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Heterodimeric JAK-STAT Activation as a Mechanism of Persistence to JAK2 Inhibitor Therapy

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Abstract

The identification of somatic activating mutations in *JAK2*[1–4] and in the thrombopoietin receptor (*MPL*)[5] in the majority of myeloproliferative neoplasm (MPN) patients led to the clinical development of JAK2 kinase inhibitors[6,7]. JAK2 inhibitor therapy improves MPN-associated splenomegaly and systemic symptoms, but does not significantly reduce or eliminate the MPN clone in most MPN patients. We therefore sought to characterize mechanisms by which MPN cells persist despite chronic JAK2 inhibition. Here we show that JAK2 inhibitor persistence is

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Author contributions PK and RLL conceived the project. PK, NB, OK and RLL designed experiments. PK, NB, OK, TM, MA, FL, OAW, LL, AW, SM and AG performed experiments. PK, NB, TH, MG and MA analyzed data. LMS, AM, BLE and GC provided reagents. ZE and SV provided patient samples. PK, NB and RLL wrote the paper with input from SV, ZE, OK, BLE, BEB and SDN.

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associated with reactivation of JAK-STAT signaling and with heterodimerization between activated JAK2 and JAK1/TYK2, consistent with activation of JAK2 in *trans* by other JAK kinases. Further, this phenomenon is reversible, such that JAK2 inhibitor withdrawal is associated with resensitization to JAK2 kinase inhibitors and with reversible changes in JAK2 expression. We saw increased JAK2 heterodimerization and sustained JAK2 activation in cell lines, murine models, and patients treated with JAK2 inhibitors. RNA interference and pharmacologic studies demonstrate that JAK2 inhibitor persistent cells remain dependent on JAK2 protein expression. Consequently, therapies that result in JAK2 degradation retain efficacy in persistent cells and may provide additional benefit to patients with JAK2-dependent malignancies treated with JAK2 inhibitors.

Keywords

tyrosine kinase inhibitors; JAK2; MPN; resistance; persistence

The development of targeted therapies has improved outcomes for patients with kinase-mutant malignancies[8–11] however acquired resistance due to mutations in the target kinase[12–14] or in other pathways that render cancer cells insensitive to kinase inhibitor therapy[15–18] remain important clinical concerns. Although JAK inhibitors are now being used to treat MPN patients, to date JAK inhibitor treatment has not been associated with significant reductions in disease burden in most MPN patients[6,7]. To understand mechanisms by which MPN cells survive despite chronic JAK kinase inhibition, we performed saturation mutagenesis[19] and next-generation sequencing in cells exposed to two structurally different JAK2 inhibitors, INCB18424 and JAK Inhibitor I. We identified second-site mutations in <30–50% of cells exposed to JAK2 inhibitors (Supplementary Table 1). Full length resequencing of clones proliferating in the presence of INCB18424 or JAK Inhibitor I confirmed the absence of second-site *JAK2* mutations in the majority of surviving clones, and we did not identify second-site *JAK2* mutations in granulocytes from 5 MPN patients treated with INCB18424. By contrast, control experiments with mutagenized BCR-Abl cells exposed to imatinib identified >20 known, clinically relevant imatinib resistance alleles[19,20] (data not shown).

These data and clinical experiences to date suggest that the failure of JAK2 inhibitors to reduce disease burden is not due to acquired drug resistance but rather due to persistent growth and signaling in the setting of chronic JAK2 kinase inhibition. We therefore investigated the basis by which JAK2-dependent cells persist despite chronic JAK2 kinase inhibition. We cultured SET-2/UKE-1 (*JAK2V617F* positive leukemia) cells and Ba/F3 cells expressing JAK2V617F (EporVF) or MPLW515L (WL) cells with INCB18424 or JAK inhibitor I for 4–6 weeks. In each case, we found that JAK2/MPL-mutant cells could survive and proliferate at inhibitor concentrations sufficient to prevent growth of parental cells (Figure 1a **and** Supplementary Figures 1a **and** 2a). JAK2 inhibitor persistent ($JAK2^{Per}$) $JAK2^{Per}$ cells were resistant to INCB18424-induced apoptosis (Supplementary Figure 3). *JAK2* resequencing confirmed the absence of second-site mutations in all $JAK2^{Per}$ cell lines. $JAK2^{Per}$ cells were also insensitive to structurally divergent JAK inhibitors, including TG101348, a JAK2-selective inhibitor in late-stage clinical trials (Figure 1b **and**

Supplementary Figures 1b, 1c, 2b **and** 4). These data indicate that JAK2^{Per} cells are insensitive to different JAK inhibitors regardless of prior exposure to that inhibitor.

