without the need for a cooperating mutation[42,48-51]. Consistent with this, our patient's *JAK2*^{ex13InDel} allele frequency was highest in eosinophils, which indicates that the mutation *per se* enhances eosinophil expansion following the establishment of clonal hematopoiesis by an as-yet undetermined pre-*JAK2* mutational event (Figure 1D). While it is possible an antecedent genetic event promotes the eosinophilic hematopoiesis observed with JAK2^{ex13InDel}, our functional studies suggest that the signaling properties of JAK2^{ex13InDel} drive hypereosinophilia. Several attempts to test this in primary CD34^{+} cells were unsuccessful, as we were unable to trace eosinophilic differentiation due to consistent loss of reporter signal from transduced cells (Supplemental Figure 7). Further investigation will be required to characterize the JAK2^{ex13InDel} interactome and its unique features compared to JAK2^{V617F} to precisely delineate the relationship between JAK2^{ex13InDel} and eosinophilic lineage bias.

Our patient's hypereosinophilia partially responded to ruxolitinib, however dose intensity was limited by thrombocytopenia (Figure 1C). Although thrombocytopenia is a well-known side effect of ruxolitinib[52], in our patient it may likely reflect a feature of this clonal myeloid disorder. This is supported by abnormal megakaryocyte morphology, and the fact that platelet counts improved on 5-azacitidine. As such, the preceding diagnosis of ITP was probably incorrect. We identified three additional cases of *JAK2* insertion/deletion mutations within the JH2 domain, one identical to patient 1 (Leu583-Ala586DelInsSer) and two with deletion of the identical four amino acids, but insertion of proline or glutamine rather than serine (Leu583_Ala586DelInsPro/Gln). All four patients had eosinophilia. In contrast, *JAK2*^{V617F} is rare in patients with idiopathic hypereosinophilia, accounting for only 4% of cases in large series[18]. The fact that, to the best of our knowledge, no additional insertion/deletion mutation in this region of the JAK2 JH2 domain have been described strongly suggests a specific genotype-phenotype correlation, in contrast to the phenotypic promiscuity of JAK2^{V617F}. Moreover, two of three cases with available data, including both with Leu583-Ala586DelInsSer, were associated with a polycythemia and low EPO, suggesting that *JAK2*^{ex13InDel} may cause a previously unrecognized clinical syndrome that combines features of PV and CEL. Like JAK2^{V617F}, JAK2^{ex13InDel} promotes EEC formation (Figure 1D), yet only JAK2^{ex13InDel} is capable

of co-opting βc family cytokine receptor signaling to induce cytokine-independent STAT5 activity in HEK293 cells (Figure 5A). Due to concomitant polycythemia and eosinophilia, *JAK2*^{ex13InDel} patients may have an especially high risk for thrombosis, warranting screening with echocardiogram and/or CT scans to detect occult vascular complications, such as the cardiac thrombus in our patient. Although this is the first characterization of a *JAK2*^{ex13InDel} somatic mutation, the routine use of NGS in MPN patients may reveal additional cases and shed further light on the spectrum of clinical phenotypes associated with JAK2 insertion/deletion mutations. As insertion/deletion mutations are more difficult to detect by NGS, and the JAK2 mutations described here are subclonal, similar to most *JAK2*^{V617F} PVs, careful inspection of sequencing traces is warranted.

CONFLICTS OF INTEREST AND DISCLOSURES

Michael W. Deininger reports research funding from and is a paid advisory board member and/or consultant for the following companies: Blueprint, Pfizer, Inc., Takeda, Ascentage Pharma, TRM, Humana.

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Stefan N. Constantinescu is the co-founder of MyeloPro Research and Diagnostics, GmbH, Vienna, Austria.

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FIGURE LEGENDS

Figure 1. (**A)** Structural layout of the JAK2 kinase from N-terminus to C-terminus. Critical domains are labeled in red. The amino acid sequences of the pseudokinase (JH2) domain of $JAK2^{WT}$, $JAK2^{V617F}$ and $JAK2^{ex13InDel}$ are highlighted. Note the deletion of residues 583-586 in $JAK2^{ex13InDel}$ and insertion of an in-frame serine residue. Tyrosine 114 in the FERM domain is critical for interactions with cytokine receptors. **(B)** Trends in the patient's blood counts in response to ruxolitinib and 5-azacitidine treatment. **(C)** Peripheral blood mononuclear cells (MNCs) from the patient and a healthy control were cultured in the absence or presence of graded concentrations of erythropoietin. In the absence of erythropoietin, ten endogenous erythroid colonies (EECs) were observed to grow out from patient-derived MNCs. **(D)** Patient-derived EECs were plucked and genotyped using *JAK2* allele-specific polymerase chain reaction followed by fragment length analysis. Seven of eight patient-derived EECs that were genotyped exhibited heterozygosity for *JAK2*^{ex13InDel}.

Figure 2. (A) Parental Ba/F3 cells were transduced with human JAK2-GFP retroviral constructs for expression of $JAK2^{WT}$, $JAK2^{V617F}$ or $JAK2^{ex13InDel}$ and cultured in WEHI conditioned medium as a source of IL-3. At 48h following transduction, IL-3 withdrawal led to rapid selection of $JAK2^{ex13InDel}$ expressing cells (n=3). **(B)** Parental Ba/F3 cells and GFP-sorted Ba/F3 cells expressing hJAK2 constructs were plated at equal numbers and grown ± IL-3. All cells proliferated in the presence of IL-3 (not shown), while only those cells containing $JAK2^{ex13InDel}$ exhibited exponential growth in the absence of IL-3 (n=3). **(C)** Parental Ba/F3 cells and $JAK2^{WT}$ and $JAK2^{V617F}$ expressing Ba/F3 cells display growth sensitivity to varying concentrations of IL-3, while growth of Ba/F3 $JAK2^{ex13InDel}$ cells is not affected by IL-3 concentration (n=3). **(D)** To assess the effect of JAK2 inhibition on Ba/F3 cells, we performed cell proliferation experiments with JAK inhibitors. The IC_{50} of ruxolitinib was increased ten-fold in $JAK2^{ex13InDel}$ Ba/F3 cells compared to controls and $JAK2^{V617F}$ Ba/F3 cells. The IC_{50} of momelotinib was increased two-fold in $JAK2^{ex13InDel}$ Ba/F3 cells (n=3).

Figure 3. (A) (upper left) Residues L583-A586 are located in the N-lobe of the pseudokinase domain of JAK2 (JH2) that faces the catalytically active kinase domain (JH1). **(upper right, light blue)** L583-A586 form the loop between the N-terminal JH2 αC and the β3 strand in $JAK2^{WT}$. This loop is structurally close to V617 and the JH2 αC, which is altered in the presence of the V617F mutation. **(lower right)** Compared to the loose conformation of JH2 αC in $JAK2^{WT}$, the JH2 αC of $JAK2^{V617F}$ exhibits a rigid helix **(lower left)**. A strikingly similar conformational change in JH2 αC is observed in the $JAK2^{ex13InDel}$ mutant. **(B)** To demonstrate that E596 is also critical for $JAK2^{ex13InDel}$ activity, we cultured Ba/F3 cells expressing $JAK2^{E596R}$, $JAK2^{E596R/V617F}$ and $JAK2^{ex13InDel/E596R}$ ± IL-3. $JAK2^{ex13InDel/E596R}$ did not demonstrate IL-3 independent growth (n=3).

Figure 4. (A) Ba/F3 cells cultured in the presence of IL-3 displayed activation of STAT5 and ERK1/2. In the absence of IL-3, STAT5 and ERK1/2 activation was maintained only by $JAK2^{ex13InDel}$ expressing cells. Additional

proteins did not demonstrate differential JAK2^{ex13InDel} activation in the absence of IL-3. **(B)** In the absence of IL-3, JAK2 phosphorylation was detectable only in JAK2^{ex13InDel} expressing cells.

