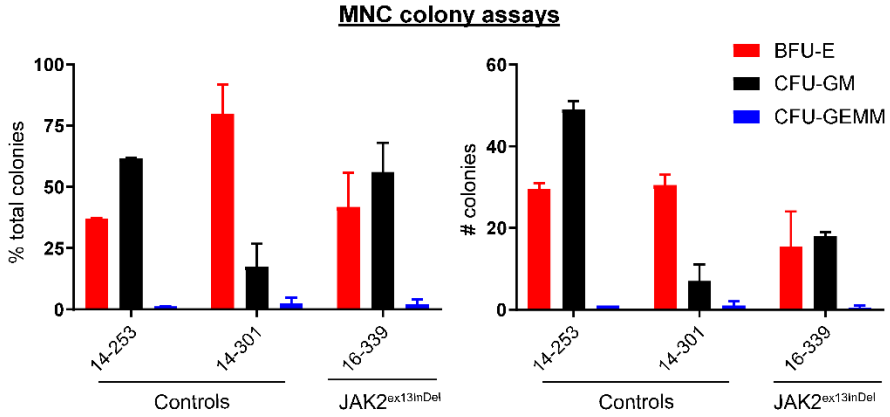
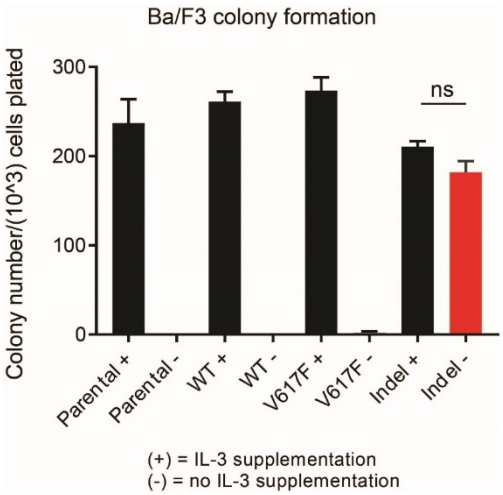
SUPPLEMENTARY INFORMATION

Results

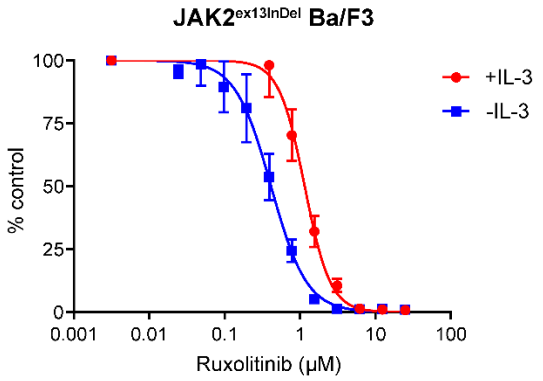
Supplemental Figure 1. **Patient-derived mononuclear cell colony assays**. Equal numbers of mononuclear cells derived from two healthy controls (samples 14-253 and 14-301) and our patient with JAK2^{ex13InDel} were plated in methylcellulose-based medium containing cytokines. Colonies were typed and counted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



