Published in final edited form as:

Sci Transl Med. 2016 April 13; 8(334): 334ra53. doi:10.1126/scitranslmed.aad3001.

Triple negative breast cancers with amplification of JAK2 at the 9p24 loci demonstrate JAK2-specific dependence

Justin M. Balko^{1,2,4,*,#}, Luis J. Schwarz^{1,#}, Rebecca S. Cook^{2,4}, Monica V. Estrada^{3,4}, Jennifer M. Giltane^{3,4}, Melinda E. Sanders^{3,4}, Violeta Sánchez¹, Phillip T. Dean¹, Kai Wang⁵, Susan E. Combs⁶, Donna Hicks², Joseph A. Pinto⁷, Melissa D. Landis⁸, Jenny C. Chang⁸, Franco D. Doimi⁹, Henry Gómez⁹, David L. Rimm⁶, Roman Yelensky⁵, Vincent A. Miller⁵, Phillip J. Stephens⁵, and Carlos L. Arteaga^{1,2,4,*}

¹Department of Medicine, Vanderbilt-Ingram Cancer Center; Vanderbilt University, Nashville, TN 37232

²Department of Cancer Biology, Vanderbilt-Ingram Cancer Center; Vanderbilt University, Nashville, TN 37232

³Department of Pathology, Microbiology & Immunology, Vanderbilt-Ingram Cancer Center; Vanderbilt University, Nashville, TN 37232

⁴Breast Cancer Research Program, Vanderbilt-Ingram Cancer Center; Vanderbilt University, Nashville, TN 37232

⁵Foundation Medicine, Cambridge, MA 02142

⁶Departments of Pathology and Medicine, Yale University, New Haven, CT

⁷Oncosalud, Lima, Perú

⁸Houston Methodist Cancer Center, Houston TX 77030

⁹Instituto Nacional de Enfermedades Neoplásicas (INEN), Lima, Perú

Abstract

Amplifications at 9p24 have been identified in breast cancer and other malignancies, but the genes within this locus casually associated with oncogenicity or tumor progression remain unclear. Targeted next-generation sequencing of post-chemotherapy triple-negative breast cancers (TNBC) identified a group of 9p24-amplified tumors, which contained focal amplification of the Janus kinase-2 (JAK2) gene. These patients had markedly inferior recurrence-free and overall survival compared to patients with TNBC without JAK2 amplification. Presence of JAK2/9p24 amplifications occurred at higher rates in chemotherapy-treated TNBCs than in untreated TNBCs or basal-like breast cancers, or in other subtypes. Similar rates of JAK2 amplification were confirmed in patient-derived TNBC xenografts. In patients where longitudinal specimens were available, JAK2 amplification was selected for during neoadjuvant chemotherapy and eventual

*Co-corresponding authors. Carlos L. Arteaga, Vanderbilt University Medical Center, 2200 Pierce Ave, 777 PRB, Nashville, TN 37232-6307, carlos.artea@vanderbilt.edu, Phone: (615) 936-3524, Fax: (615) 343-7602, Justin Balko, Vanderbilt University Medical Center, 2200 Pierce Ave, 777 PRB, Nashville, TN 37232-6307, justin.balko@vanderbilt.edu, Phone: (615) 875-8666, Fax: (615) 343-7602

#Authors contributed equally to this work

metastatic spread, suggesting a role in tumorigenicity and chemoresistance, phenotypes often attributed to a cancer stem-like cell population. In TNBC cell lines with JAK2 copy gains or amplification, specific inhibition of JAK2-STAT6 signaling reduced mammosphere formation and cooperated with chemotherapy in reducing tumor growth *in vivo*. In these cells, inhibition of JAK1-STAT3 signaling had little effect or, in some cases, counteracted JAK2-specific inhibition. Collectively, these results suggest that JAK2-specific inhibitors are more efficacious than dual JAK1/2 inhibitors against JAK2-amplified TNBCs. Furthermore, JAK2 amplification is a potential biomarker for JAK2-dependence which, in turn, can be used to select patients for clinical trials with JAK2 inhibitors.

Introduction

Triple-negative breast cancer (TNBC) is the more lethal subtype of this disease, the majority of which exhibit basal-like gene expression. Currently, TNBC lacks clinically approved molecularly targeted therapies, and are primarily treated with traditional chemotherapy and surgery. Although neoadjuvant (pre-surgery) chemotherapy can be effective in eradicating TNBCs in the breast in approximately 30% of patients, many tumors do not respond or respond partially, eventually recurring as distant metastases. We and others have identified potential molecular mechanisms of drug resistance and poor outcome in TNBC¹⁻³. Some of these alterations are potentially 'actionable' or targetable with molecularly directed therapies in clinical development.

We previously detected actionable alterations by targeted next-generation sequencing (tNGS) in the residual cancer of a cohort of patients who did not achieve a pathological complete response (pCR) to neoadjuvant chemotherapy (NAC)². Of the alterations identified in this study, amplifications or gains at the 9p24.1 locus, including Janus kinase 2 (JAK2), occurred at higher rates than in previous reports of untreated TNBC, suggesting a causal association with chemotherapy resistance. The JAK/STAT pathway is known to be mutated or otherwise altered in other tumor types, including myeloproliferative disease, and has been shown to have a central role in driving normal and cancer stem cell growth^{4,5}.

The mammalian JAK-STAT signaling pathway comprises four Janus kinase domain-containing proteins: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), and seven Signal Transducer and Activators of Transcription (STATs), STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6⁶. Deregulation of this pathway has been previously implicated in the promotion of oncogenic phenotypes, including tumorigenesis, invasion, metastasis, proliferation, survival, angiogenesis, anti-apoptosis and immune evasion^{7,8}. In breast cancer, the JAK-STAT pathway has been shown to be altered by the following mechanisms: 1) downregulation of phosphotyrosine specific phosphatases⁹⁻¹¹, 2) elevation of the JAK/STAT-activating ligand IL-6¹²⁻¹⁴, 3) activation of other upstream oncogenic pathways, such as ErbB1, c-Src¹⁵ or PI3K/mTOR¹⁶, and 4) down-regulation of STAT3 negative regulators such as *suppressor of cytokine signaling-3* (SOCS3)¹⁷.