Figure 5. (A-C) HEK293 cells expressing βc were transfected with the α chains of the IL-3R, IL-5R or CSF2R (GM-CSF receptor) together with a luciferase-based STAT5 reporter (Spi_Luc), and JAK2^{WT}, JAK2^{V617F} or JAK2^{ex13InDel}. STAT5 transcriptional activity was measured in the presence or absence of the respective cytokines at 10 ng/mL (n=3). Data were analyzed with a two-way ANOVA and Tukey's correction for multiple comparisons (*p<0.05, **p<0.001). **(D)** The FERM domain mutation Y114A was introduced into JAK2^{ex13InDel}. Ba/F3 cells expressing JAK2$^{Y114A/ex13InDel}$ cells were cultured ± IL-3. JAK2 double-mutant cells did not proliferate upon IL-3 withdrawal (n=3). **(E)** Parental and Ba/F3 cells expressing JAK2^{WT}, JAK2^{V617F} and JAK2^{ex13InDel} grown with IL-3 supplementation were washed to remove IL-3 and replated ± IL-3. Cells were harvested after four hours. **(upper panel)** βc immunoprecipitates were resolved on SDS-PAGE and incubated with a phosphotyrosine antibody. A representative experiment out of three independent repeats is shown. **(lower panel)** βc immunoprecipitates were incubated with a JAK2 antibody. A representative experiment out of three independent repeats is shown.

Table 1. Clinical characteristics of JAK2 insertion/deletion variants.

	Age/Sex	Variant (DNA)	Variant	VAF	Additional mutations	Clinical phenotype	Clinical features
Patient 1	69 F	c.1747_1756delinsT	Leu583_Ala586delinsSer	10%	None	PV/CEL -Epo 2.5IU/L -Hct 48.3%	Findings -LV thrombus Treatment course -AbE improved on ruxolitinib
Patient 2	82 F	c.1748_1757delinsA	Leu583_Ala586delinsGln	16.70%	DNMT3A c.1728delT (VAF 32%)	CEL	Findings -Cortical basal degeneration Treatment course -started on co-careldopa; deceased
Patient 3	30 M	c.1747_1756delinsT	Leu583_Ala586delinsSer	44%	None	PV/CEL -Epo 1.4IU/L -Hct 57%	Findings -Visual symptoms Treatment course -Hct improved on pegylated-IFN but no improvement in AbE
Patient 4 (Pardanani et al, 2016)	Unknown	c. 1748_1756del	Leu583_Ala586delinsPro	N/A	N/A	CEL, unknown if PV	Unknown

AbE - absolute eosinophil count; CEL - chronic eosinophilic leukemia; Epo – erythropoietin; Hct- hematocrit; IFN – interferon; LV - left ventricular; NGS – next generation sequencing; PV - polycythemia vera; VAF - variant allele frequency

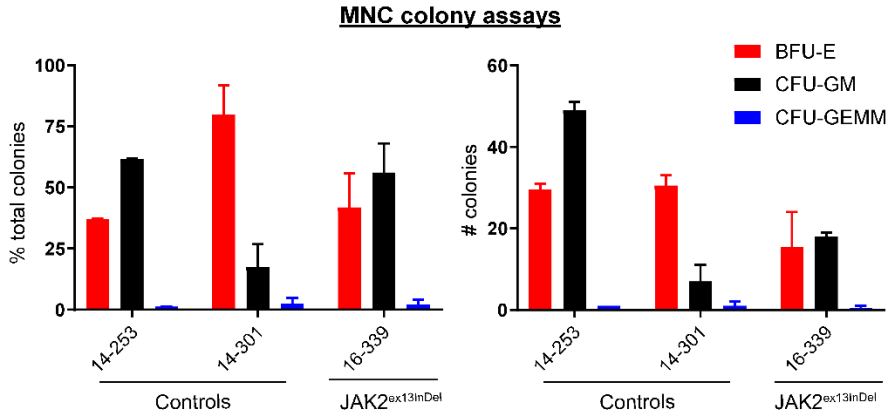
Table 2. Summary of clonality and genotyping studies

Cell type / source	**Clonality studies with informative loci**		**JAK2 genotype (transcript %)**	
	MPP1 [G/T]	G6PD [C/T]	$JAK2^{ex13InDel}$	$JAK2^{WT}$
Granulocytes	[1/99]	[99.95/0.05]	39	61
Neutrophils	[3/97]	[99.75/0.25]	15	85
Eosinophils	[0.1/99.9]	[99.99/0.01]	45	55
Platelets	[4/96]	[99.5/0.5]	N/A	N/A
Lymphocytes	N/A	N/A	0	100

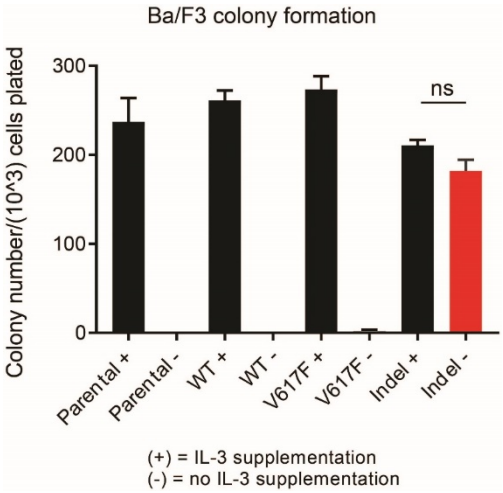
SUPPLEMENTARY INFORMATION

Results

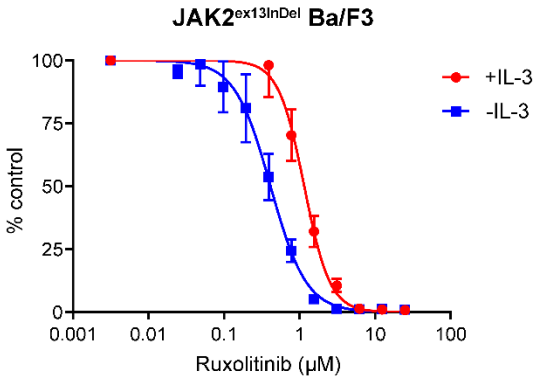
Supplemental Figure 1. **Patient-derived mononuclear cell colony assays**. Equal numbers of mononuclear cells derived from two healthy controls (samples 14-253 and 14-301) and our patient with JAK2^{ex13InDel} were plated in methylcellulose-based medium containing cytokines. Colonies were typed and counted after 10 days.



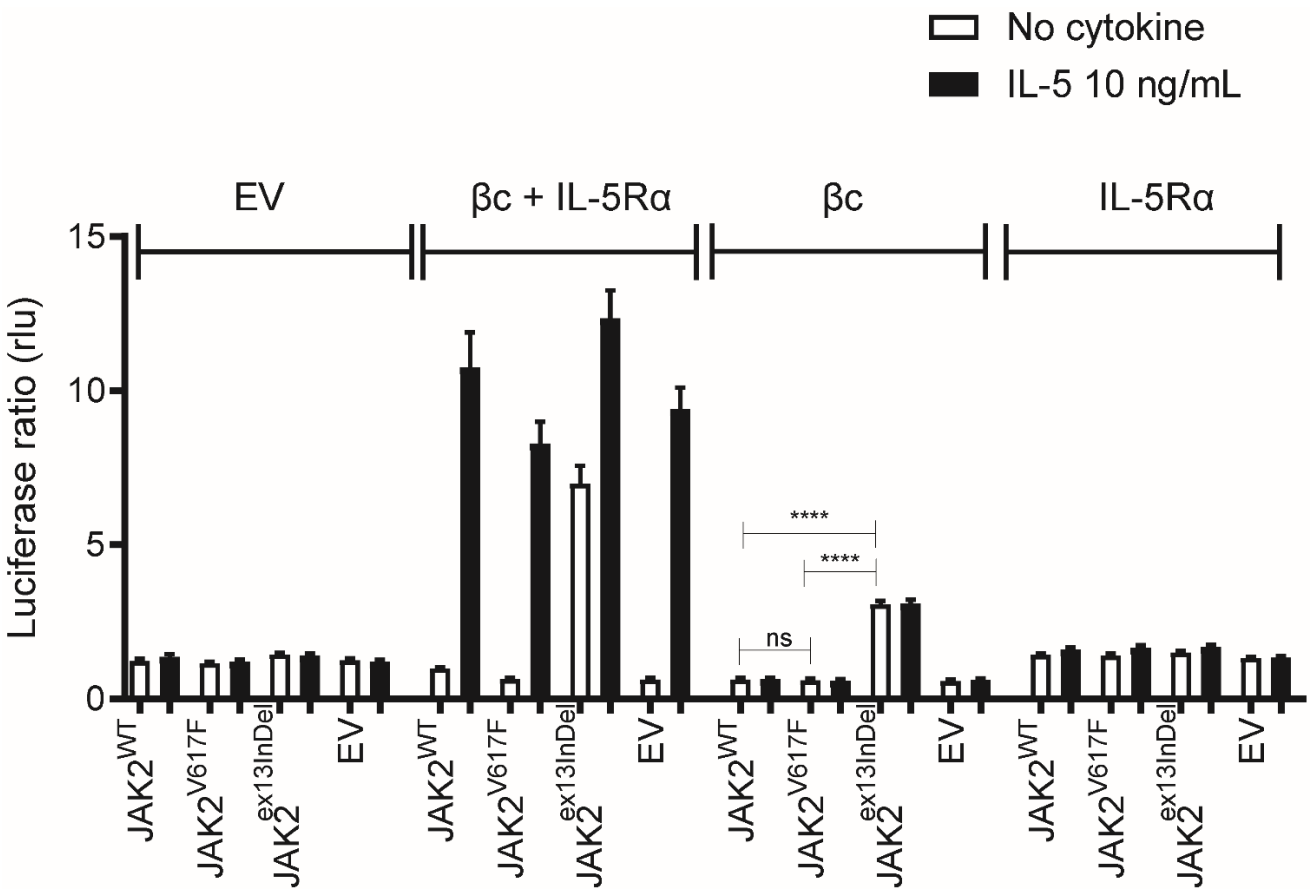
Supplemental Figure 2. Ba/F3 colony assays. Ba/F3 parental and mutant cells were plated in methylcellulose-based colony-forming assays +/- IL-3 supplementation. Colonies were counted at one week and normalized to initial cells plated.