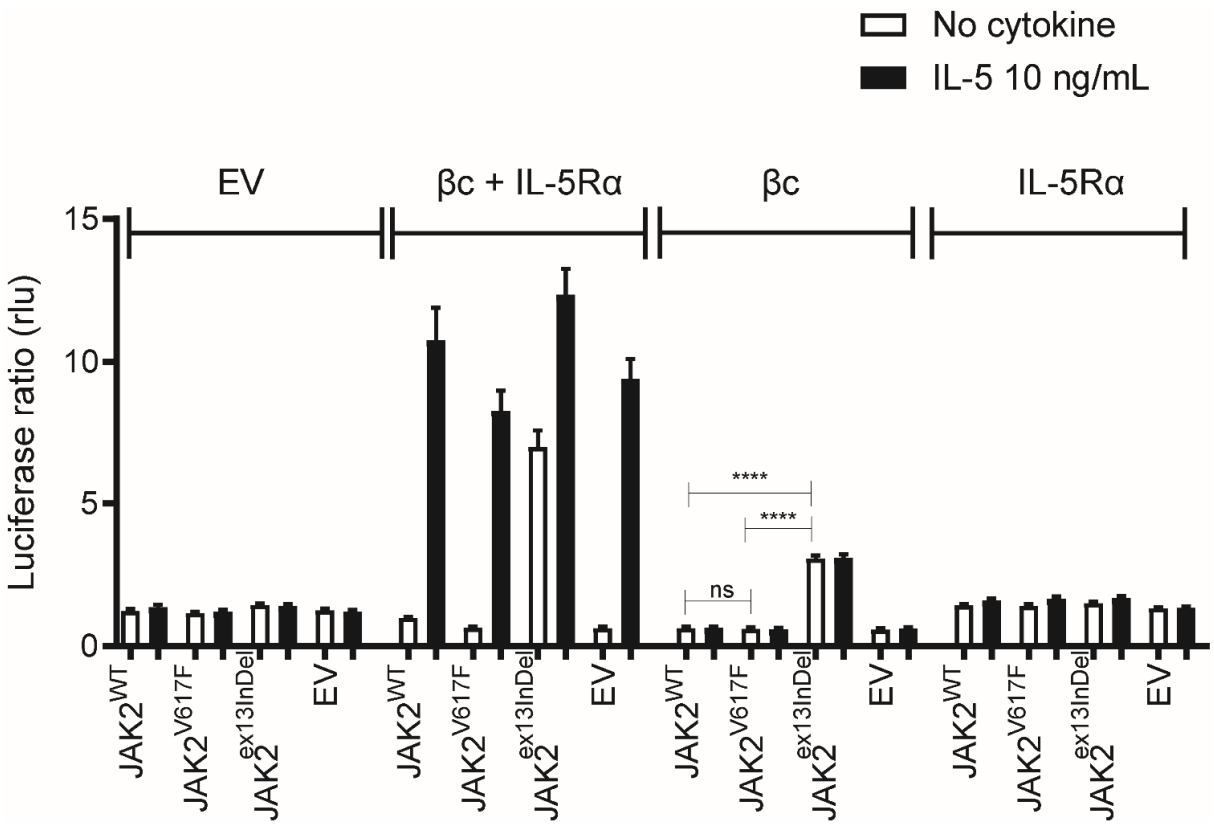
Supplemental Figure 2. Ba/F3 colony assays. Ba/F3 parental and mutant cells were plated in methylcellulose-based colony-forming assays +/- IL-3 supplementation. Colonies were counted at one week and normalized to initial cells plated.



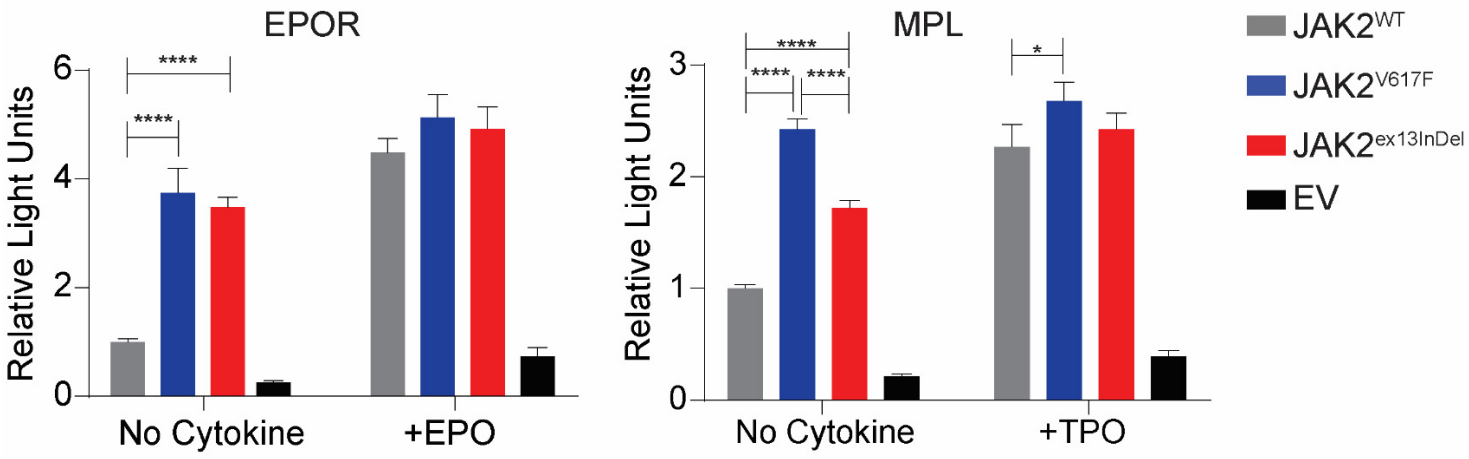
Supplemental Figure 3. **JAK2^{ex13InDel} cell proliferation studies ± IL-3**. JAK2^{ex13InDel} Ba/F3 cells were seeded at 2000 cells/well in 96-well plates in the presence of graded concentrations of ruxolitinib in medium with or without IL-3 supplementation. Cell proliferation was measured at 72 hours (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.