These data are consistent either with selection of a subpopulation of pre-existing, persistent cells, as previously posited for EGFR inhibitor-insensitive "drug-tolerant persisters" (DTPs)[21], or with acquisition of persistence by naïve, inhibitor-sensitive cells. To distinguish between these possibilities, we derived single cell clones of inhibitor naïve JAK2/MPL mutant cell lines. Each clonally derived naïve cell line was sensitive to JAK inhibitors and retained the capacity to become persistent over time to different JAK inhibitors (Supplementary Figure 5 **and data not shown**). These data depict a general capacity for persistence in the absence of clonal selection.

Next, we assessed signaling downstream of JAK2 in JAK2^{Per} cells. We observed dose-dependent inhibition of downstream signaling in naïve cells treated with INCB18424 or JAK Inhibitor I, but not in INCB18424^{Per} (Figure 2a **and** Supplementary Figure 6a) or JAK Inhibitor I^{Per} cells (Supplementary Figure 6b). Similarly, *ex vivo* treatment of granulocytes from chronically treated INCB18424 patients demonstrated sustained downstream signaling at inhibitor concentrations that inhibited signaling in naive MPN patient samples (Figure 2b). We then asked whether persistence was associated with constitutive JAK2 activation. We observed persistent phosphorylation of JAK2 in JAK2^{Per} cells (Supplementary Figures 2c **and** 6c). Further, gene expression analysis showed that expression of known JAK-STAT target genes were maintained in JAK^{Per} cells, whereas these genes were suppressed with acute treatment of inhibitor naïve, parental cells (Supplementary Figure 7).

Given that JAK inhibitors should inhibit JAK2 autophosphorylation, we reasoned that other kinases might associate with and phosphorylate JAK2 in persistent cells. Although EpoR and MPL predominantly signal through JAK2,[22] previous studies have shown that many cytokine receptors signal through JAK kinase heterodimers[23]. We therefore assessed the activation status of JAK1, JAK3, and TYK2 in naïve and persistent SET-2 and WL cells. We observed increased phosphorylation of JAK1 in JAK2^{Per} cells compared to parental cells while TYK2 was constitutively phosphorylated in both parental and JAK2^{Per} cells (Figure 2c). Accordingly, immunoprecipitation studies demonstrated that JAK1 and TYK2 associated with phosphoJAK2 in JAK2^{Per} SET-2, WL (Figure 2d) and UKE-1 (Supplementary Figure 2d) cells, but not in the respective parental cells. Most importantly, we saw similar association between phosphoJAK2 and JAK1/TYK2 in INCB18424 treated patient samples but not in inhibitor naïve patient samples (Figure 2e **and** Supplementary Table 2).

Next, we asked whether the JAK^{Per} cells were insensitive to JAK inhibitors. *In vitro* kinase assays revealed that the JAK^{Per} heterodimer complex could phosphorylate MBP at concentrations of INCB18424 sufficient to inhibit JAK2 kinase activity in naïve SET-2 cells (Supplementary Figure 8). These data suggest that the heterodimer complex in JAK^{Per} cells retains kinase activity that is relatively insensitive to JAK inhibitors. To determine if JAK1-mediated phosphorylation of JAK2 was insensitive to INCB18424, we co-expressed a constitutively active mutant form of JAK1 (JAK1V658F)[24] with kinase-dead JAK2 (JAK2K882E) in JAK2-deficient γ2A cells. We observed persistent JAK2 phosphorylation

in JAK1V658F/JAK2K882E γ2A cells exposed to INCB18424 at concentrations sufficient to inhibit JAK2 autophosphorylation (Supplementary Figure 9).

We then investigated whether JAK2 inhibitor persistence was reversible. We removed INCB18424 or JAK inhibitor I for 2–4 weeks which led to JAK inhibitor resensitization (Figure 3a **and** Supplementary Figures 10a **and** 10b). Resensitized ($JAK2^{Resens}$) cells were sensitive to all 3 JAK inhibitors, suggesting MPN patients may respond to retreatment or to a different JAK2 inhibitor after brief treatment withdrawal. JAK1/TYK2 association with phosphoJAK2 was lost in $JAK2^{Resens}$ cells (Figure 3b **and** Supplementary Figure 10c), and activated JAK2 levels were lower in $JAK2^{Resens}$ cells (Supplementary Figure 10d).