Herein, we demonstrate that a fraction of TNBCs harbor amplifications at the 9p24.1 locus, which includes JAK2. Copy number of the 9p24 amplicon increased during selection by chemotherapy and the development of metastasis, suggesting a role in tumor progression and

therapeutic resistance. We found that JAK2 drives a JAK1/STAT3-independent signaling program that can be overcome using JAK2-specific inhibitors in combination with chemotherapy in order to reduce tumor-initiating stem-like cells and to suppress tumor growth and progression. These findings have important implications for the targeted development of selective JAK2 inhibitors in patients with TNBC and basal like breast cancer (BLBC).

Results

JAK2/9p24 amplifications in post-NAC TNBC

We previously reported an integrated molecular analysis (immunohistochemistry, gene expression and tNGS) of a series of 74 post-NAC TNBCs². Patient demographics are reported in Table 1. From these data we identified 7 patients (11%) with amplifications in the JAK2 locus (9p24), a frequency higher over that in publically available patient cohorts of primary untreated TNBC or basal-like breast cancer. All patients with *JAK2* amplifications (*JAK2*^{AMP}) detected by tNGS were confirmed by FISH with a 100% concordance rate (Figure 1A–B). All of these amplifications included the JAK2 gene, although the majority (88%) also included the immune checkpoint ligand PD-L1 (*CD274*) (Supplemental Figure 1A). Frequently, multiple genes along an amplicon can contribute to oncogenic progression^{18,19,20}. Supporting this idea, quantitative immunofluorescence for PD-L1 revealed that putative *JAK2*^{AMP} tumors also tended to have high PD-L1 expression, suggesting *CD274* may also be an important gene within this amplicon (Supplemental Figure 1B).

Interestingly, patients harboring *JAK2*/9p24 amplifications had poor Miller-Payne scores²¹ in response to NAC, suggesting these were highly chemotherapy-resistant tumors (57% category I in *JAK2*^{AMP} versus 16% category I in *JAK2*^{NML}) (Table 2). Consistent with this finding, *JAK2*^{AMP} patients also experienced significantly inferior recurrence-free (RFS) and overall survival (OS) (Figure 1C–D), with all patients with JAK2-amplified tumors being deceased within <20 months following definitive surgery. Although amplifications at 9p24 have been previously noted^{10,22}, the genes within this amplicon that are associated with tumor virulence are unclear. The JAK/STAT pathway is known to be deregulated in a number of tumor types, with recent reports suggesting functional significance of JAK/STAT signaling in inflammatory and triple-negative breast cancer⁴. Gene expression of *interleukin-6* (*IL6*), an inflammatory cytokine involved in cell proliferation^{23,24} and wound healing²⁵ that can be secreted both from the tumor and immune milieu to activate JAK/STAT signaling, was also significantly higher (p=0.009) in *JAK2*^{AMP} cancers, suggesting a possible autocrine or paracrine loop within these tumors (Figure 1E). RNA *in situ* hybridization analysis of a *JAK2*^{AMP} tumor suggested evidence of paracrine signaling, as individual cells tended to express either *IL6* or *JAK2* but not both (Figure 1F).

JAK2/9p24 amplifications are enriched by chemotherapy treatment

In examining patient data from primary untreated BLBC (TCGA), we found that *JAK2* amplification occurred at a higher rate within our population of chemotherapy-treated TNBC (10% vs. 2%; p=0.08; Fig 2A). When examining amplifications and gains, *JAK2* alterations

were more frequent in BLBC compared to luminal breast cancers (Figure 2B). *JAK2* copy number was a significant predictor of *JAK2* mRNA expression in both tumors and breast cancer cell lines, suggesting that copy number alterations can affect gene expression (Supplemental Figure 2A–B). Collectively, these data suggested that *JAK2* gains or amplifications are predominant in TNBC and BLBC and are selected for by chemotherapy. Supporting this notion, we examined *JAK2* copy number in two patients where serial specimens were available. *JAK2* copy number was normal in the diagnostic biopsies in both patients, but increased markedly in the post-NAC biopsy, and was further elevated in DNA extracted from subsequent metastatic recurrences (Figure 2C).

To determine if *JAK2* amplifications were also present in experimental models of breast cancer, we performed FISH for *JAK2* in sections from 22 patient-derived TNBC xenografts (PDXs). The characteristics of these xenografts have been previously described²⁶. Using a *JAK2*:centromere 9 ratio ≥ 2 as evidence of gene amplification, two of 22 (9%) xenografts were amplified at the *JAK2* locus. This similar frequency to our patient cohort supports the concept that *JAK2*/9p24-amplified tumors are highly aggressive and thus have improved cross-species engraftment rates (Figure 2D). Interestingly, two PDXs in this cohort were derived from the same patient before and after chemotherapy. Careful inspection of *JAK2* copies at the single cell level demonstrated a significant enrichment of tumor cells with *JAK2* copy gains in the post-treatment PDX (2277) compared to the pre-treatment PDX (2147) ($p=0.004$; Figure 2E).

JAK2 mediates STAT6, but not STAT3 activation in breast cancer cells

To understand the significance of *JAK2*/9p24 gains and amplifications at the cellular level, we queried the Cancer Cell Line Encyclopedia (CCLE) for breast cancer cell lines with alterations in *JAK2* copy number. As observed in the TCGA data, *JAK2* copy gains and amplifications were more common in TNBC and BLBC cell lines (Figure 3A). We chose several of these cell lines, representing deep deletion (MDA-231, HCC1143), copy gain (HCC70, HCC38), and amplification (MDA-436, HCC1954). We also utilized SUM159PT cells, which were not included in the CCLE, but have been previously characterized as reliant on JAK/STAT signaling⁴. FISH analysis failed to demonstrate amplification of the 9p24 locus in SUM159PT, but confirmed gain/amplification in HCC70, HCC38, and MDA-436 (Figure 3B). *JAK2* and baseline p-STAT3 (its downstream effector) expression levels as measured by immunoblot correlated in general with *JAK2* gene amplification.