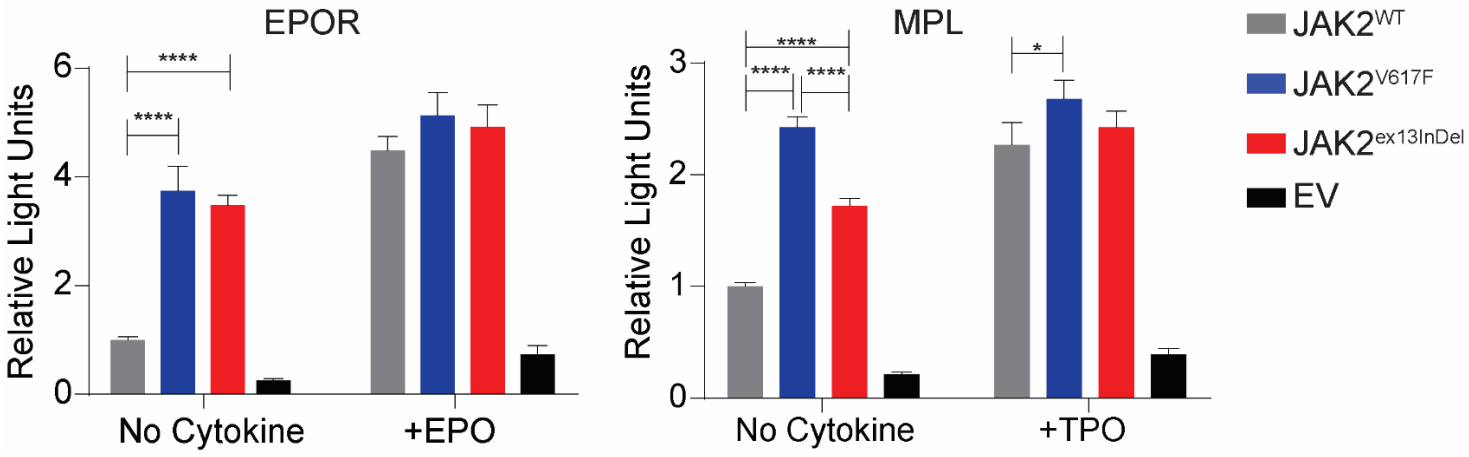
Supplemental Figure 3. **JAK2^{ex13InDel} cell proliferation studies ± IL-3**. JAK2^{ex13InDel} Ba/F3 cells were seeded at 2000 cells/well in 96-well plates in the presence of graded concentrations of ruxolitinib in medium with or without IL-3 supplementation. Cell proliferation was measured at 72 hours (n=3) . The IC_{50}s of ruxolitinib in the presence of IL-3 and absence of IL-3 were 1.41µM and 0.42µM, respectively.



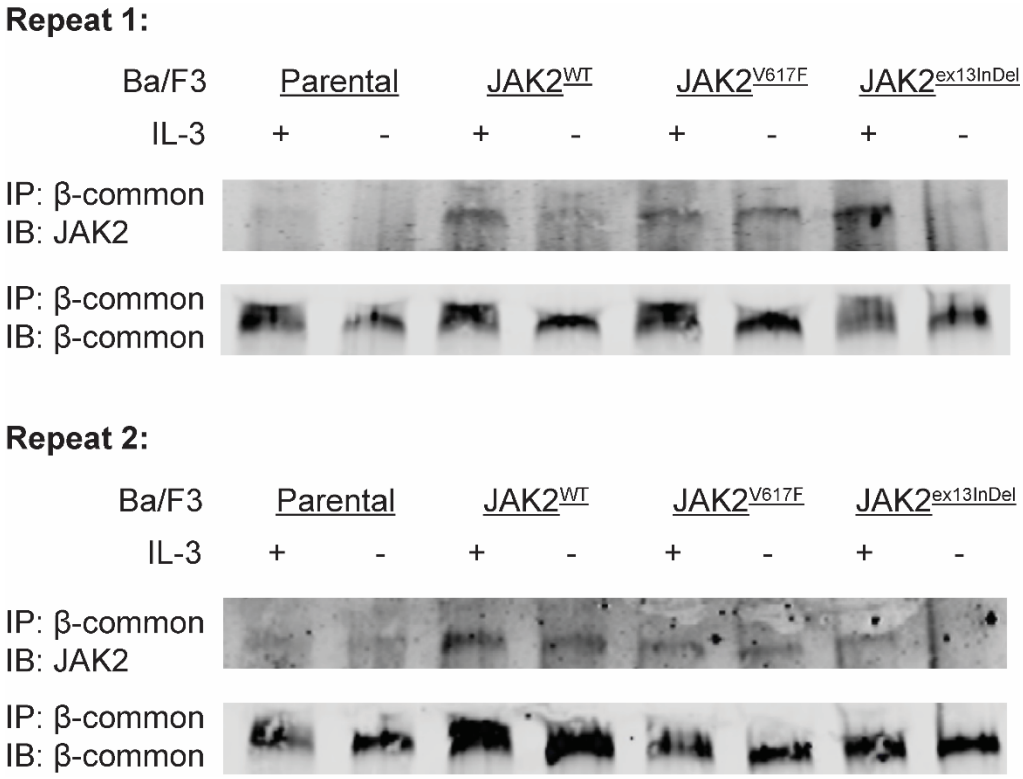
Supplemental Figure 4. STAT5 luciferase activity with components of the IL-5 receptor. Luciferase-based STAT5 transcriptional activity was measured ± IL-5 in the presence or absence of various components of the IL-5 receptor (no receptor components, βc chain + α chain, βc alone, or α chain alone). Data were analyzed with a two-way ANOVA and Tukey's correction for multiple comparisons (****p<0.0001).