Previous work attributed persistence in EGFR inhibitor-insensitive DTPs[21] to engagement of alternate survival pathways. By contrast, JAK^{Per} cells were characterized by JAK-STAT pathway reactivation (Figure 2). We therefore hypothesized that changes in the epigenetic regulation of JAK2 might contribute to JAK inhibitor persistence. *JAK2* mRNA (Supplementary Figure 11**)** and JAK2 protein (Figure 3c **and** Supplementary Figures 2e **and** 10e) levels were higher in $JAK2^{Per}$ cells compared to parental cells, and were reduced in $JAK2^{Resens}$ cells. ChIP-Seq analysis of naïve *JAK2*-mutant SET-2 cells (M.A., O.A.W., B.E.B., R.L.L., unpublished data) revealed that the *JAK2* locus is characterized by H3K4-trimethylation, a histone modification associated with active promoters, and by H3K9-trimethylation, a mark more typically associated with inactive heterochromatin (Supplementary Figure 12a **and** Supplementary Table 3). ChIP-PCR analysis of the *JAK2* locus demonstrated a significant increase in H3K4me3 and a reduction in H3K9me3 in $JAK2^{Per}$ cells compared to parental cells (Figure 3d) consistent with a change to a more active chromatin state at the *JAK2* locus. Global H3K4me3 levels in naïve and persistent cells, however, remained unchanged consistent with specific effects on H3K4me3 at the *JAK2* locus in persistent cells (Supplementary Figure 12b).

Given that JAK2 protein levels and particularly phosphoJAK2 levels increased with persistence, we asked whether JAK2 inhibitor persistence was also associated with post-transcriptional stabilization of total/activated JAK2. We have previously shown that JAK2 levels rapidly decline with cycloheximide treatment in JAK2-mutant cells[25]. We noted a time-dependent decrease in phosphoJAK2 and total JAK2 levels in naïve and resensitized WL/SET-2 cells, however cycloheximide exposure did not result in a significant decline in JAK2, or more notably in phosphoJAK2, in IN^{Per} cells (Figure 3e **and** Supplementary Figure 13). These data suggest that chronic inhibitor treatment results in stabilization of activated JAK2, which combined with increased *JAK2* mRNA expression facilitates the formation of heterodimers.

We then assessed whether this phenomenon was observed *in vivo*. We treated mice engrafted with MPLW515L-mutant murine bone marrow[26] with vehicle or with INCB18424. INCB18424 treatment was associated with reduced splenomegaly; however the proportion of malignant cells was not reduced with JAK inhibitor treatment as previously described (Supplementary Figure 14a)[26]. INCB18424 treatment was associated with an increase in *JAK2* mRNA and JAK2 protein expression (Supplementary Figure 14b**)** similar to that observed in $JAK2^{Per}$ cells. We also observed an increase in *JAK2* granulocyte mRNA

levels in INCB18424 treated patients without clinical/molecular responses, compared to patients with clinical/molecular responses to INCB18424 (p=0.05) (Figure 3f **and** Supplementary Table 2). Finally, consonant with the expression data, we noted increased JAK2 phosphorylation and increased association between JAK1 and JAK2 in hematopoietic cells from INCB18424 treated PMF mice (Supplementary Figures 14c and 14d).

We asked whether JAK2^{Per} cells remain JAK2 dependent. JAK2 silencing inhibited proliferation (Figure 4a), JAK2 activation, and downstream signaling (Figure 4b) in naive and JAK2^{Per} SET-2 cells consistent with a requirement for JAK2 expression in JAK2^{Per} cells. These data are consistent with previous studies in prolactin receptor cellular systems demonstrating that catalytically inactive JAK2 can serve as a scaffold for transactivation and downstream signaling[27]. However this had not previously been implicated in JAK-mutant/dependent malignancies or in the response to JAK kinase inhibitors. Knockdown of JAK1/TYK2 increased sensitivity of SET-2 IN^{Per} and SET-2 J^{Per} cells to INCB18424 and JAK Inhibitor I, respectively (Figure 4c **and** Supplementary Figures 15a, 15b and 15c), while the parental cells remained unaffected by JAK1/TYK2 knockdown (Supplementary Figure 15d). Further, JAK1/TYK2 knockdown led to decreased downstream signaling and reduced JAK2 phosphorylation in the persistent cells **(**Supplementary Figures 15e and 15f).