Treatment with ruxolitinib, a JAK1/2 inhibitor with relative IC_{50} of 3.3 nM and 2.8 nM for JAK1 and JAK2, respectively²⁷, down-regulated STAT3 phosphorylation at tyrosine (Y) 705 (the putative JAK phosphorylation site) in all cell lines tested, while it had little effect on an alternative STAT family member and JAK effector, STAT6 (Fig. 3C). To explore whether the effects of ruxolitinib were specific to JAK1 or JAK2 inhibition, we tested increasing concentrations of it against SUM159PT, HCC38, and MDA-436 cells next to the *JAK2* inhibitor NVP-BSK805, with >20 fold selectivity over JAK1 and JAK3 ($JAK2$ IC_{50} = 0.5 nM, JAK1, 31.6 nM; JAK3 18.7 nM; and TYK2 10.8 nM)²⁸. Treatment with ruxolitinib inhibited STAT3 phosphorylation but not STAT6 phosphorylation in dose-dependent fashion, whereas BSK805 only inhibited STAT6 phosphorylation at all doses. Interestingly,

ruxolitinib treatment seemed to cause reciprocal activation of STAT6 at higher doses (Fig. 3D). To confirm these results, we determined how specific siRNA knockdown of JAK1 (siJAK1), JAK2 (siJAK2), both JAK1 and JAK2 (siJAK1/2), or treatment with ruxolitinib would alter STAT3 and STAT6 phosphorylation in four TNBC cell lines (Figure 3E). Downregulation of JAK1 with siRNA (siJAK1) reduced Y705 pSTAT3 but not Y641 pSTAT6 whereas downregulation of JAK2 did the opposite, selectively abrogating STAT6 phosphorylation. These data suggest ruxolitinib treatment reduces STAT3 signaling via inhibition of JAK1 but not JAK2 (Figure 3E).

STAT3 is the most studied downstream effector of STAT signaling in breast cancer⁸. In order to determine the transcriptional effect of STAT3 inhibition in these cell lines, we performed microarray analysis in HCC1143 (low JAK2), HCC70 (JAK2-gained), HCC38 (JAK2-gained) and SUM159PT (JAK2-overexpressing) after 4 or 24 h of JAK inhibition using ruxolitinib. STAT3 phosphorylation was completely inhibited in all of the cell lines, (Supplemental Figure 3A). Surprisingly, at a false-discovery rate of 5%, no genes were significantly altered in HCC1143, HCC70, and HCC38, while only three genes were altered in SUM159PT (Supplemental Figure 3B–C). These results suggest that transcriptionally, STAT3 has little function in these cell lines in *in vitro* conditions. Alternatively, STAT3 may only function within a minor population (i.e., cancer stem-cell like population) which may dilute the transcriptional effects observed. Consistent with the microarray results, ruxolitinib treatment (<10 μ M) did not inhibit cell proliferation or viability *in vitro*, either alone (Supplemental Figure 4A) or in combination with the chemotherapy drugs adriamycin and docetaxel (Supplemental Figure 4B).

JAK2-specific effects on mammosphere potential

Since JAK/STAT signaling is known to play a role in stem-cell functionality and self-renewal^{4,24}, we determined whether specific siRNA knockdown of JAK1 (siJAK1), JAK2 (siJAK2), both JAK1 and JAK2 (siJAK1/2), or treatment with ruxolitinib would affect mammosphere formation, a marker of the tumor-initiating or cancer stem cell-like population. We found that in both HCC38 (JAK2-gained) and MDA-436 (JAK2-amplified), siJAK1 enhanced mammosphere formation, while siJAK2 reduced mammosphere-forming ability (Supplemental Figure 5). Surprisingly, the effect of JAK1 knockdown with siRNA in HCC38 cells had a dominant effect, thus abrogating the effect of JAK2 knockdown even when both siRNAs (siJAK1/2) were used in combination. Consistent with this result, ruxolitinib (JAK1/2 inhibitor)-treated HCC38 cells also exhibited increased mammosphere formation compared to untreated cells (Supplemental Figure 5). To confirm this observation, we utilized JAK2 short-hairpin RNAs in a doxycycline-inducible vector (Figure 4E). To select for cells with mammosphere forming capacity, we used paclitaxel, a chemotherapeutic known to spare CSCs with tumor-initiating capacity^{29,30}. In both HCC38 and MDA-436 cells, doxycycline-induced inhibition of JAK2, abrogated the enrichment of mammosphere-forming, drug-resistant cells spared by paclitaxel (Figure 4A–D). Pharmacological inhibition with the JAK2-specific inhibitor BSK805 produced similar results in both cell lines, further suggesting the inhibition of mammospheres is a JAK2 specific effect (Figure 4F and Supplemental Figure 6).

Finally, we asked whether the addition of BSK805 to paclitaxel would improve therapeutic efficacy *in vivo*. In mice bearing MDA-436 or HCC38 xenografts, the addition of BSK805 to paclitaxel markedly reduced tumor growth. Two of seven HCC38 tumors were completely eliminated and did not recur up to 30 days after withdrawing treatment (Figure 5 A–B). Lysates from paclitaxel-treated xenografts exhibited higher levels of P-STAT3 and P-STAT6 compared to untreated controls. However, lysates from tumors treated with the combination of paclitaxel and BSK805 exhibited markedly reduced levels of P-STAT6 but not P-STAT3 (Figure 5C). Dissociated cells from tumors harvested at the completion of treatment were evaluated in mammosphere assays. Tumor cells from xenografts treated with paclitaxel exhibited increased mammosphere formation compared to cells from untreated tumors or cells from tumors treated with paclitaxel and BSK805 (Figure 5D–E), suggesting inhibition of JAK2 suppressed chemotherapy-resistant CSCs with tumor-initiating capacity. We also evaluated the efficacy of JAK2 inhibition in combination with paclitaxel, or as a single agent against TNBC PDX-4013 (Figure 5F). This PDX was identified as JAK2-amplified (Figure 2D and 5G). BSK805 treatment, alone or in combination with paclitaxel, substantially reduced tumor growth over vehicle control. Collectively, these results suggest that therapeutic benefit may be obtained from the addition of JAK2-specific inhibitors to chemotherapy in *JAK2* gained/amplified breast cancers.

Discussion

We report herein a series of studies supporting the role of JAK2 in triple negative breast cancer. JAK2 amplification was more frequent in TNBC treated with chemotherapy than in newly diagnosed untreated tumors. In addition, JAK2 copy number increased in a limited number of patients where serial biopsies collected at different times in their TNBC progression were available. Notably, the rate of JAK2 amplification in a panel of serially passed patient-derived breast xenografts was similar to that observed in residual TNBC after neoadjuvant chemotherapy, altogether supporting a role for JAK2 in drug resistance and tumor-initiating or cancer stem-like capacity.

