



Published in final edited form as:

Leukemia. 2023 August ; 37(8): 1686–1697. doi:10.1038/s41375-023-01959-0.

MAPK-negative feedback regulation confers dependence to JAK2^{V617F} signaling

Meenu Kesarwani¹, Zachary Kincaid¹, Mohammad Azhar¹, Jacob Menke¹, Joshua Schwieterman¹, Sekhu Ansari¹, Angela Reaves¹, Michael E. Deininger², Ross Levine³, H. Leighton Grimes^{4,5,6}, Mohammad Azam^{1,5,6,✉}

¹Division of Pathology, Cincinnati Children's Hospital, Cincinnati, OH, USA.

²Versiti Blood Research Institute and Medical College of Wisconsin, Milwaukee, WI, USA.

³Center for Hematologic Malignancies, and Molecular Cancer Medicine Service, Memorial Sloan-Kettering Cancer Center, New York, NY, USA.

⁴Division of Immunobiology, Cincinnati Children's Hospital, Cincinnati, OH, USA.

⁵Department of Pediatrics, University of Cincinnati, Cincinnati, OH, USA.

⁶Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital, Cincinnati, OH, USA.

Abstract

Despite significant advances in developing selective JAK2 inhibitors, JAK2 kinase inhibitor (TKI) therapy is ineffective in suppressing the disease. Reactivation of compensatory MEK-ERK and PI3K survival pathways sustained by inflammatory cytokine signaling causes treatment failure. Concomitant inhibition of MAPK pathway and JAK2 signaling showed improved in vivo efficacy compared to JAK2 inhibition alone but lacked clonal selectivity. We hypothesized that cytokine signaling in JAK2^{V617F} induced MPNs increases the apoptotic threshold that causes TKI persistence or refractoriness. Here, we show that JAK2^{V617F} and cytokine signaling converge to induce MAPK negative regulator, DUSP1. Enhanced DUSP1 expression blocks p38 mediated p53 stabilization. Deletion of *Dusp1* increases p53 levels in the context of JAK2^{V617F} signaling that causes synthetic lethality to *Jak2*^{V617F} expressing cells. However, inhibition of *Dusp1* by a small molecule inhibitor (BCI) failed to impart *Jak2*^{V617F} clonal selectivity due to pErk1/2 rebound

Reprints and permission information is available at <http://www.nature.com/reprints>

✉ Correspondence and requests for materials should be addressed to Mohammad Azam, Mohammad.Azam@cchmc.org.

AUTHOR CONTRIBUTIONS

MK, ZK, MA, JM, JS, SA, AR, performed the experiments and analyzed the data. MED, RL, and HLG provided patient samples, mouse models, retroviral constructs and guided the experimental design. MK and M Azam designed all experiments. MK and M Azam wrote the manuscript.

COMPETING INTERESTS

MA is a recipient of the bridge award from the American society of hematology (ASH). RLL is on the supervisory board of Qiagen and is a scientific advisor to Imago, Mission Bio, Bakx, Zentalis, Ajax, Auron, Prelude, C4 Therapeutics and Isoplexis. He has received research support from Abbvie, Constellation, Ajax, Zentalis and Prelude. He has received research support from and consulted for Celgene and Roche and has consulted for Syndax, Incyte, Janssen, Astellas, Morphosys and Novartis.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41375-023-01959-0>.

caused by off-target inhibition of *Dusp6*. Ectopic expression of *Dusp6* and BCI treatment restored clonal selectivity and eradicated the *Jak2^{V617F}* cells. Our study shows that inflammatory cytokines and JAK2^{V617F} signaling converge to induce DUSP1, which downregulates p53 and establishes a higher apoptotic threshold. These data suggest that selectively targeting DUSP1 may provide a curative response in JAK2^{V617F}-driven MPN.

INTRODUCTION

The clinical success of ABL tyrosine kinase inhibitor (TKI) therapy in treating Ph+ chronic myeloid leukemia (CML) prompted to identify the oncogenic lesions in Ph- MPN for therapeutic intervention. This led to the discovery of activating mutations in *JAK2* [1, 2], *MPL* [3] and *CALR* [4]. Interestingly, all these mutations converge in activating the JAK-STAT signaling, which provided a strong rationale for targeting JAK2 kinase activity using small molecule kinase inhibitor. However, unlike the cytotoxic effect of ABL inhibitors in CML, JAK2 inhibition imparted a cytostatic response and failed to eradicate the malignant clones or induce molecular remission [5]. Clonal persistence and adaptation to JAK2-tyrosine kinase inhibitors (TKIs) are the primary cause of treatment failure, which is mainly driven by the activation of survival pathways by inflammatory cytokines.