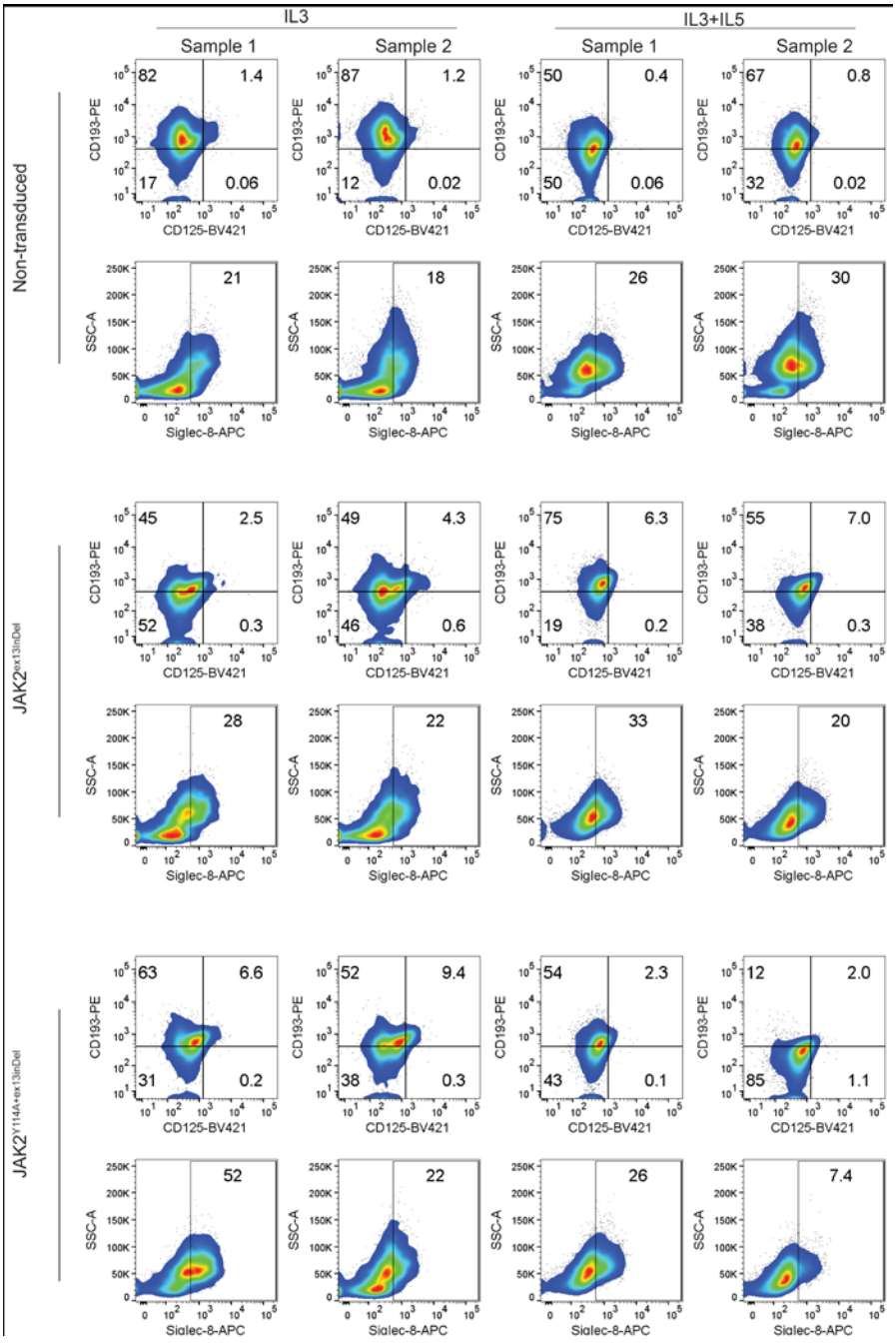
Supplemental Figure 5. Luciferase-based STAT5 transcriptional assays in HEK293 cells using homodimeric type I cytokine receptors. HEK293 cells were transfected with either EPOR or TPOR with a luciferase-based STAT5 reporter (Spi_Luc), and JAK2^{WT}, JAK2^{V617F} or JAK2^{ex13InDel}. STAT5 transcriptional activity was measured in the presence or absence of the respective cytokines at 10U/mL (EPO) or 10 ng/mL (TPO) (n=3 for each receptor). Data were analyzed with a two-way ANOVA and Tukey's correction for multiple comparisons (*p<0.05, ****p<0.0001).



Supplemental Figure 6. Additional JAK2 and β-common co-immunoprecipitation experiments in Ba/F3 cell lines. Parental Ba/F3 and JAK2-mutant cells were cultured ± IL-3. Immunoprecipitation was performed on lysates with a β-common antibody and eluted samples were subjected to immunoblot analysis.



Supplemental Figure 7. Cord blood eosinophil differentiation studies. CD34^{+} cells were purified from human umbilical cord blood from two donors and either cultured directly or transduced with JAK2^{ex13InDel} or JAK2$^{Y114A+ex13InDel}$ (Y114A mutation is known to inhibit receptor box 1/2 motif binding). Cells were cultured for 21 days with IL-3 or IL-3 and IL-5 then analyzed by flow cytometry for eosinophil markers CD125, CD193, and Siglec-8. Gates were drawn on FSC high, singlets that were negative for 7-AAD. Gates were set with samples containing no fluorophores, only 7-AAD, and fluorescence minus one controls for PE, BV125, and APC. Note that no or very few GFP$^{\pm}$ cells were observed (not shown) and that the plots reflect the bulk cultures, which showed evidence for acquisition of eosinophilic differentiation markers, particularly Siglet-8.



Additional patient information

Patient #2

Local ID: E12556 (W1617130)

Mutation: NM_004972:c.1748_1757delinsA, p.(Leu583_Ala586delinsGln)

VAF: 16.7% (peripheral blood)

Absent in cultured T-cells

Myeloid panel analysis (Illumina TruSight Myeloid Panel):

Gene: DNMT3A

Transcript: NM_175629.2

Nucleotide Change: c.1728delT

Protein Change: p.(Lys577Argfs*74)

VAF: 32%

Total Depth: 1842

An 82 year old lady was referred by her primary provider for persistent eosinophilia which had been present for at least the past 5 years. She had a past medical history of hypertension for which she was taking amlodipine, and a total hip replacement. She had no history of allergy, tropical travel, inflammatory or autoimmune conditions. She denied any cardiorespiratory or gastrointestinal symptoms and had no history of rashes.

Clinical examination was entirely unremarkable. Her CBC was as follows (normal ranges, normal range are in parenthesis):

Hemoglobin 13 g/dL (12-15)

WBC 11.7 x 10^9/L (4-10)

Platelets 214 x 10^9/L (150-400)

Neutrophils 5.28 x 10^9/L (2-7)

Lymphocytes 1.87 x 10^9/L (1-3)

Eosinophils 3.87 x 10^9/L (0.02-0.5)

Monocytes 0.64 x 10^9/L (0.2-1.0)

Basophils 0.04 x 10^9/L (0.02-0.1)

The blood film showed mature eosinophils with no left shift. The renal and liver function were normal as was the serum LDH and tryptase (8 mcg/L, NR 2-14). A bone marrow aspirate was hypercellular with some hypolobated megakaryocytes, normal erythropoiesis but with marked eosinophilia and eosinophilic precursors. Mast cells appeared prominent. There was no increase in blasts. The trephine biopsy was hypercellular with a prominent

eosinophil population and grade 1/3 reticulin staining. The marrow was negative for BCR-ABL transcripts and KITD816V. FISH was negative for PDGFR a-FIP1L1. Cytogenetics were normal.

Further investigations for eosinophilia related end-organ damage included a chest X-ray (normal). During additional work-up, she presented to the Emergency Department with acute confusion and drowsiness on a background of several months history of decline in upper limb coordination and word finding difficulties. She had an extrapyramidal type tremor and was found to be bradykinetic with bilateral upper limb increased tone and ataxia. A MRI brain did not highlight any acute changes but there were chronic white matter ischemic changes and an old infarct in the cerebellum. A diagnosis of likely cortical basal degeneration was made and she was commenced on Co-careldopa. Over the ensuing three months, she continued to deteriorate neurologically and passed away.

Patient #3

Local ID: E13502

Mutation: NM_004972:c.1747_1756delinsT (p.Leu583_Ala586delisnSer)

VAF: 39.8% (peripheral blood)

Confirmed in second blood sample (44.0% VAF)

Absent in cultured T-cells

No additional mutations by myeloid panel analysis

This 30-year old man consulted his family doctor in October 2017 with a 3-month history of intermittent visual disturbances. His CBC showed erythrocytosis and eosinophilia (Hb 18.7 g/dL, Hct 57 %, WBC 22.1 x 10^9/L, neutrophils 2.2 x 10^9/L, eosinophils 15.7 x 10^9/L). He had no other symptoms, no history of exotic travel, and was a non-smoker. His spleen was just palpable but examination was otherwise unremarkable. He was first seen by Hematology in January 2018, and his CBC (shown below) demonstrated erythrocytosis, eosinophilia and neutrophilia, confirmed on blood film. His liver and renal function, serum tryptase and inflammatory markers were normal and routine auto-antibodies were negative. His serum erythropoietin was markedly suppressed (1.4 IU/L, NR 5.0-25.0 IU/L). Strongyloides serology was negative as was fecal microscopy for ova, cysts and parasites. MR imaging of his head was normal and whole-body FDG PET-CT imaging demonstrated mild, non-avid splenomegaly (14 cm cranio-caudally) but no FDG-avid lymphadenopathy. Lung function testing and echocardiography were normal.

Peripheral blood testing for *JAK2* V617F (by RT-PCR), *JAK2* exon 12 mutations (by high resolution melt curve assay), FIP1L1-PDGFRA (by RT-PCR) and *KIT* D816V (by RT-PCR) was negative. The bone marrow aspirate was non-diagnostic but the trephine was hypercellular and showed disordered erythropoiesis, with dysplastic

and megaloblastic changes. The myeloid series was left-shifted and note was made of increased eosinophils and their precursors, as well as a few spindle-shaped mast cells. Reticulin was normal.