The *JAK2* gene is located in 9p23-24, which also contains other candidate oncogenes such as GASC1 (also known as JMJD2C/KDM4C), UHRF2, KIAA1432, C9orf123 and PD-L1 (CD274)^{10,31}. In our study, 88% of the JAK2 amplified TNBC also overexpressed PD-L1 (Suppl. Fig. 1A,B). Nodular sclerosis Hodgkin's lymphoma (NSHL) also exhibits amplification of 9p24.1, which contains both JAK2 and PD-L1 loci. In these cells, JAK2 induces transcription of the PD-1 ligand via STAT1, and is associated with increased sensitivity to JAK2 inhibitors. A similar association in TNBC remains to be explored as well as the possibility that breast tumors with an operative JAK2-PD-1 axis would be more sensitive to immune checkpoint inhibitors³². Rui *et al.* explored the cooperation of different genes within the 9p24 amplicon in primary mediastinal B-cell lymphoma (PMBL) and NSHL, showing a synergistic antitumor effect of JAK2 and JMJD2C inhibition³³. These data suggest that this amplicon contains multiple oncogenes that can act cooperatively or independently. Thus, in order to define the role of JAK2 in TNBC, we performed genetic and pharmacological inhibition of JAK2 in JAK2-amplified breast cancer cells and xenografts as well as PDX's.

We treated JAK2 gene-amplified HCC-38, HCC-70 and MDA-436 TNBC cells and JAK2-overexpressing cells SUM-159PT cells with ruxolitinib, an ATP-competitive inhibitor of JAK1 and JAK2, approved for the treatment of intermediate or high-risk myelofibrosis³⁴. Treatment with ruxolitinib alone or in combination with chemotherapy, at doses that eliminated P-STAT3, did not affect gene expression (Suppl. Fig. 3B,C), cell viability (Suppl. Fig. 4A,B) or mammosphere formation (Suppl. Fig. 5A,B) in all three cell lines with JAK2 amplification (Fig. 3C, Suppl. Fig. 3A). These results are concordant with the findings of Barbie *et al.* where treatment with ruxolitinib had no effect in a different panel of TNBC cell lines³⁵. Since STAT3 is activated/phosphorylated by JAK1 or JAK2³⁶, we performed siRNA knockdown of JAK1 and JAK2 in HCC-70, HCC-38 and MDA-436 cells. Knockdown of JAK1 inhibited P-STAT3 but not P-STAT6, whereas knockdown of JAK2 inhibited P-STAT6 but not P-STAT3 (Fig. 3E). These findings support STAT3 activation by JAK1, as previously shown by Britschgi *et al.* in MDA-468, MDA-231 and 4T1 TNBC cells³⁷.

STAT6 is part of an IL-4/IL-13/STAT6 axis, which promotes T helper 2 (Th2) cytokines that favor mammary gland development. By day 5 of gestation, most of the ductal/luminal cells in the mammary gland of pregnant mice exhibit nuclear P-Stat6. Conversely, *Stat6* deficient mammary glands exhibit delayed ductal and lobuloalveolar development⁵. In PMBL and NSHL, where 30–50% of the tumors are JAK2 amplified³⁸, a JAK2-STAT6 connection has been described in JAK2^{AMP} tumors. In JAK2^{AMP} PMBL, JAK2 is constitutively active, independent of IL3/IL-14 ligands, and phosphorylates STAT6. This constitutive activation is blocked by the JAK2 inhibitor AG490³⁹. Further suggesting a link between JAK2 amplification and dependence, treatment with fedratinib, a selective ATP-competitive inhibitor of JAK2, reduces cellular viability and proliferation in JAK2^{AMP} NSHL and PMBL cells, with a positive correlation between JAK2 copy number and the drug's antitumor effect⁴⁰. Unfortunately, the clinical development of fedratinib was discontinued due to Wernicke's encephalopathy produced by deficit in thiamine uptake⁴¹. Whether this toxicity is due to JAK2 inhibition, is not known.

Marotta *et al.* recently reported the role of the IL-6/JAK2/STAT3 pathway on growth of stem cell-like human breast cancer cells. This association was preferentially active in CD44⁺/CD24⁻ cells, which occur with higher frequency in basal-like breast cancers, and was confirmed in a panel of TNBC cell lines. Different to our study herein, this panel did not include cells with JAK2 amplification⁴. In JAK2^{AMP} HCC-38 and MDA-436 cells, knockdown of JAK2 but not JAK1 inhibited mammosphere formation in the presence and absence of the chemotherapeutic drug paclitaxel (Suppl. Fig. 5). Further, paclitaxel induced mammosphere formation in these JAK2^{AMP} cell lines and this effect was dampened by knockdown of JAK2 using a doxycycline-inducible short hairpin (Fig. 4A–E). Consistent with these data, treatment with NVP-BSK-805, a small molecule inhibitor with >20-fold selectivity of JAK2 over JAK1, inhibited P-STAT6 and not P-STAT3 (Fig. 3D) as well as mammosphere formation (Fig. 4F, Suppl. Fig. 5 and Suppl. Fig. 7). Similar results were observed *in vivo* where HCC-38 and MDA-436 cells harvested from tumors treated with paclitaxel and BSK-805 exhibited markedly reduced P-STAT6 levels and mammosphere-forming capacity compared to cells from tumors that had been treated with paclitaxel alone (Fig. 5). Taken together, these results support a connection among JAK2, STAT6 and a stem cell-like phenotype.

To our knowledge, this is the first report of JAK2 gene amplification in primary breast cancers. These data support a process of JAK2-supported clonal evolution/selection after neoadjuvant therapy and upon tumor progression with the acquisition of drug resistance and poor patient outcome. In concordance with previous reports, JAK2 is involved in cancer cell “stemness”. This role appears limited to breast cancers with JAK2 gene amplification and has similarities with other hematologic neoplasias with altered with JAK2-STAT6 signaling. Based on these observations, we propose JAK2 is a therapeutic target in JAK2 amplified TNBC that can be blocked with inhibitors with predominant activity against JAK2-STAT6 such as NVP-BSK-805. Because of its more limited activity against JAK2 and STAT6, ruxolitinib is likely to be ineffective against JAK2 amplified TNBC.

Methods

Patients and Tumor Specimens

Surgically resected tumor samples (N = 111) were from patients with TNBC diagnosed and treated with NAC at the Instituto Nacional de Enfermedades Neoplásicas (Lima, Perú). Clinical and pathological data were retrieved from medical records under an institutionally approved protocol (INEN 10-018). Tumors were determined to be triple-negative if they were negative for ER, PR, and HER2 overexpression measured by IHC. The results were further verified by comparison with the NGS results.