We next assessed whether novel therapeutic approaches might reverse JAK inhibitor persistence. We previously reported HSP90 inhibitors increase JAK2 degradation *in vitro* and *in vivo*.[25] JAK2^{Per} and parental cells were equally sensitive to HSP90 inhibition by PU-H71, (Figure 4d and Supplementary Figure 16a) and PU-H71 treatment led to JAK2 degradation and inhibited signaling in JAK2^{Per} cells (Figure 4e). Currently available, type I JAK inhibitors are conformation dependent and can only engage activated JAK2.[28] We therefore tested the effects of BBT-594, a Type II inhibitor which retains the ability to bind inactive JAK2,[28] in JAK2^{Per} cells. BBT-594 inhibited the proliferation, JAK activation, and signaling of naïve and JAK^{Per} cells to a similar extent (Figure 4f and Supplementary Figures 16b and 16c).

Taken together, our results suggest that kinase inhibitor persistence can occur through reversible changes in JAK2 expression and transphosphorylation (Supplementary Figure 17). We show that persistent JAK2 activation in the setting of JAK inhibitor exposure allows cells to survive without reducing dependence on JAK2 expression. Consequently, treatments which lead to JAK2 degradation (HSP90 inhibitors or HDAC inhibitors)[29,30] or which retain the ability to inhibit JAK2 in persistent cells have the potential to improve therapeutic efficacy in MPN patients.

Methods summary

Generation of JAK2 Inhibitor-persistent Cells

Cells were cultured continuously in increasing concentrations of INCB18424 or JAK Inhibitor I for 4–6 weeks. Cells were considered resistant when the IC_{50} values of the persistent derivatives was at least twice the IC_{50} of parental cells (verified by *in vitro* inhibitor assays). Persistent cells were cultured continuously in the presence of the JAK2

inhibitor. For resensitization experiments, inhibitor was withdrawn from the media and cells were cultured in the absence of the drug for 2–4 weeks.

Knockdown of JAK2 and TYK2 in Human Cell Lines

shRNA for JAK2 was either purchased from the High Throughput Drug Screening Facility at Memorial Sloan-Kettering Cancer Center, or were a kind gift from Dr. Louis Staudt. shRNA against TYK2 was a kind gift from Dr. Thomas Look. Whenever required, shRNA oligonucleotides were cloned into pLKO lentiviral systems. Cell lines were transfected with lentivirus, and selected using puromycin. siRNA targeting either JAK1 or TYK2 were purchased from Invitrogen, and used according to manufacturer's instructions.

Murine model and Analysis of Mice

The MPLW515L murine BMT assay was performed as described previously[5]. Sick mice were randomized to receive INCB18424 twice daily at 60 and 90 mg/kg or vehicle (0.5% methylcellulose) by oral gavage. Mice were treated for 28 days or until any one of several criteria for sacrifice were met, including moribundity, >10% body weight loss, and palpable splenomegaly extending across the midline. Animal care was in strict compliance with Memorial Sloan-Kettering Cancer Center guidelines. Bone marrow and spleen cells were strained and viably frozen in 90% FCS and 10% DMSO.

Additional references, methods and information are in the attached supplement.

Full Methods

Reagents and cell lines

The pan JAK inhibitor, JAK Inhibitor I, was purchased from Calbiochem (Cat. No. 420097). The JAK1/2 specific inhibitor, INCB18424 was purchased from Chemietek (Indianapolis, IN, USA). PU-H71 8-(6-iodobenzo[d][1.3]dioxol-5-ylthio)-9-(3-(isopropyl amino)propyl)-9H-purine-6-amine was synthesized as previously reported[31]. BBT-594 was a kind gift from Dr. Thomas Radimerski. 1mM stock aliquots were prepared in DMSO and diluted in appropriate media prior to use. Antibodies used for Western blotting and immunoprecipitation included phosphorylated and total JAK2, STAT3, MAPK, AKT and phosphoSTAT5 (Cell Signaling Technologies, Beverly, MA, USA), Total STAT5 antibody was purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Actin from EMD Chemicals (Darmstadt, Germany). JAK1 and TYK2 antibodies were purchased from BD Transduction, San Diego; CA. Pan phospho-tyrosine antibody was purchased from Millipore, MA, USA. The generation and maintenance of Ba/F3 hMPLW515L and Ba/F3 EpoR-V617F cells has been described previously[5]. The *JAK2V617F* positive human leukemic cell line SET-2 was grown in RPMI-1640 with 20% heat-inactivated serum; whereas, UKE-1 (also *JAK2V617F* positive) cells were grown in RPMI-1640 with 10% fetal calf serum, 10% horse serum, and 1μM hydrocortisone (Sigma, Cat. No. H6909). Cycloheximide was purchased from Sigma.