A diagnosis of polycythemia vera with eosinophilia was made, and the patient initially underwent fortnightly phlebotomies, then commenced on pegylated interferon. His hemoglobin had fallen but his eosinophil count remained elevated. He remained systemically well but continued to have very occasional visual disturbances for which he was under investigation by neurology.

Supplemental Methods

Expression plasmids. MSCV-IRES-GFP (MIG2) empty vector was purchased from Addgene (a gift from Tannishtha Reya, Addgene plasmid # 20672 ; http://n2t.net/addgene:20672 ; RRID:Addgene_20672). First, we inserted two unique cutting sites (MluI and SacII) within the MCS of MIG2 using synthesized oligos between EcoRI and XhoI sites. Full length WT-hJAK2 and V617F-hJAK2 were amplified by PCR. The Leu583-Ala586DelInsSer mutation was introduced by PCR-driven overlap extension. All PCR fragments were digested with MluI/SacII, column purified, ligated in the MluI/SacII digested MIG2-vector and transformed in chemically competent E. coli. All plasmids produced were verified by Sanger sequencing. The E596R mutation was introduced by PCR-driven overlap extension using previously generated plasmids as template. The PCR for this step was designed to amplify a 1639-bp (1630-bp for InDel) region between EcoRI and BamHI sites. All PCR products were digested with BsiWI, BamHI and XcmI, gel purified and ligated in BsiWI/BamHI digested vector. The FERM domain mutation Y114A was introduced via homology driven PCR and the produced fragment was ligated into MIG2-hJAK2InDel vector using MluI and BsiWI restriction sites. All plasmids produced were verified by Sanger sequencing (see below).

Mutagenesis. Site-directed mutagenesis was completed by the overlapping-extension PCR amplification method using primers containing the desired mutations (see below). The reaction was performed using 1 µL of PfuTurbo DNA polymerase 2.5 U/µL following the manufacturer's protocol. The coding region of each construct was verified by Sanger sequencing. The three JAK2 constructs were cloned in the bicistronic retroviral vector pMX-IRES-GFP1, and verified by Sanger sequencing.

Generation of Ba/F3 lines. 293T cells grown in DMEM supplemented with 10% FBS were seeded in 6-well plates at 6.5 x 10^5 cells/well 24 hours before transfection. For generation of pseudoviral particles, cells were co-transfected with packaging vector (2 µg EcoPak) and MIG2-JAK2^{WT} and all variant plasmids (2 µg), respectively in Opti-MEM using Lipofectamine 2000 and Plus Reagent. Six hours after transfection, cell culture media was replaced with DMEM supplemented with 10% FBS and 1% BSA. Viral supernatant was harvested 72 hours after transfection, filtered (0.45 um) and used for transduction without additional processing. Parental Ba/F3 cells were transduced with the human JAK2 retroviral constructs and sorted for GFP positivity after infection.

Immunoblot and immunoprecipitation. Parental and JAK2-containing Ba/F3 cells were washed three times in RPMI, cultured ± 10% WEHI CM and harvested for immunoblot after 4 hours. Cell pellets were washed once with cold PBS, then lysed in radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin; with 1 mM PMSF added immediately before use). Equal amounts of protein were separated by SDS-PAGE (Bio-Rad) and transferred to nitrocellulose membrane. Antibodies used: β-actin, anti-ERK1/2, anti-pERK1/2, anti-p38, anti-pp38, anti-JAK2, anti-pTyr, anti-SHP2, anti-pSHP2, anti-pSTAT5^{Y694} (all from Cell Signaling Technology, Danvers, MA), and anti-STAT5 (BD Biosciences, San Jose, CA). Images were obtained using Licor Odyssey CLx Infrared Imaging System. For immunoprecipitation, cell lysis was performed with Triton-XP 100 lysis buffer containing proteinase inhibitor (cOmplete™), phosphatase inhibitor (PhosSTOP™) and PMSF. Protein A/G beads (Thermo Fisher, Waltham, MA) were incubated with cell lysates and antibodies at 4 °C overnight and protein was eluted with sample buffer prior to gel loading.

EEC colony assay. Erythroid colonies (BFU-E) were grown from peripheral blood mononuclear cells without EPO and in the presence of EPO at 15 mU, 30 mU, 60 mU and 3 U/mL[2,3]. Individual BFU-Es were harvested and RNA was isolated for the transcription-based clonality assay. gDNA was genotyped for the presence of JAK2InDel and JAK3R925S[1-3].

***Dual-luciferase reporter assay*.** STAT5 transcriptional activity was measured in HEK293 cells by dual-luciferase reporter assays with the luciferase-based STAT5 reporter gene Spi_Luc[26]. Cells were transfected with cytokine receptors (βc plus IL-3Rα, IL-5Rα or GM-CSFRα; EPOR; MPL), JAK2, Spi_Luc, pRL-TK (*Renilla* luciferase-expressing plasmid used as internal control) and reporter genes (Firefly luciferase-expressing plasmid) as indicated using Lipofectamine™ 2000 (Invitrogen) in Optimem. Standard protocol was followed as previously described[27]. Optimem medium was changed 4 h after transfection and replaced by DMEM medium supplemented with 10 % FBS ± cytokines. Cells were lysed 24 hours after transfection. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI) following manufacturer's instructions. Emitted light was recorded on a luminescence microplate reader (Perkin-Elmer, Norwalk, CT). Assay results are expressed in relative light units, (average of firefly activity divided by average of *Renilla* luciferase activity). Assays were performed in triplicate.

Primary cell colony assay. Equal numbers of patient-derived mononuclear cells were plated in duplicate in MethoCult H4435 (StemCell Technologies). Colonies were typed and counted after 10 days.

Cord blood eosinophil differentiation. Human CD34$^+$ cells were purified with an AutoMacs Pro Cell Separator (Miltenyi Biotech, Bergisch Gladbach, Germany). Umbilical cord blood was purchased from the St. Louis Cord Blood Bank, now part of the University of Colorado (Denver). Whole cord blood was treated to lyse red blood cells and mononuclear cells were purified with Ficoll-Paque Premium (1.078 g/mL, GE Healthcare,

Chicago, IL). For the first 3 days cells were cultured in serum free StemSpan™ SFEM II (Stemcell technologies, Seattle, WA) supplemented with 1% penicillin-streptomycin and SCF (50 ng/mL), FLT-3 ligand (50 ng/mL), GM-CSF (0.1 nM), IL-3 (0.1 nM) and IL-5 (0.1 nM). Cells were transduced with lentivirus generated using pCDH-CMV-JAK2^{ex13InDel} -EF1α-EGFP or pCDH-CMV-JAK2$^{Y114A+ex13InDel}$ -EF1α-EGFP plasmids and sorted for GFP at 48h after infection on a FACSARIA (BD Biosciences, San Jose, CA). After sorting, the cells were cultured with IL-3 or IL-3+IL5. A non-transduced population was also maintained under the same condition. Culture medium was changed every 3 days and the culture was maintained for 21 days.

Primers for JAK2 expression plasmids:

hJAK2-MluI-For: 5'-GATTCACGCGTATGGGAATGGCCTGCCTTAC-3',

hJAK2-SacII-Rev: 5'-CTCGAGCCGCGGTCATCCAGCCATGTTATCCCTTATT-3',

hJAK2-InDel-BRev: 5' TTCTGTGTGAAACTTTTAAAAGAACTTCTGTTTCATGCAGTTGA-3',

hJAK2-InDel-CFor: 5'-TCAACTGCATGAAACAGAAGTTCTTTTAAAAGTTTCACACAGAA-3',

hJAK2-EcoRI-A-For: 5'-ACGGTGGAATTCAGTGGTCAAGA-3',

hJAK2-E596R-C-For: 5'-TCAGAGTCTTTCTTTAGGGCAGCAAGTATGAT-3',

hJAK2-E596R-B-Rev: 5'-ATCATACTTGCTGCCCTAAAGAAAGACTCTGA-3',

hJAK2-BamH1-D-Rev: 5'-ACTGTGTAGGATCCCGGTCTTCA-3'.

Additional primers for sequencing were:

hJAK2-600-For, 5'- CTATAACTCTATCAGCTACAAGACATTCTT-3',

hJAK2-1400-For, 5'-ACAAAGAAGAACTTCAGCAGTCT-3',

hJAK2-2256-For, 5'-ACCTCTAAGTGCTCTGGATTCTCAAAG-3' ,

hJAK2-3000-For, 5'-CTTGCCACAAGACAAAGAATACT-3'.