Fluorescence in situ hybridization (FISH)

JAK2 copy number was quantified by comparing the ratio of JAK2 to CEN9 probe signals. Tumor cells were examined directly using an Olympus AX70 epifluorescence microscope equipped with narrow band pass filters. Each slide was initially scanned at low power to identify appropriate areas of tumor tissue with clearly defined nuclei. The 100× objective was then used to score signals in 40–50 non-overlapping tumor cell nuclei to determine the average number of JAK2 and centromere 9 copies per cell. The risk of sampling error due to tissue heterogeneity was minimized by scoring at least 5 tumor areas for each specimen. In two cases, fewer nuclei were interpretable and these counts were normalized to a value for 40 cells. The ratio of the JAK2 and centromere 9 copy number averages was used to determine the presence of JAK2 gene amplification. Specimens with a JAK2:centromere 9 ratio ≥ 2 were scored as positive for JAK2 gene amplification. Specimens were considered to have JAK2 copy number gain if they had an average of ≥ 3 JAK2 signals.

RNA in situ hybridization

RNA *in situ* hybridization was performed of 5µm FFPE sections using RNAscope reagents (Advanced Cell Diagnostics, Hayward CA) and probes specific for human *JAK2* and *IL6* using the manufacturer's recommended protocol.

Automated quantitative analysis (AQUA) for PD-L1

PD-L1 expression levels were quantified in tissue microarrays (TMA) using AQUA as described previously⁴². AQUA allows exact and objective measurement of fluorescence intensity within a defined tissue area, as well as within subcellular compartments. Briefly, a series of monochromatic high-resolution images were captured using an epifluorescent

microscope platform and signal intensity of the target of interest was measured according to a previously described algorithm. For each TMA histospot, images were obtained for each fluorescence channel, DAPI (nuclei), Alexa 546 (cytokeratin) or Cy5 (target probe). In order to distinguish tumor from stroma, an epithelial tumor “mask” was created by dichotomizing the pan-cytokeratin (CK) signal. Target protein was quantified in the tumor (CK positive), the stroma (absence of CK positivity) or the total tissue area (all DAPI-positive cells)⁴³.

Immunoblot analysis

Immunoblot analysis was performed as previously described⁴⁴ using antibodies for β -actin (#4970), calnexin (#2679), JAK1(#3344), JAK2(#3230, p-STAT3 (Y705) (#9145), p-STAT6 (Y641) (#9364), and STAT3(#12640), all of which were purchased from Cell Signaling Technologies. Immunoreactive bands were detected by enhanced chemiluminescence following incubation with horseradish peroxidase-conjugated secondary antibodies (Promega).

Chemicals and inhibitors

BSK805 was obtained from Novartis and solubilized in DMSO for cell culture, or suspended in hydroxypropylmethylcellulose and tween 80. Ruxolitinib for cell culture was purchased from SelleckChem. Doxycycline was purchased from Sigma. Paclitaxel for injection was obtained from the Vanderbilt University Hospital Outpatient Pharmacy.

Cell lines

MDA-436 and MDA-231 were obtained from ATCC and grown in DMEM + 10% fetal bovine serum (FBS). HCC38, HCC1954, HCC1143 and HCC70 were also obtained through ATCC (ICBP50 panel) and cultured in RPMI + 10% FBS. SUM159PT cells were a gift from the laboratory of Jennifer Pietenpol (Vanderbilt University). Cell lines were obtained either directly from the ATCC, or had been recently fingerprinted for previous publication⁴⁵.

siRNA knockdown

Cells in 60-mm dishes were transfected with a siRNA targeting JAK1 (s7647 and s7648, Ambion), JAK2 (s7648, s7650, Ambion) or non-silencing control using Dharmafect4 (Dharmacon) transfection reagent according to the manufacturer's protocol.

Lentiviral vector transduction

shRNAs for JAK2 clone IDs V2LHS_61653 and V2LHS_61649 were obtained from the Open Biosystems shRNA library and cloned into pIND-CLucZ, a gift from Thorsten Stiewe (Addgene plasmid # 53224). Purified plasmid was transfected into 293FT cells along with psPAX2 and pMD2G in order to generate lentivirus. Conditioned media was applied to target cells (MDA-436 and HCC38) in the presence of polybrene, for two days, prior to puromycin selection. Expression of *JAK2* in the presence or absence of doxycycline (100 ng/mL) was confirmed by qRT-PCR using previously published methods⁴⁶ and primer sequences of: forward: 5'-TCTTTCTTTGAAGCAGCAAG-3'; reverse: 5'-CCATGCCAACTGTTTAGCAA-3'

Viability assays

Viability was ascertained by sulfarhodamine B (SRB), as previously described^{1,2,44,46}.

Mammosphere assays

Mammosphere assay conditions have been previously described^{31,45}. Briefly, single cell suspensions were seeded in 6-well ultra-low attachment plates (Corning) in serum-free DMEM/F12 with 20 ng/ml rhEGF (R&D Systems), hydrocortisone (Sigma) and 1× B27 (Invitrogen). Cell viability was assessed with Thiazolyl Blue Tetrazolium Bromide staining and the number of mammospheres measuring >100 µm was determined in an automated fashion using the GelCount mammalian cell colony counter (Oxford Optronix).

Xenografts

Female athymic mice were injected with HCC-38 or MDA-436 cells in the number 4 mammary fat pad. After four weeks, mice bearing tumors 150 mm³ were randomized to treatment with vehicle, paclitaxel (20 mg/kg/d i.p. daily for 4 days) or paclitaxel + BSK805 (100 mg/kg/day, p.o.). Tumor diameters were measured using calipers twice per week and volume in mm³ calculated with the formula: volume = width² × length/2. For patient-derived xenografts, 2×2mm tumor samples were serially passaged in SCID-beige mice by fatpad transplantation under general anesthesia.

Microarray analysis

For microarray experiments, cells were treated for 4 or 24 h in the presence of DMSO or ruxolitinib (1 µM) and harvested on ice for RNA purification. Total RNA was purified using a Maxwell-16 (Promega) instrument. The experiment was repeated for 3 independent biological replicates prior to microarray hybridization to Affymetrix Human Gene 1.0 ST arrays. Data were normalized in R using RMA and filtered based on variance across all samples and collapsed to the gene level using the genefilter package prior to analysis by one-way ANOVA (for each cell line). P-values were corrected for false discovery rate (FDR) using the Benjamini and Hochberg method⁴⁷. Significant genes (FDR<5%) were contrasted among the treatment groups for each cell line using the Tukey's post-hoc test. Microarray data have been deposited at the Gene Expression Omnibus (GEO), with accession GSE70508.