Knockdown of JAK1, JAK2 and TYK2 using siRNA or shRNA

siRNA against JAK1 and TYK2 were purchased from Invitrogen, and used according to the manufacturer's instructions. The two siRNA oligonucleotides used for JAK1 knockdown were GCACAGAAGACGGAGGAAAUGGUAU (JAK1VHS41387) and GCCUUAAGGAAUAUCUUCCAAAGAA (JAK1VHS41388). siRNA sequence for TYK2 included a combination of two oligonucleotides (TYK2VHS41729 UUCUCAUGGACUGUCUUCAGAAUGG) and TYK2VHS41246 (GCAGCAAGUAUGAUGAGCAAGCUUU). Scrambled siRNA was purchased from Dharmacon (D-001206-13-20). Cells were transfected with scrambled siRNA, siJAK1, siTYK2 or both. Viability assays were set up 24 hours post transfection and harvested after 48 hours. Cells were harvested after 72 hours post transfection to verify knockdown and assess downstream signaling. Persistent cells were cultured in the presence of inhibitor during the entire experiment. shRNA against JAK2 and TYK2 were kind gifts from Dr. Louis Staudt and Dr. Thomas Look respectively. shRNA target sequences used for knockdown of JAK2 were shRNA #1 CTCTTCGAGTGGATCAAATAA and shRNA #2 GCAGAATTAGCAAACCTTATA. The target sequence for shRNA against TYK2 was CGTGAGCCTAACCATGATCTT. Lentiviral particles were generated using standard procedures. Cells were spinfected with virus and selected using puromycin. Cell viability was monitored using trypan blue (for JAK2 knockdown studies), and cells were harvested 10 days after selection in puromycin. $JAK2^{Per}$ cells were cultured in the presence of respective inhibitors during the entire experiment.

In Vitro Inhibitor Assays, Western Blot Analysis and Immunoprecipitations

Viable cells were plated at 10,000 cells/well in 96 well tissue culture treated plates in 200μL media with increasing concentrations of the JAK2 inhibitor or PU-H71 in triplicate. 48 hour inhibitor assays were assessed using the Cell viability luminescence assay (CellTiter-Glo®, Promega, Cat. No. G7571). Results were normalized to growth of cells in media containing an equivalent volume of DMSO. The effective concentration at which 50% inhibition in proliferation occurred was determined using Graph Pad Prism 5.0 software.

For Western blot analysis, cells were harvested after treatment, and processed as described previously[26]. For immunoprecipitation experiments, cells were harvested either at steady state conditions or after 4h incubation with a JAK2 inhibitor. Protein was normalized using the Bradford dye, and 500–1000 μg of total protein was incubated the appropriate antibody overnight, followed by incubation with protein-G agarose beads (EMD Chemicals) for a further 2 hours. Post incubation, cells were washed thrice with cold PBS and boiled with Laemmli buffer for 12 minutes. Supernatant was loaded onto gels and separated as previously described[26].

Quantitative RT-PCR Analyses

Total RNA was extracted using using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the Verso cDNA Kit (Thermo Scientific). Quantitative PCR was performed using FastStart Universal SYBR Green Master (Roche) with the following primer sequences:

mouse JAK2 F: GATGGCGGTGTTAGACATGA,

mouse JAK2 R: TGCTGAATGAATCTGCGAAA,

mouse β actin F: GATCTGGCACCACACCTTCT,

mouse β actin R: CCATCACAATGCCTGTGGTA,

human JAK2 F: TCTTTCTTTGAAGCAGCAAG,

human JAK2 R: CCATGCCAACTGTTTAGCAA,

human HPRT1 F: AGATGGTCAAGGTCGCAAG,

human HPRT1 R: GTATTCATTATAGTCAAGGGCATATC.