Analysis of samples from the University of Southampton

Genotyping of JAK2 exon 13. 173 samples from patients with FIP1L1-PDGFRA negative eosinophilia referred to Wessex Regional Genetics Laboratory for FIP1L1-PDGFRA fusion testing were retrospectively screened for JAK2 exon 13 mutations. Primers covering JAK2 exon 13 (Table A) were designed for custom targeted amplicon NGS. Primers were tested using qPCR and Bioanlyzer to confirm performance and specificity. Indexed JAK2

exon 13 amplicons were generated using 60 ng of patient DNA per reaction, and purified using AmpureXP beads. Amplicons were pooled to generate final sequencing libraries consisting of ~40 patients. Libraries were assessed using Bioanalyzer and Qubit. Sequencing runs were performed on the Illumina MiSeq platform using Nano v2 2x250 reagents. VCF files were generated using the WRGL in-house genotyping pipeline, and were annotated using web-based bioinformatics tools Galaxy, ANNOVAR and Ensembl VEP. Confirmation of JAK2 exon 13 InDels detected by NGS was performed by fragment analysis and Sanger sequencing (primer sequences below).

F_01 and R_01 were used in amplicon library generation.

JAK2_13_F_01	GTATTTTCTTGTTCCTACTTCGTTC
JAK2_13_R_01	TTTAAACAGCATAAACTACATGAACA

F_02 and R_02 were used for Sanger sequencing.

JAK2_13_F_02	TGTTCCTACTTCGTTCTCCATCT
JAK2_13_R_02	AGCACATCTTTAAACAGCATAAACT

F_03 and R_02 used for fragment analysis.

JAK2_13_F_03	[6FAM]ACGGTCAACTGCATGAAACAREFERENCES

SUPPLEMENTAL REFERENCES

1. Liu X, Constantinescu SN, Sun Y, et al: Generation of mammalian cells stably expressing multiple genes at predetermined levels. Anal Biochem 280:20-8, 2000

2. Kralovics R, Stockton DW, Prchal JT: Clonal hematopoiesis in familial polycythemia vera suggests the involvement of multiple mutational events in the early pathogenesis of the disease. Blood 102:3793-6, 2003

3. Nussenzveig RH, Swierczek SI, Jelinek J, et al: Polycythemia vera is not initiated by JAK2V617F mutation. Exp Hematol 35:32-8, 2007

Figure 1.

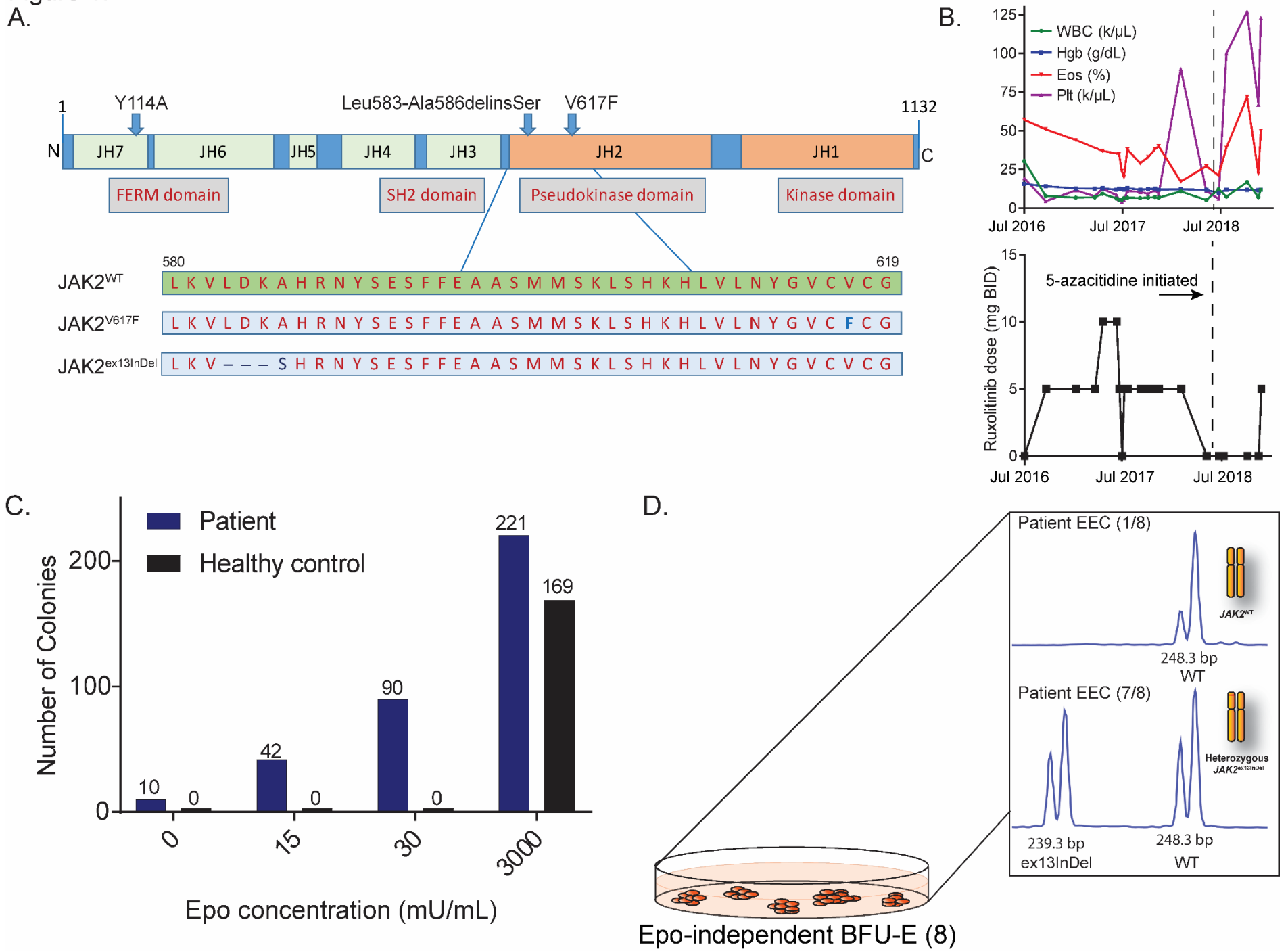

A.
1
Y114A
Leu583-Ala586delinsSer
V617F
1132
N
JH7
JH6
JH5
JH4
JH3
JH2
JH1
C
FERM domain
SH2 domain
Pseudokinase domain
Kinase domain
580
619
JAK2WT L K V L D K A H R N Y S E S F F E A A S M M S K L S H K H L V L N Y G V C V C G
JAK2V617F L K V L D K A H R N Y S E S F F E A A S M M S K L S H K H L V L N Y G V C F C G
JAK2ex13InDel L K V – – – S H R N Y S E S F F E A A S M M S K L S H K H L V L N Y G V C V C G
B.
WBC (k/μL)
Hgb (g/dL)
Eos (%)
Plt (k/μL)
125
100
75
50
25
0
Jul 2016
Jul 2017
Jul 2018
5-azacitidine initiated
Ruxolitinib dose (mg BID)
15
10
5
0
Jul 2016
Jul 2017
Jul 2018
C.
Patient
Healthy control
Number of Colonies
200
100
0
10
0
42
0
90
0
221
169
0
15
30
3000
Epo concentration (mU/mL)
D.
Patient EEC (1/8)
JAK2WT
248.3 bp
WT
Patient EEC (7/8)
Heterozygous
JAK2ex13InDel
239.3 bp
ex13InDel
248.3 bp
WT
Epo-independent BFU-E (8)

Figure 2.