Genomic data analysis

Genomic data from the TCGA breast⁴⁸ and Cancer Cell Line Encyclopedia⁴⁹ were accessed via the cBio portal⁵⁰.

Statistical analysis

In cell proliferation assays, significant differences were determined by repeated measures ANOVA with Bonferroni post hoc-test, except as noted below. Paired *t*-tests were used to determine significant differences in siRNA proliferation assays, Caspase-Glo 3/7 assay, real-time qPCR assays and immunohistochemistry (IHC) histoscores. A p-value of <0.05 was considered statistically significant. Bar graphs show mean ± SD, unless otherwise stated in

the figure legend. For all comparisons, statistical significance is noted by * $p < 0.05$; ** $p < 0.01$, and *** $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by the Department of Defense Breakthrough Award BC131494 (JMB, RSC, MES, and JMG), a grant from the IBC Network Foundation (JMB), and Susan G. Komen for the Cure Foundation Grant SAC100013 (CLA). Other sources of support include NIH/NCI K99/R00-CA181491 (JMB), Breast Cancer Specialized Program of Research Excellence (SPORE) P50 CA098131, and Vanderbilt-Ingram Cancer Center Support Grant P30 CA68485.

References

1. Balko JM, Cook RS, Vaught DB, et al. Profiling of residual breast cancers after neoadjuvant chemotherapy identifies DUSP4 deficiency as a mechanism of drug resistance. *Nat Med*. 2012; 18:1052–1059. [PubMed: 22683778]
2. Balko JM, Giltane JM, Wang K, et al. Molecular profiling of the residual disease of triple-negative breast cancers after neoadjuvant chemotherapy identifies actionable therapeutic targets. *Cancer Discov*. 2014; 4:232–245. [PubMed: 24356096]
3. Bauer JA, Chakravarthy AB, Rosenbluth JM, et al. Identification of markers of taxane sensitivity using proteomic and genomic analyses of breast tumors from patients receiving neoadjuvant paclitaxel and radiation. *Clin Cancer Res*. 2010; 16:681–690. [PubMed: 20068102]
4. Marotta LL, Almendro V, Marusyk A, et al. The JAK2/STAT3 signaling pathway is required for growth of CD44(+)CD24(–) stem cell-like breast cancer cells in human tumors. *Journal of Clinical Investigation*. 2011; 121:2723–2735. [PubMed: 21633165]
5. Khaled WT, Read EKC, Nicholson SE, et al. The IL-4/IL-13/Stat6 signalling pathway promotes luminal mammary epithelial cell development. *Development*. 2007; 134:2739–2750. [PubMed: 17611223]
6. Shuai K, Liu B. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol*. 2003; 3:900–911. [PubMed: 14668806]
7. Sonnenblick A, Uziely B, Nechushtan H, et al. Tumor STAT3 tyrosine phosphorylation status, as a predictor of benefit from adjuvant chemotherapy for breast cancer. *Breast Cancer Res Treat*. 2013; 138:407–413. [PubMed: 23446809]
8. Yu H, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nature Reviews Cancer*. 2014; 14:736–746. [PubMed: 25342631]
9. Shields BJ, Wiede F, Gurzov EN, et al. TCPTP regulates SFK and STAT3 signaling and is lost in triple-negative breast cancers. *Mol Cell Biol*. 2013; 33:557–570. [PubMed: 23166300]
10. Wu J, Liu S, Liu G, et al. Identification and functional analysis of 9p24 amplified genes in human breast cancer. *Oncogene*. 2012; 31:333–341. [PubMed: 21666724]
11. Yip SS, Crew AJ, Gee JM, et al. Up-regulation of the protein tyrosine phosphatase SHP-1 in human breast cancer and correlation with GRB2 expression. *International journal of cancer Journal international du cancer*. 2000; 88:363–368. [PubMed: 11054664]
12. Bachelot T, Ray-Coquard I, Menetrier-Caux C, Rastkha M, Duc A, Blay JY. Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. *Br J Cancer*. 2003; 88:1721–1726. [PubMed: 12771987]
13. Selander KS, Li L, Watson L, et al. Inhibition of gp130 signaling in breast cancer blocks constitutive activation of Stat3 and inhibits in vivo malignancy. *Cancer Res*. 2004; 64:6924–6933. [PubMed: 15466183]

14. Lieblein JC, Ball S, Hutzen B, et al. STAT3 can be activated through paracrine signaling in breast epithelial cells. *BMC Cancer*. 2008; 8:302. [PubMed: 18939993]
15. Garcia R, Bowman TL, Niu G, et al. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. *Oncogene*. 2001; 20:2499–2513. [PubMed: 11420660]
16. Yokogami K, Wakisaka S, Avruch J, Reeves SA. Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR. *Curr Biol*. 2000; 10:47–50. [PubMed: 10660304]
17. Sutherland KD, Lindeman GJ, Choong DY, et al. Differential hypermethylation of SOCS genes in ovarian and breast carcinomas. *Oncogene*. 2004; 23:7726–7733. [PubMed: 15361843]
18. Yang ZQ, Streicher KL, Ray ME, Abrams J, Ethier SP. Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer. *Cancer research*. 2006; 66:11632–11643. [PubMed: 17178857]
19. Yang ZQ, Liu G, Bollig-Fischer A, Giroux CN, Ethier SP. Transforming properties of 8p11-12 amplified genes in human breast cancer. *Cancer research*. 2010; 70:8487–8497. [PubMed: 20940404]
20. Sinclair CS, Rowley M, Naderi A, Couch FJ. The 17q23 amplicon and breast cancer. *Breast Cancer Res Treat*. 2003; 78:313–322. [PubMed: 12755490]
21. Ogston KN, Miller ID, Payne S, et al. A new histological grading system to assess response of breast cancers to primary chemotherapy: prognostic significance and survival. *Breast*. 2003; 12:320–327. [PubMed: 14659147]
22. Liu G, Bollig-Fischer A, Kreike B, et al. Genomic amplification and oncogenic properties of the GASC1 histone demethylase gene in breast cancer. *Oncogene*. 2009; 28:4491–4500. [PubMed: 19784073]
23. Sansone P, Storci G, Tavoroli S, et al. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Invest*. 2007; 117:3988–4002. [PubMed: 18060036]
24. Hartman ZC, Poage GM, den Hollander P, et al. Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. *Cancer research*. 2013; 73:3470–3480. [PubMed: 23633491]
25. Lin ZQ, Kondo T, Ishida Y, Takayasu T, Mukaida N. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *J Leukocyte Biol*. 2003; 73:713–721. [PubMed: 12773503]
26. Zhang X, Claerhout S, Prat A, et al. A renewable tissue resource of phenotypically stable, biologically and ethnically diverse, patient-derived human breast cancer xenograft models. *Cancer Res*. 2013; 73:4885–4897. [PubMed: 23737486]
27. Quintas-Cardama A, Vaddi K, Liu P, et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood*. 2010; 115:3109–3117. [PubMed: 20130243]
28. Baffert F, Regnier CH, De Pover A, et al. Potent and Selective Inhibition of Polycythemia by the Quinoxaline JAK2 Inhibitor NVP-BSK805. *Mol Cancer Ther*. 2010; 9:1945–1955. [PubMed: 20587663]
29. Calcagno AM, Salcido CD, Gillet JP, et al. Prolonged drug selection of breast cancer cells and enrichment of cancer stem cell characteristics. *J Natl Cancer Inst*. 2010; 102:1637–1652. [PubMed: 20935265]
30. Bhola NE, Balko JM, Dugger TC, et al. TGF-beta inhibition enhances chemotherapy action against triple-negative breast cancer. *Journal of Clinical Investigation*. 2013; 123:1348–1358. [PubMed: 23391723]
31. Green MR, Monti S, Rodig SJ, et al. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. *Blood*. 2010; 116:3268–3277. [PubMed: 20628145]
32. Errico A. Immunotherapy: PD-1-PD-L1 axis: efficient checkpoint blockade against cancer. *Nature reviews Clinical oncology*. 2015; 12:63.