Chromatin Immunoprecipitation (ChIP) assay

We performed ChIP-qPCR and ChIP-Seq analysis in SET2 naïve and JAK2-inhibitor persistent cells using a previously described ChIP method[32]. Briefly, chromatin from fixed cells was fragmented to a size range of 200–700 bases with a Branson 250 Sonifier. Solubilized chromatin was immunoprecipitated with antibody against H3K4me3 (Abcam 8580), H3K9me3 (Abcam 8898) and H3K27me3 (Upstate 07-449). Each of these antibodies was validated by Western blots and peptide competitions as previously described[32]. Antibody–chromatin complexes were pulled-down using protein A-Sepharose, washed and then eluted. After cross-link reversal and Proteinase K treatment, immunoprecipitated DNA was extracted with phenol-chloroform, ethanol precipitated, and treated with RNase. ChIP DNA was quantified using PicoGreen. For ChIP-qPCR, primer sequences for qPCR tiling primers across the JAK2 promoter region are listed in Supplementary Table 3. qPCR was performed on ABI-7500 instrument. For ChIP-Seq in native SET2 cells, ChIP DNA and input controls were sequenced using the Illumina Genome Analyzer.

In vitro kinase assays

Protein was harvested from naïve and IN^{Per} SET-2 cells and used for *in vitro* kinase assays. Endogenous JAK2 protein was precipitated with anti-JAK2 antibody (Santa Cruz, Cat. No. sc-34480) and Protein G Sepharose gel. For JAK2 activity assay, the immunoprecipitated JAK2 was incubated with Myelin Basic Protein (MBP) in a buffer containing 25 mM Tris-HCl (pH7.5), 10mM $MgCl_2$, 5 μM ATP and 2 mM DTT. The reaction was incubated at room temperature with 1 and 10 nM INCB18424 for an hour and stopped by adding the SDS sample loading buffer. Samples were run in reducing conditions on SDS-PAGE gels and immunoblotted using a pan phospho tyrosine antibody (Millipore).

Patient samples

The Institutional Review Boards of Memorial Sloan Kettering Cancer Center and MD Anderson Cancer Center approved sample collection and all experiments. Informed consent was obtained from all human subjects prior to study. Granulocytes were extracted using standard procedures from patient samples and viably frozen prior to use.

Gene expression analyses

Ba/F3 WL cells were treated with either DMSO or 0.8 μM INCB18424 for 4 hours in triplicates. IN^{Per} WL cells were also treated for 0.8 μM INCB18424 for 4 hours in triplicates after which cells were harvested in Trizol. RNA was extracted from the cells and analyzed for gene expression using Affymetrix microarray version MOE 430 2.0. Data was analyzed using the Partek GS Version 6.5 software. Data has been deposited in the Gene Expression Omnibus under accession number GSE38335.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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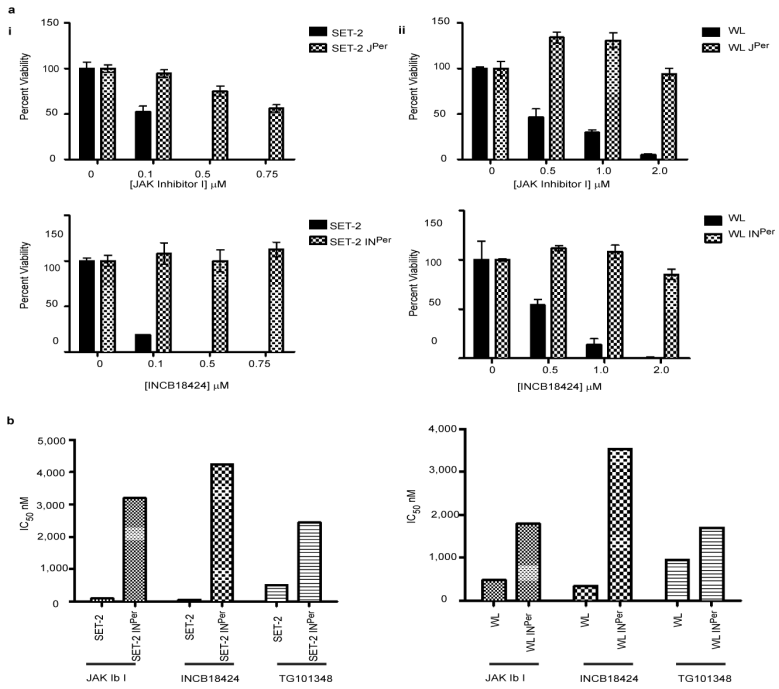

Figure 1. Generation of JAK2 inhibitor-persistent cells

a) Proliferation of naïve and persistent SET-2 (i) and WL (ii) cells with JAK2 inhibitors. Data are from wells plated in triplicate (S.D.), and are representative of 3 independent experiments. b) IC_{50} values of SET-2 IN^{Per} and WL IN^{Per} cells exposed to INCB18424, TG101348, and JAK Inhibitor I.