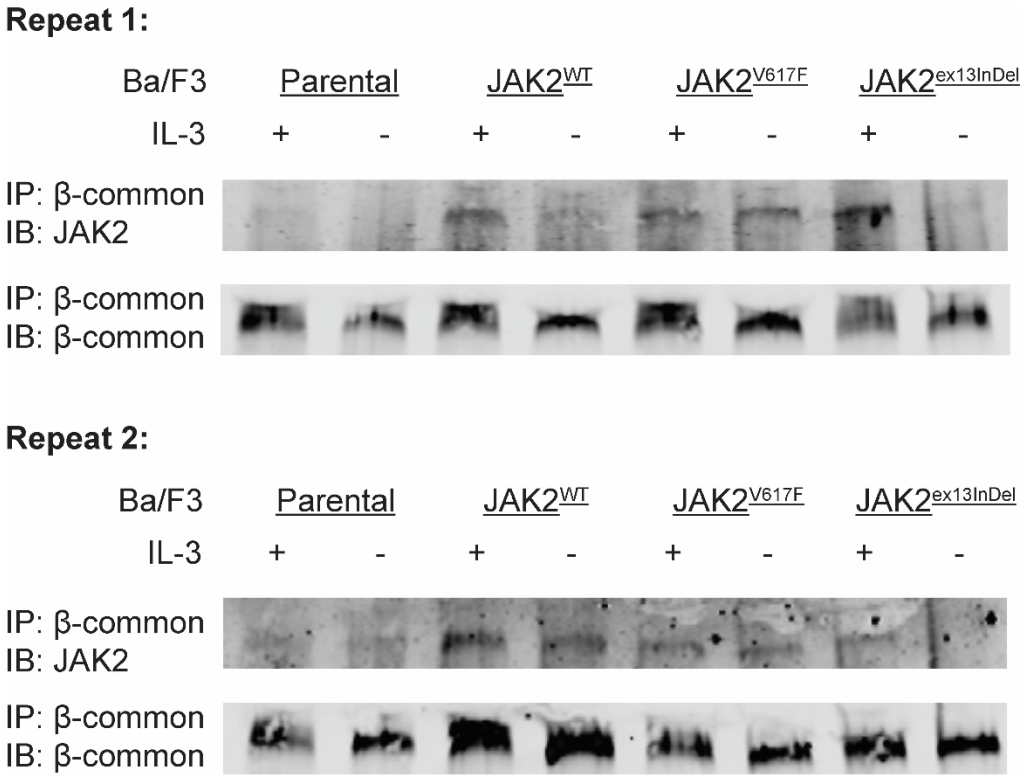
Supplemental Figure 4. STAT5 luciferase activity with components of the IL-5 receptor. Luciferase-based STAT5 transcriptional activity was measured ± IL-5 in the presence or absence of various components of the IL-5 receptor (no receptor components, βc chain + α chain, βc alone, or α chain alone). Only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



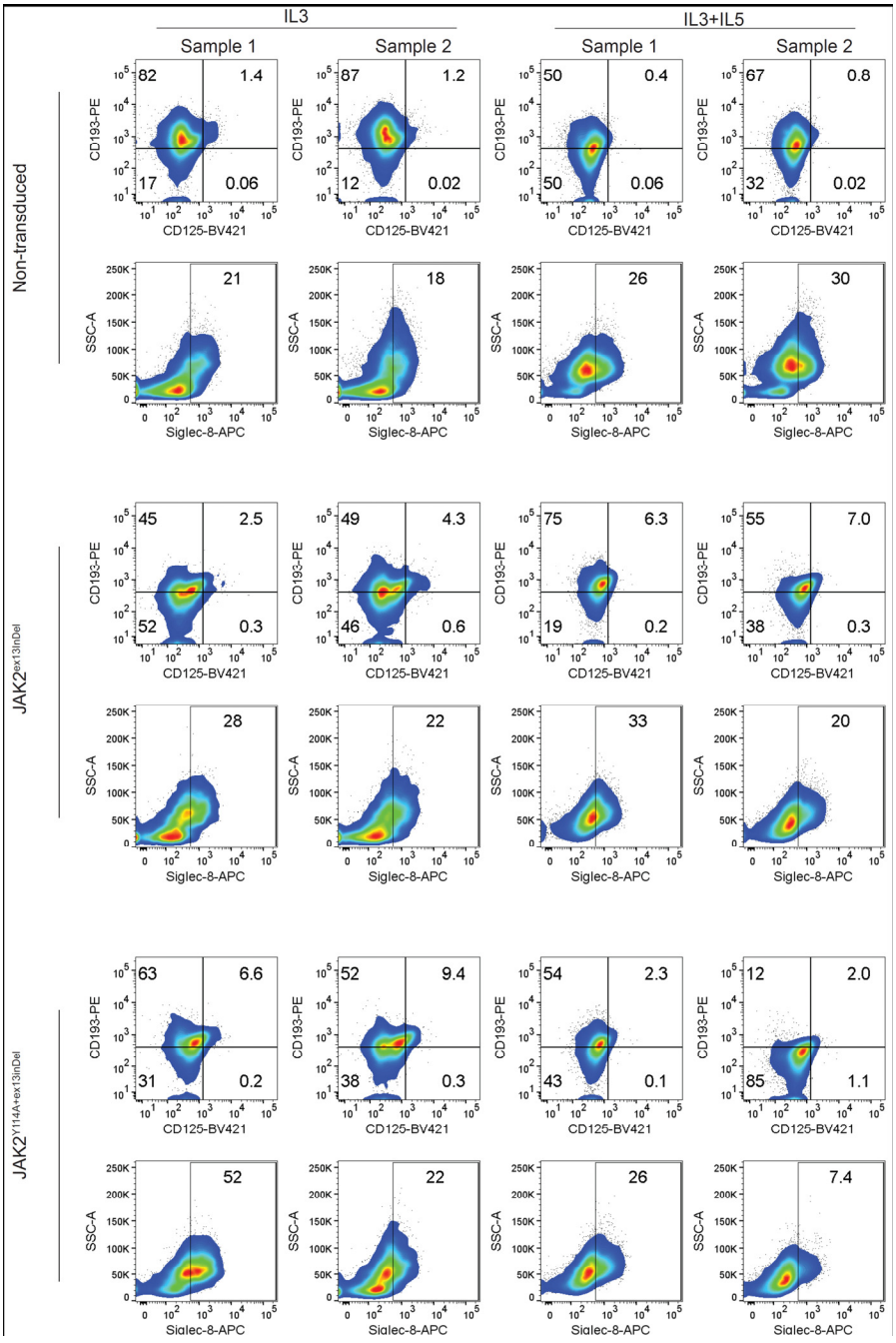
Supplemental Figure 5. Luciferase-based STAT5 transcriptional assays in HEK293 cells using homodimeric type I cytokine receptors. HEK293 cells were transfected with either EPOR or TPOR with a luciferase-based STAT5 reporter (Spi_Luc), and JAK2^{WT}, JAK2^{V617F} or JAK2^{ex13InDel}. STAT5 transcriptional 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^{+} cells were observed (not shown) and that the plots reflect the bulk cultures, which showed evidence for acquisition of eosinophilic differentiation markers, particularly Siglec-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 female 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