33. Rui L, Emre NC, Kruhlak MJ, et al. Cooperative epigenetic modulation by cancer amplicon genes. *Cancer cell*. 2010; 18:590–605. [PubMed: 21156283]
34. Verstovsek S, Mesa RA, Gotlib JR, et al. Results of COMFORT-I, a randomized double-blind phase III trial of JAK 1/2 inhibitor INCB18424 (424) versus placebo (PB) for patients with myelofibrosis (MF). *J Clin Oncol*. 2011; 29
35. Barbie TU, Alexe G, Aref AR, et al. Targeting an IKBKE cytokine network impairs triple-negative breast cancer growth. *The Journal of clinical investigation*. 2014; 124:5411–5423. [PubMed: 25365225]
36. Barton BE, Karras JG, Murphy TF, Barton A, Huang HF. Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines. *Molecular cancer therapeutics*. 2004; 3:11–20. [PubMed: 14749471]
37. Britschgi A, Andraos R, Brinkhaus H, et al. JAK2/STAT5 inhibition circumvents resistance to PI3K/mTOR blockade: a rationale for cotargeting these pathways in metastatic breast cancer. *Cancer cell*. 2012; 22:796–811. [PubMed: 23238015]
38. Mullally A, Ebert BL. Janus Reveals Another Face: The Biologic Rationale for Targeting Janus Kinase 2 in Lymphoma. *J Clin Oncol*. 2012; 30:4168–4170. [PubMed: 22965959]
39. Guiter C, Dusanter-Fourt I, Copie-Bergman C, et al. Constitutive STAT6 activation in primary mediastinal large B-cell lymphoma. *Blood*. 2004; 104:543–549. [PubMed: 15044251]
40. Hao Y, Chapuy B, Monti S, Sun HH, Rodig SJ, Shipp MA. Selective JAK2 inhibition specifically decreases Hodgkin lymphoma and mediastinal large B-cell lymphoma growth in vitro and in vivo. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2014; 20:2674–2683. [PubMed: 24610827]
41. Zhang Q, Zhang Y, Diamond S, et al. The Janus kinase 2 inhibitor fedratinib inhibits thiamine uptake: a putative mechanism for the onset of Wernicke's encephalopathy. *Drug Metab Dispos*. 2014; 42:1656–1662. [PubMed: 25063672]
42. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med*. 2002; 8:1323–1327. [PubMed: 12389040]
43. Brown JR, Wimberly H, Lannin DR, Nixon C, Rimm DL, Bossuyt V. Multiplexed quantitative analysis of CD3, CD8, and CD20 predicts response to neoadjuvant chemotherapy in breast cancer. *Clin Cancer Res*. 2014; 20:5995–6005. [PubMed: 25255793]
44. Balko JM, Jones BR, Coakley VL, Black EP. MEK and EGFR inhibition demonstrate synergistic activity in EGFR-dependent NSCLC. *Cancer Biol Ther*. 2009; 8:522–530. [PubMed: 19305165]
45. Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011; 121:2750–2767. [PubMed: 21633166]
46. Balko JM, Schwarz LJ, Bhola NE, et al. Activation of MAPK pathways due to DUSP4 loss promotes cancer stem cell-like phenotypes in basal-like breast cancer. *Cancer Res*. 2013
47. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological)*. 1995; 57:289–300.
48. TCGA. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012; 490:61–70. [PubMed: 23000897]
49. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012; 483:603–607. [PubMed: 22460905]
50. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012; 2:401–404. [PubMed: 22588877]

Statement of Summary

Triple-negative breast cancers with JAK2 amplification demonstrate poor prognosis, but may respond to JAK2-specific inhibitors in combination with chemotherapy.

Figure 1. *JAK2*^{AMP} is associated with a lower response to NAC and poor patient survival
A) Validation by FISH of *JAK2* amplifications detected by NGS in tumor samples. **B)** Representative images of *JAK2* FISH9p24 region (*JAK2*) in red and for centromere9 centromere in green. Four cases are depicted, with the upper left demonstrating a patient tumor with normal (2 copies) of *JAK2*. The remaining 3 cases demonstrated *JAK2* gains/amplification. **C,D)** Kaplan-Meier curves assessing recurrence free survival (RFS) and overall survival (OS) in patients with *JAK2*^{AMP} and *JAK2*^{NML} cancers (PFS, HR=0.19, 95% CI, 0.05 to 0.73; OS, HR=0.1, 95% CI, 0.02 to 0.44) **E)** *IL6* mRNA in *JAK2*^{AMP} and

JAK2^{NML} tumors, quantified by nanoString analysis. **F)** RNA *in situ* analysis of a *JAK2*^{AMP} tumor for *IL6* (red) and *JAK2* (green).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. Chemotherapy enriches the JAK2^{AMP} tumor cell population