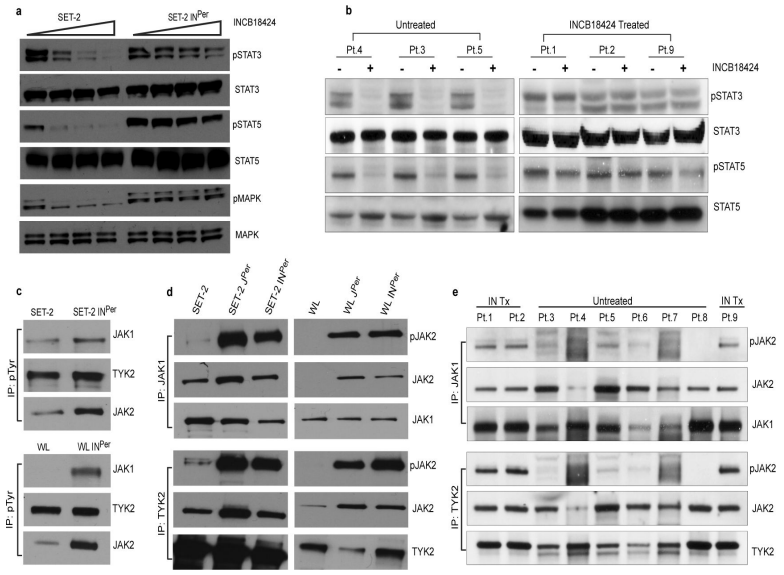

Figure 2. Inhibitor-persistent cells and INCB18424 treated patient granulocytes show continual JAK-STAT signaling and JAK2 activation via transphosphorylation by JAK1/TYK2

a) SET-2 and SET-2 IN^{Per} cells were washed and incubated with increasing concentrations of INCB18424 for 4 hours and western blotted. b) Granulocytes from naïve and INCB18424-treated patients were incubated *ex vivo* with DMSO or 150 nM of INCB18424 for 6 hours and western blotted. c) Increased phosphorylation of JAK1 in persistent cells and constitutive TYK2 phosphorylation in both naïve and persistent cells. d) Increased association between phosphoJAK2 and both TYK2/JAK1 in SET-2 JAK^{Per} cells and increased association between JAK2 and both TYK2/JAK1 in WL JAK^{Per} cells. e) JAK1/TYK2 association with phosphoJAK2 in granulocytes from 3 INCB18424 treated patients, which is not observed in INCB18424 naïve MPN samples.

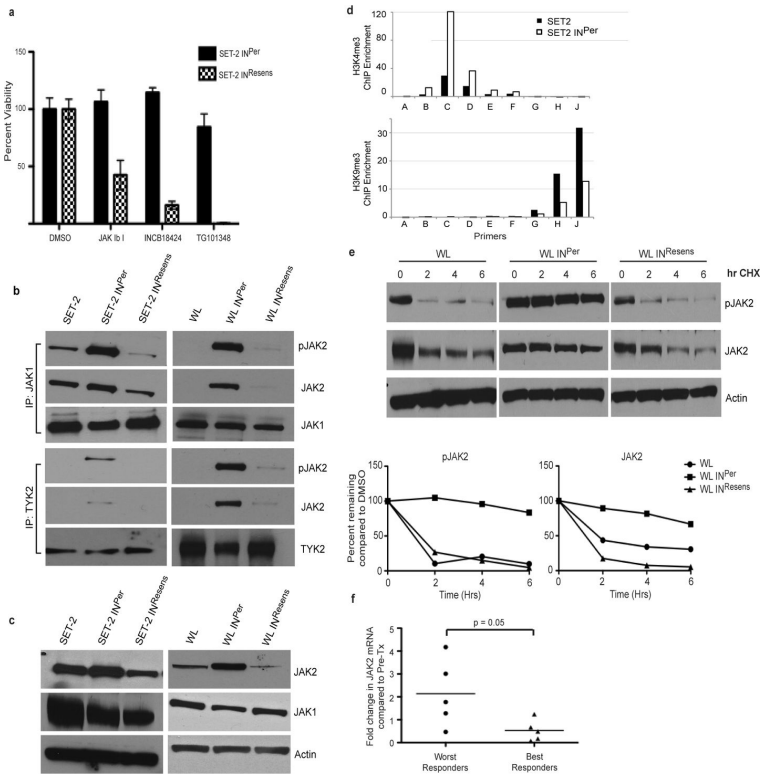

Figure 3. JAK2-Inhibitor persistence is reversible and JAK2 levels correlate with persistence/ resensitization