Fluorescence activated cell sorting of primary cells. Leukocytes from Patient 1 were incubated with fluorescent labeled antibodies [APC anti-human Siglec-8 (Biolegend, Cat #347106 clone 7C9) and PE/Cy7 anti-human CD16 (BD Pharmingen Cat# 557744 clone 3G8)], washed and sorted on a FACSARIA (BD Biosciences, San Jose, CA) as follows: eosinophils (CD16-, Siglec-8 +, high side scatter), neutrophils (CD16+, Siglec-8-, high side scatter, lymphocytes (CD16-, Siglec-8-, low side scatter).

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 Stbl3 E. coli (Invitrogen). 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 μm) 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]. Opti-MEM medium was removed 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-Rev: 5' TTCTGTGTGAAACTTTTAAAAGAACTTCTGTTTCATGCAGTTGA-3',

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

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

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

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

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

hJAK2-Y114A-Rev: 5'-GAGGAAAGTAAAATCTTATTCTTGCGAGTACATTATGCCTGGTTGACTCA-3'

hJAK2-Y114A-For: 5'-TGAGTCAACCAGGCATAATGTACTCGCAAGAATAAGATTTTACTTTCCTC-3'

hJAK2-BsiWI-Rev: 5'-CTCCTACTTCTCTTCGTACGCCTTTAAA-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'.

Gag-For, 5'-ATCCTCCCTTTATCCAGCCCTC-3'.

IRES-Rev, 5'-GCCCTCACATTGCCAAAAGACGG-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 2×250 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]-ACGGTCAACTGCATGAAACA

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

247

Supplemental Table 1. Percentage of Ba/F3 GFP-positive cells 48 hours following transduction.

Genotype / Experiment #	Experiment 1	Experiment 2	Experiment 3
Ba/F3 hJAK2 WT	1.38	2.82	4
Ba/F3 hJAK2 V617F	2.79	3	6
Ba/F3 hJAK2 ex13InDel	2.38	3.1	3

Supplemental Table 2. Complete blood count data for Patient 3.

Parameter / Date	April 2018	February 2018	January 2018
Hemoglobin (g/dL)	16	18.2*	18.4*
HCT %	50.1%*	55.1%*	56.7%*
Platelet count x 10^9/L	305	255	283
White cell count x 10^9/L	19.70*	22.58*	24.64*
Basophils x 10^9/L	0.06	0.07	0.12*
Eosinophils x 10^9/L	7.96*	14.27*	5.69*
Lymphocytes x 10^9/L	3.15	3.16	3.45
Monocytes x 10^9/L	0.59	0.61	0.71
Neutrophils x 10^9/L	7.94*	4.47	14.66*

(* indicates above normal range)