Constitutive cytokine and JAK2 signaling also activate MAPK pathway by activating the PI3 kinase. Consequently, inhibition of MAPK or PI3K with JAK2 inhibitors synergistically suppressed the proliferation of JAK2^{V617F} expressing MPN cells [6, 7]. In preclinical mouse model, combinatorial targeting of JAK2 with MEK/ERK or PI3K in vivo showed modest improvement compared to JAK2 inhibition alone, as most mice exhibited persistent disease [8–11]. Given that both MEK and JAK2 inhibitors exert cytostatic response in vivo, combinatorial targeting of both proteins would least likely exert clonal selectivity. The underlying mechanism driving cytostatic response has yet to be fully understood. Nonetheless, constitutive cytokine signaling induced persistent MAPK activity is suspected for the cytostatic response.

The therapeutic efficacy of TKI was attributed to be a function of oncogene-dependence where acute inhibition of driver-oncogene by TKI results in apoptotic cell death while sparing normal cells [12–14]. While TKI therapy in CML kills most leukemic cells, it is not curative because leukemic stem cells (LSCs) are intrinsically resistant to treatment resulting in minimal residual disease (MRD). Even the most potent TKIs are ineffective in eliminating the LSCs [15]. Although the molecular mechanisms underlying the failure of TKI to eradicate these persistent cells are not fully understood, growth-factor signaling is implicated in rescuing these cells during TKI therapy in leukemia and solid organ tumors [16–20]. We have shown that the growth-factor signaling in the context of oncogenic signaling induces *c-Fos* and *Dusp1* (Dual specificity phosphatase 1) expression, together these genes modulate the AP1 transcriptional network and the activity of p38 kinase, which establishes a higher apoptotic threshold resulting in LSC persistence and TKI refractoriness [21].

Failure of JAK2 TKIs to completely suppress the overproduction of inflammatory cytokines, a cardinal feature of MPN development and transition to AML, drives clonal persistence

and TKI refractoriness [22]. We reasoned that, like LSCs in CML where growth-factor signaling increases the apoptotic threshold, inflammatory cytokine signaling in MPNs drives a higher apoptotic threshold. Consequently, these cells are intrinsically resistant to TKI treatment. Here, we show that MPN cells derived from the primary patients exhibit increased expression of *DUSP1* but not the *c-FOS*. Using mouse genetic models, we show that ablation of *DUSP1* is synthetic lethal to MPN development. Biochemical analysis revealed that *JAK2^{V617F}* signaling downregulates p53 expression by *DUSP1* mediated inhibition of p38 activity. Phosphorylation of p53 at Serine 15 prevents its interaction with Mdm2. Reduced p53 phosphorylation at Serine 15 due to p38 blockade enhances p53 degradation resulting in reduced expression. Consequently, *DUSP1* deletion activates p38 that results in enhanced p53 stabilization and selective demise of *Jak2^{V617F}* clones. Our study provides evidence that MAPK-negative feedback confers dependence to *JAK2^{V617F}*-induced MPN, and its targeting may provide a curative response.

METHODS

Available as supplementary material.

RESULTS

To test the effect of growth-factor signaling on TKI response, BaF3 cells stably expressing MPN genes (*Jak2^{V617F}*, *MPL^{W515L}* and *CALR^{mut}*) were examined to determine the efficacy of ruxolitinib in the presence and absence of IL3. Parental BaF3 cells require IL3 for survival, expression of oncogenes renders growth-factor independent growth that allows evaluation of targeted therapy. As expected, ruxolitinib treatment inhibited the proliferation of *BaF3-Jak2^{V617F}*, *BaF3-MPL^{W515L}* and *BaF3-CALR^{mut}* cells with IC50 values of 79 and 46, and 57 nM, respectively (Fig. 1A). The addition of growth factor (IL3) conferred resistance to ruxolitinib resulting in increased IC50 values similar to parental BaF3 cells. Immunoblotting of phospho-JAK2 and phospho-STAT5 revealed complete inhibition of JAK2 and STAT5 in both conditions (Fig. 1B). Thus, providing evidence that resistance to ruxolitinib is not due to growth-factor-mediated JAK2 reactivation. Similarly, primary bone marrow derived c-Kit⁺ cells expressing *Jak2^{V617F}*, *MPL^{W515L}* and *CALR^{mut}* grown with hematopoietic cytokines (SCF, FLT3 ligand, IL3, IL6 and TPO) exhibited higher IC50 values for ruxolitinib compared to the control, pMSCV-ires-GFP expressing cells (Fig. 1C). Altogether these data provide evidence that growth-factor signaling confers resistance to ruxolitinib.