a) Percent viability of SET-2 IN^{Per} and SET-2 IN^{Resens} cells at 0.25μM JAK Inhibitor I, 0.25μM INCB18424 and 2μM TG101348, data are from wells plated in triplicate (S.D.), and are representative of 3 independent experiments. b) Loss of JAK1/TYK2 association with phosphoJAK2 in SET-2 and WL IN^{Resens} cells. c) Reversible changes in JAK2 levels in IN^{Per} cells compared to naïve and IN^{Resens} SET-2 and WL cells. d) ChIP-PCR of *JAK2* locus shows increased H3K4me3 and decreased H3K9me3 marks in SET-2 IN^{Per} cells. e) PhosphoJAK2 and total JAK2 levels are degraded upon cycloheximide treatment (500 μg/ml for 2, 4 and 6 hours) in naïve and resensitized WL cells, but not in IN^{Per} cells. f) Higher *JAK2* levels in INCB18424 treated MPN granulocytes by qRT-PCR compared to those in a small cohort of best responders.

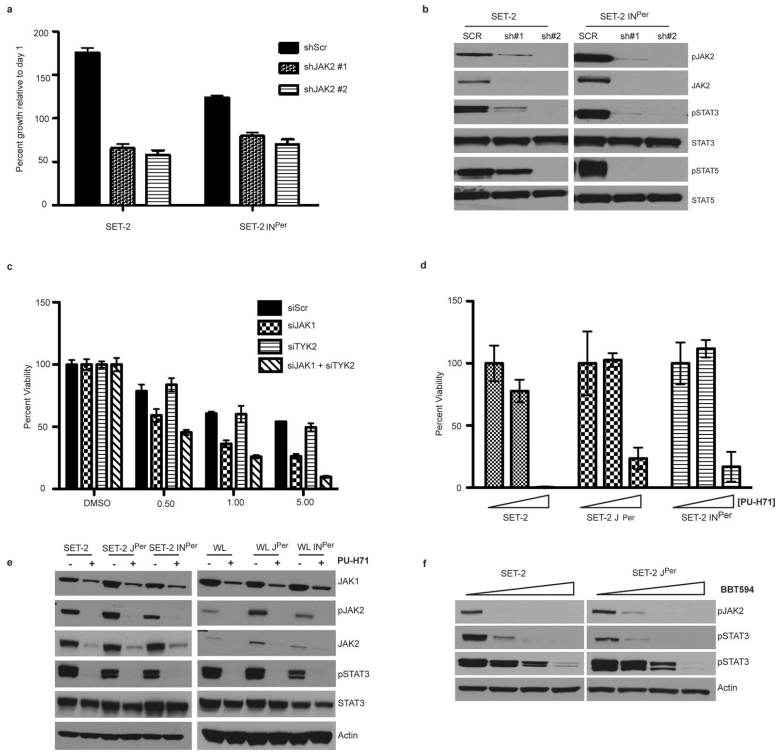

Figure 4. Transphosphorylation of JAK2 by JAK1/TYK2 contributes to persistence and persistent cells can be targeted using Type II JAK2 inhibitors or HSP90 inhibition

a) SET-2 cells were transfected with scrambled pLKO or JAK2 shRNA. Viability after 10 days of puromycin selection relative to cell numbers on day 1 is shown, results are from 3 biologic replicates, S.E.M. is shown. b) JAK2 knockdown inhibits signaling in puromycin selected sensitive and persistent SET-2 cells. c) IN^{Per} SET-2 cells were partially resensitized to INCB18424 following loss of JAK1 or JAK1+TYK2 using siRNA. Data are from wells plated in triplicate (S.D.), and are representative of 3 independent experiments. d) Naïve and persistent SET-2 cells are inhibited by PU-H71, data are from wells plated in triplicate (S.D.), and are representative of 3 independent experiments. e) PU-H71 degrades JAK2, inhibits signaling in SET-2 cells. Cells were treated with DMSO or 2 μM PU-H71 (SET-2) and 1 μM PU-H71 (WL) for 16 hours. f) 4 hour treatment with BBT-594 inhibits signaling in naïve and persistent SET-2 cells.