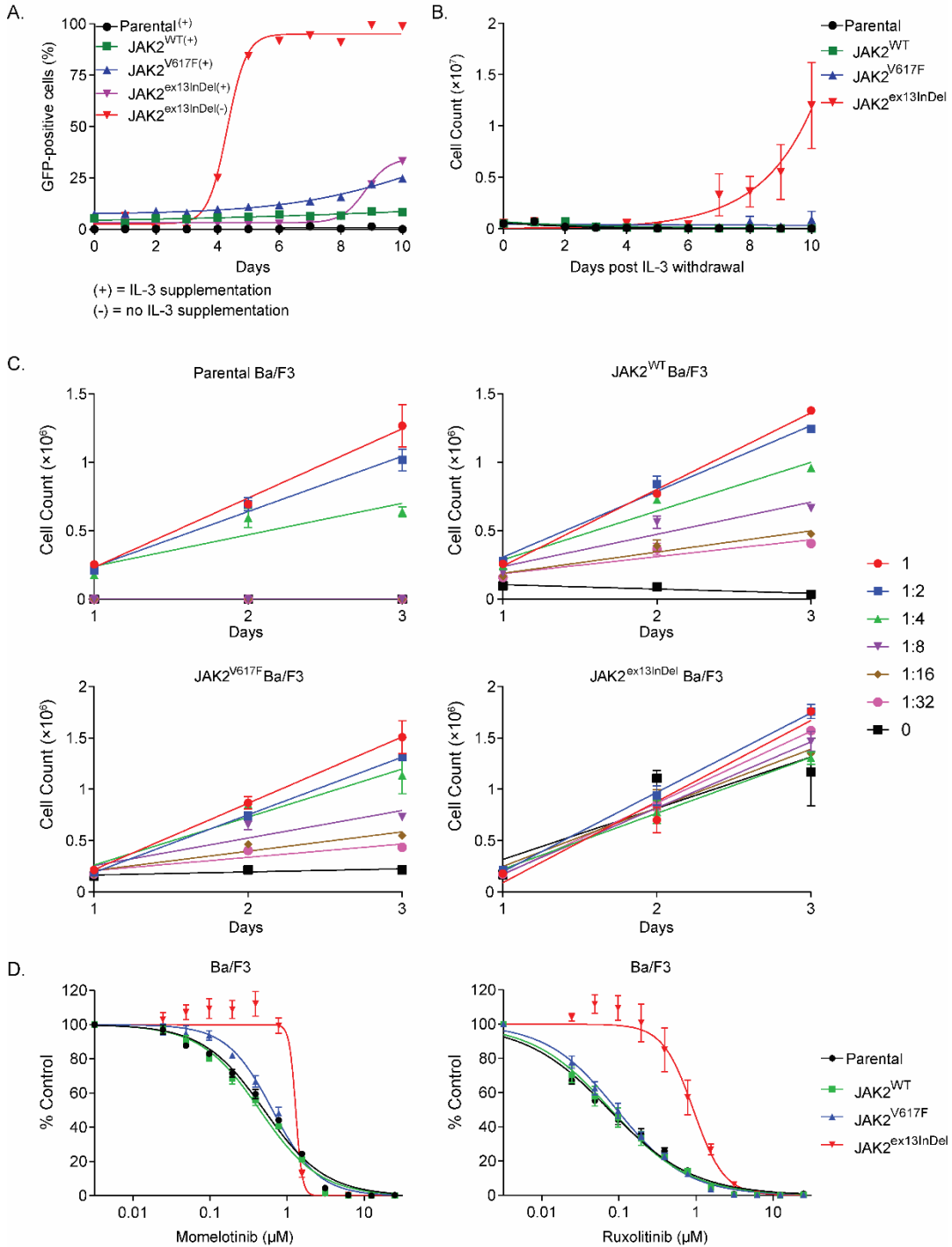

A.
GFP-positive cells (%)
Parental(+)
JAK2WT(+)
JAK2V617F(+)
JAK2ex13InDel(+)
JAK2ex13InDel(-)
Days
(+) = IL-3 supplementation
(-) = no IL-3 supplementation
B.
Cell Count (×10⁷)
Parental
JAK2WT
JAK2V617F
JAK2ex13InDel
Days post IL-3 withdrawal
C.
Parental Ba/F3
JAK2WT Ba/F3
JAK2V617F Ba/F3
JAK2ex13InDel Ba/F3
Cell Count (×10⁶)
Days
1
1:2
1:4
1:8
1:16
1:32
0
D.
Ba/F3
% Control
Momelotinib (μM)
Ruxolitinib (μM)
Parental
JAK2WT
JAK2V617F
JAK2ex13InDel

Figure 3.

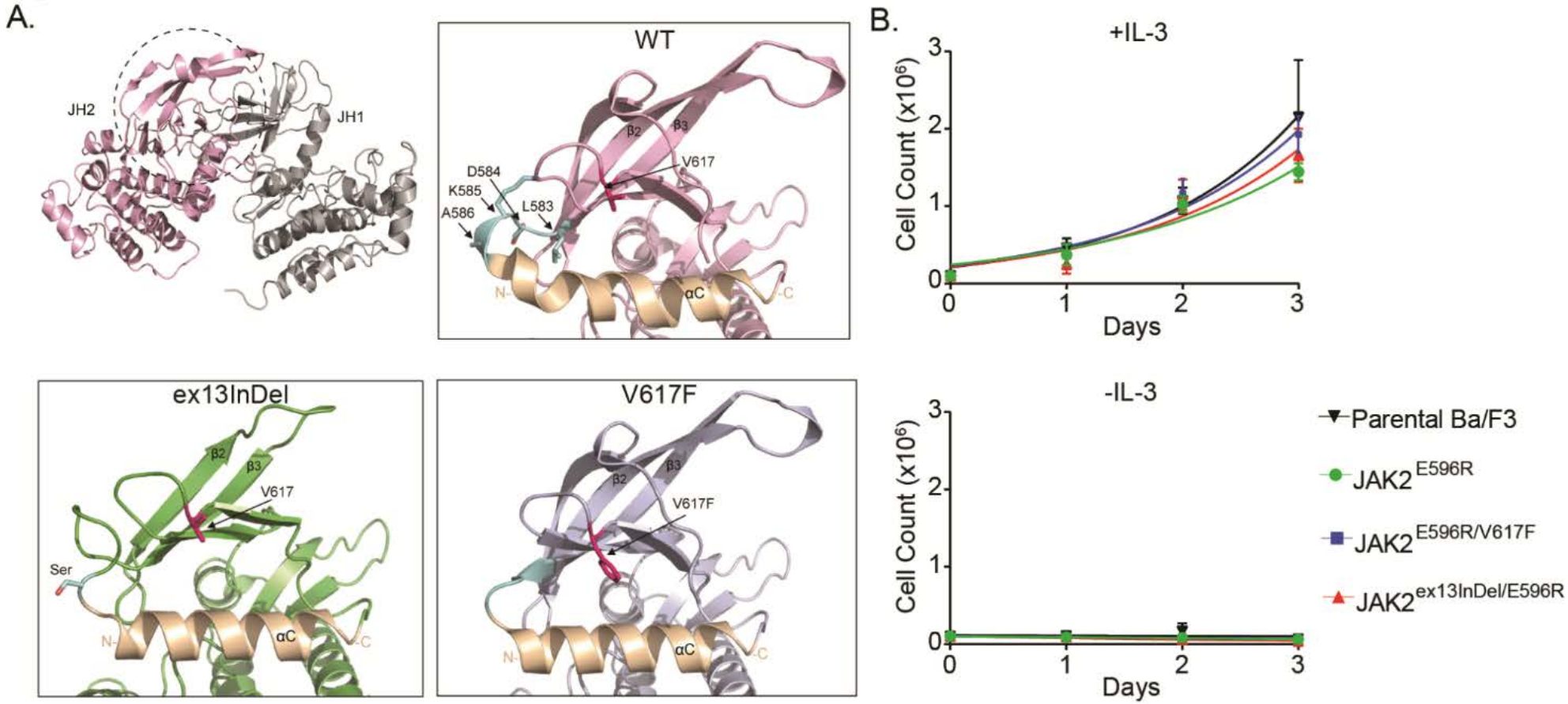

A.
JH2
JH1
WT
D584
K585
A586
L583
V617
β2
β3
αC
N-
-C
ex13InDel
Ser
V617F
B.
+IL-3
-IL-3
Cell Count (x10⁶)
Days
0
1
2
3
Parental Ba/F3
JAK2E596R
JAK2E596R/V617F
JAK2ex13InDel/E596R

Figure 4.