A) Comparison of JAK2 amplification rates in 68 post-NAC TNBCs versus TCGA (primary basal-like) **B)** Amplifications in JAK2 occur primarily in basal-like breast cancer. Data were obtained through the cBio website for TCGA data access. Luminal: n=324; Basal: n=81. **C)** Enrichment of JAK2 gene copy number in longitudinal samples (pre-therapy biopsy, post-NAC surgical specimen and metastatic biopsy in two patients with TNBC). **D)** JAK2 copy number across a series of 22 patient-derived TNBC xenografts (PDX) using a JAK2:CEP9 ratio as described in Methods. Samples in red are JAK2-amplified. Samples in patterned pink are matched PDXs from the same TNBC, established before (2147) and after (2277) chemotherapy. **E)** JAK2 FISH copy number counts (relative to centromere 9 signal) at the single cell level in matched untreated (2147) and post-chemotherapy (2277) PDXs. Clinically amplified PDX (4013) and FISH from an untreated patient (1° JAK2 amp) demonstrating high level of amplification are plotted for comparison. The population of JAK2-amp cells was enriched in the PDX model established after chemotherapy treatment. P-value represents result of a two-tailed t-test.

Figure 3. JAK2 drives a STAT3-independent program in JAK2^{AMP} TNBC cell lines

A) Analysis of CCLE data via the cBio portal to identify cell lines *JAK2* copy alterations. **B)** *JAK2* FISH in red and for chromosome 9 centromere in green for breast cancer cell lines SUM-159PT (*JAK2*^{NORMAL}), HCC-70 (*JAK2*^{GAIN}), HCC-38 (*JAK2*^{GAIN}) and MDA-436 (*JAK2*^{AMP}). **C)** Cells were treated with 10% FBS ± 1 μM ruxolitinib for 24 h and analyzed by immunoblot with the indicated antibodies. **D)** SUM159PT cells were treated increasing doses of the JAK2-specific inhibitor BSK805 or ruxolitinib for 24 h and analyzed by immunoblot. **E)** TNBC cell lines were transfected with siControl (siCON), siJAK1, siJAK2,

or treated with 1 μ M ruxolitinib. Two different sequences for each *JAK1* and *JAK2* were used to confirm specificity. Cells were harvested 72 h after siRNA transfection or 24 h after ruxolitinib treatment and analyzed by immunoblot with the indicated antibodies.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. JAK2 knockdown abrogates tumorsphere expansion after chemotherapy

A) HCC38 cells stably-transduced with doxycycline-inducible expression of shRNA targeting JAK2 (two independent sequences) or a non-targeting shRNA control (shNTC) were grown in 10% FBS and treated with vehicle (control), IC₅₀ of paclitaxel (50 nM) ± 100 ng/ml doxycycline or doxycycline for 4 days and allowed to recover in fresh media for 3 days; cells were then trypsinized and assessed for their ability to form mammospheres. **B)** On day 7, colonies were stained with MTT and photographed. Colony numbers are expressed relative to untreated controls (scale bars: 200 µm.) **C–D)** Experiments identical to

(A–B) were carried out in MDA-436 cells (paclitaxel IC_{50} 150nM). **E**) Immunoblot analysis demonstrating doxycycline-inducible knockdown of JAK2 at 72 h in HCC38 and MDA-436 cells. **F**) MDA-436 and HCC38 cells were grown in 10% FBS and treated with vehicle (control), paclitaxel (at the respective IC_{50}) $\pm$ 5 μ M BSK805 or 5 μ M BSK805 alone for 4 days and allowed to recover in fresh media for 3 days; colonies were quantitated as described above. All experiments were replicated at least twice (n=3).

Figure 5. Pharmacological JAK2 inhibition in vivo abrogates tumor-initiating potential after chemotherapy

Female athymic mice were injected with MDA-436 (A) or HCC-38 (B) cells in the number 4 mammary fat pad. Mice bearing tumors 150 mm³ were randomized to treatment with vehicle, paclitaxel (20 mg/kg/d i.p. 4 times) or paclitaxel (20 mg/kg/d i.p. 4 times) + BSK805 (100 mg/kg/day, p.o.). Tumor volumes were measured twice weekly. Two complete responses to dual therapy were achieved in mice bearing HCC38 tumors. Bars represent mean $\pm$ SEM (* p <0.001 vs. vehicle, # p <0.05 vs. paclitaxel). Inset: At the end of treatment,

tumors were dissociated to a single cell suspension and plated in a mammosphere assay. After 12 days, mammosphere growth was imaged and quantified. Differences were analyzed by one-way ANOVA with Tukey's contrasts (* $p < 0.05$; ** $p < 0.01$). **C)** Western blot analysis of tumors harvested at the end of treatment for the indicated antibodies. **D)** Representative images of mammospheres from treated tumors in (A) and (B). **E)** Quantification of mammospheres from tumors harvested at the end of treatment. **F)** SCID-beige mice implanted with PDX model PDX4013 were randomized to treatment with vehicle, paclitaxel (20 mg/kg/d i.p. 4 times), BSK805 (80 mg/kg/day, p.o.), or paclitaxel (20 mg/kg/d i.p. 4 times) + BSK805 (80 mg/kg/day, p.o.). Tumor volumes were measured twice weekly. **G)** Image of *JAK2*-FISH in the PDX4013 model demonstrating amplification.

Table 1

Clinical characteristics of cohort

	No.	%
Samples	111	
Age		
Median	48	
IQR	24–78	
Menopausal status		
Pre-menopausal	57	51.4
Post-menopausal	54	48.6
Node status		
Positive	71	64.0
Negative	40	36.0
Stage		
II	9	8.1
III	102	91.9
Anthracyclines		
Yes	106	95.5
No	5	4.5
Taxane		
Yes	58	52.3
No	53	47.7
JAK2-amplifications		
	7/68	10.2

Table 2 Clinical comparison of patients by *JAK2* status

	<i>JAK2</i>- amplified	<i>JAK2</i>- normal
No.	7	57
Median age	39.9	47.6
Pre-menopausal	No. (%)	No. (%)
	5 (71.4)	33 (57.9)
Miller & Payne		
I	4 (57.1)	9 (15.8) *
II	1 (14.2)	15 (26.3)
III	0 (0)	5 (8.8)
IV	0 (0)	6 (10.5)
V	0 (0)	5 (8.8)
Unknown	2 (28.7)	17 (29.8)
OS (median)	10.8 **	24.4
RFS (median)	7.9 **	21.8

Characteristics of patients with complete tNGS data (* $p < 0.05$, two-tailed Fisher exact test; ** $p < 0.01$, two-sided log-rank test)