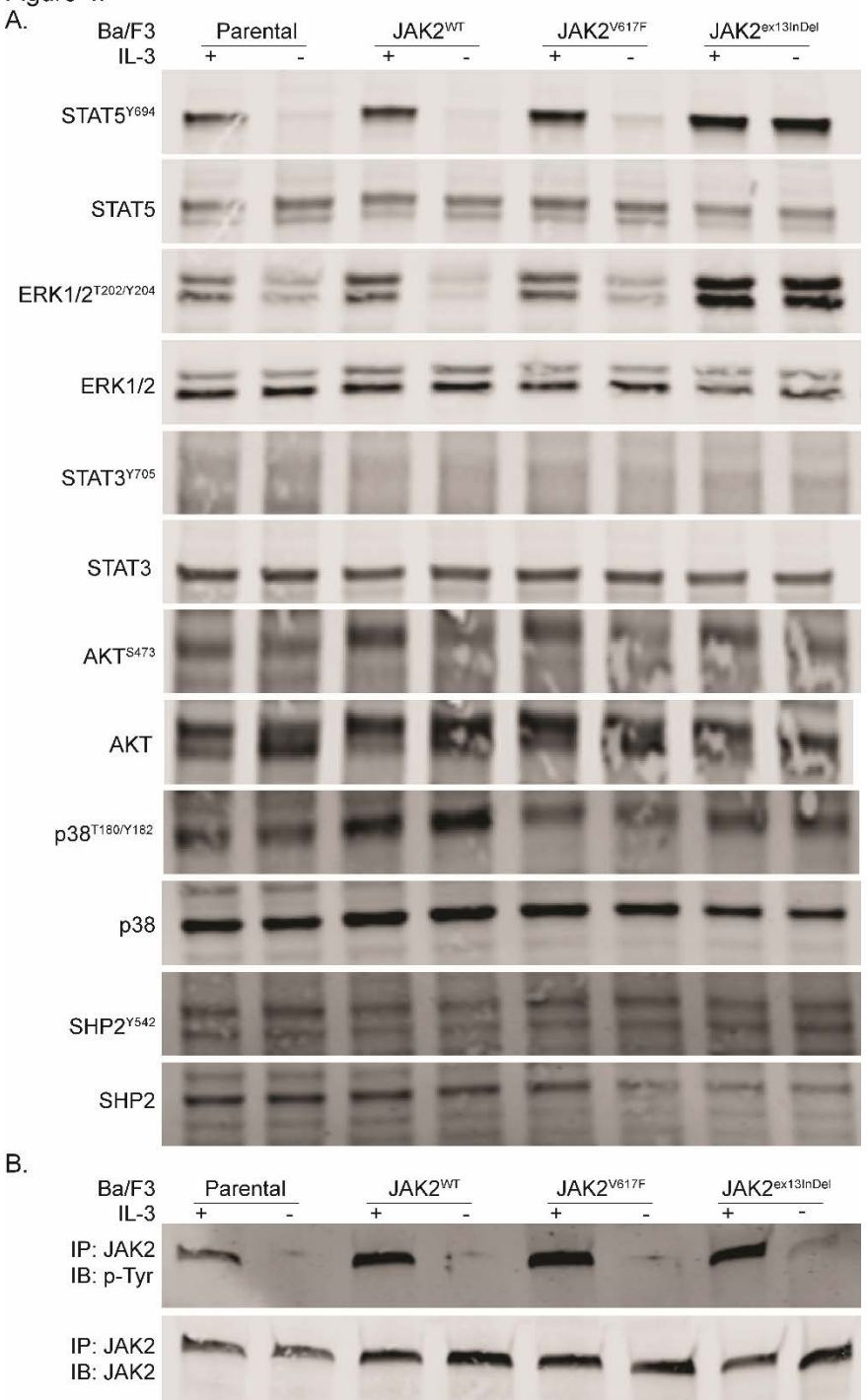
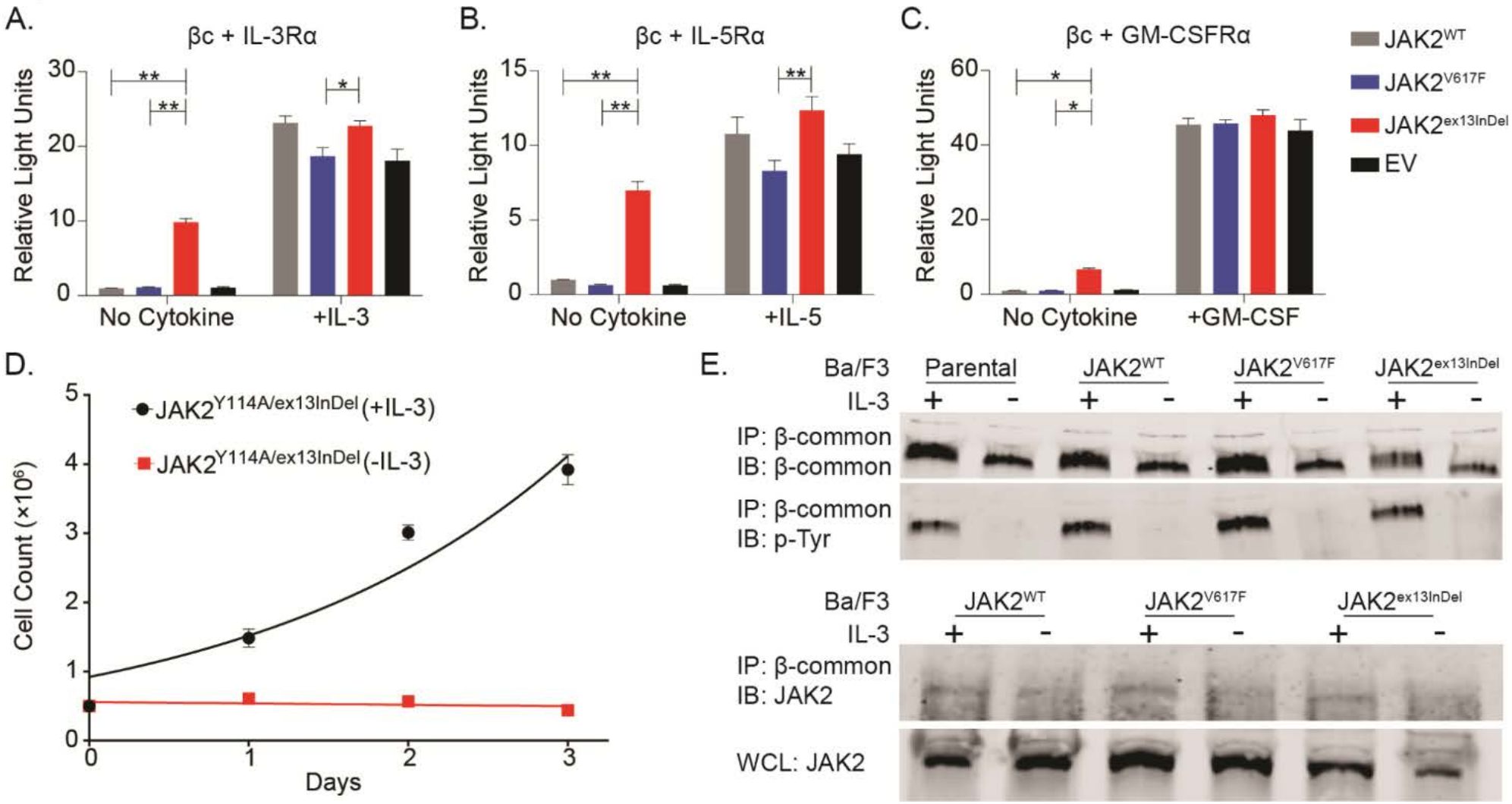

A.
Ba/F3
Parental
JAK2WT
JAK2V617F
JAK2ex13InDel
IL-3
+ - + - + - + -
STAT5Y694
STAT5
ERK1/2T202/Y204
ERK1/2
STAT3Y705
STAT3
AKTS473
AKT
p38T180/Y182
p38
SHP2Y542
SHP2
B.
Ba/F3
Parental
JAK2WT
JAK2V617F
JAK2ex13InDel
IL-3
+ - + - + - + -
IP: JAK2
IB: p-Tyr
IP: JAK2
IB: JAK2

Figure 5.


A.
βc + IL-3Rα
Relative Light Units
30
20
10
0
**
**
*
No Cytokine
+IL-3
B.
βc + IL-5Rα
Relative Light Units
15
10
5
0
**
**
**
No Cytokine
+IL-5
C.
βc + GM-CSFRα
Relative Light Units
60
40
20
0
*
*
No Cytokine
+GM-CSF
JAK2WT
JAK2V617F
JAK2ex13InDel
EV
D.
JAK2Y114A/ex13InDel (+IL-3)
JAK2Y114A/ex13InDel (-IL-3)
Cell Count (×10⁶)
5
4
3
2
1
0
0
1
2
3
Days
E.
Ba/F3
Parental
JAK2WT
JAK2V617F
JAK2ex13InDel
IL-3
+
-
+
-
+
-
+
-
IP: β-common
IB: β-common
IP: β-common
IB: p-Tyr
Ba/F3
JAK2WT
JAK2V617F
JAK2ex13InDel
IL-3
+
-
+
-
+
-
IP: β-common
IB: JAK2
WCL: JAK2