To understand the molecular mechanism driving resistance, the BM-derived Kit⁺ cells expressing *MPL^{W515L}*, *Jak2^{V617F}*, and vector (pMIG) were subjected to the whole genome RNA-seq analysis. A comparative analysis revealed altered expression of 4208 genes in *Jak2^{V617F}* and *MPL^{W515L}* expressing cells compared to the vector control (Fig. 1D, Supplementary Fig. 1A). Among these, 2796 genes (2062 induced and 736 downregulated) are commonly modulated between *MPL^{W515L}* and *Jak2^{V617F}*, and a significant number of genes were uniquely regulated by *MPL^{W515L}* (1412; 1038 induced and 374 downregulated) and *Jak2^{V617F}* (815; 479 induced and 337 downregulated) Supplementary Fig. 1A. A network analysis of genes induced explicitly by *JAK2^{V617F}* exhibited significant

enrichment to pathways previously reported to be activated by JAK2 signaling, such as signal transduction, cellular differentiation, and growth-factor signaling [23]. We observed a more significant enrichment of genes downregulating WNT and JNK pathways in *Jak2^{V617F}* expressing cells (Supplementary Fig. 1B–D). In contrast, analysis of *MPL^{W515L}*-induced genes showed enrichment to pathways implicated in interferon β -signaling, platelet activation, TLR signaling and negative regulation of apoptosis (Supplementary Fig. 1E). However, network analysis of commonly induced genes exhibited more significant enrichment in pathways driving chemokine production (one of the hallmarks of MPN), cytokine signaling, and negative regulation of MAPK pathway besides modulating the previously reported MAPK and JAK-STAT pathways (Supplementary Fig. 1F–H and Supplementary Figs. 2 and 3). To identify the genes governing TKI (ruxolitinib) resistance under growth-factor signaling, we compared the expression profile generated from the growth-factor induced TKI resistant CML cells (185 genes) [21]. Comparison of these three data sets identified 19 commonly regulated genes between them (Fig. 1D, E). Among these, expression of *Dusp1*, *Atp6v0d2*, *Scin*, and *Ifi2712a* are upregulated in *Jak2^{V617F}* and *MPL^{W515L}* cells compared to BCR-ABL expressing cells (Fig. 1E). Using CML as a model, we have shown that *FOS*, *DUSP1*, and *ZFP36* are functional mediators of growth-factor-induced TKI resistance. Depletion of *FOS* and *DUSP1* alone or together sensitized BaF3-BCR-ABL cells to imatinib, even in the presence of IL3. However, the lack of *ZFP36* conferred equal TKI sensitivity to both parental BaF3 and *BaF3-BCR-ABL* cells, suggesting that *ZFP36* is redundant. Given that the oncogenic JAK2 signaling robustly induces MAPK-ERK activation besides activating the JAK-STAT pathway, we reasoned that induced expression of *DUSP1* seemingly provides feedback regulation to MAPK signaling to suppress the apoptotic stimulation commonly associated with unrestrained MAPK/ERK activation [24–28]. Therefore, we focused subsequent analyses on *DUSP1*.

Quantitative expression analysis of bone marrow-derived LSK cells expressing *Jak2^{V617F}*, *MPL^{W515L}* and *CALR^{mut}* exhibited higher *Dusp1* expression than the control (Fig. 1F). Likewise, analysis of patient samples revealed significantly increased *DUSP1* expression (2–4-fold) in PV and PMF but not in ET compared to normal CD34⁺ cells (Fig. 1G, H). Next, genetic depletion of *Dusp1* by shRNA-mediated knockdown in parental BaF3 cells did not show any adverse effect while its depletion in BaF3-*Jak2^{V617F}*, BaF3-*MPL^{W515L}*, BaF3-*CALR^{mut}* significantly suppressed the proliferation (Fig. 1I). Importantly, *DUSP1* depletion abolished cytokine-induced TKI resistance in BaF3- cells expressing *Jak2^{V617F}*, *MPL^{W515L}* and *CALR^{mut}* (Fig. 1J). Likewise, human MPN cells, HEL and SET2, grown with hematopoietic cytokines (IL3, IL6, SCF, FLT3L and GM-CSF) exhibited induced *DUSP1* expression and resistance to ruxolitinib (Fig. 1K–N). Depletion of *DUSP1* by shRNA-mediated knockdown suppressed their proliferation and restored TKI sensitivity under growth-factor (GF) signaling (Fig. 1K–N). Together, these results suggest that growth-factor induced *DUSP1* expression causes TKI refractoriness in MPN cells.

Deletion of *Dusp1* is synthetic lethal to JAK2^{V617F}-induced MPN

Next, we examined the role of *DUSP1* in MPN development and treatment response. Bone marrow derived c-Kit⁺ positive cells from the wild-type and *Dusp1^{-/-}* mice were transduced with *Jak2^{V617F}-Ires-GFP*, *Mpl^{W515L}-Ires-GFP*, and pMSCV-*Ires-GFP* retroviruses. GFP-

positive cells sorted by FACS were used for ex vivo CFU assays and in vivo BM transplantation to monitor disease development and treatment response (Fig. 2A). Deletion of *Dusp1* significantly reduced the $JAK2^{V617F}$ (>90%) and MPL^{W515L} (>70%) induced CFUs compared to vector control, cells expressing *MSCV-Ires-GFP* (Fig. 2B). As reported earlier [3, 29], mice transplanted with wild-type Kit^+ cells expressing $Jak2^{V617F}$ developed PV exhibiting higher RBC, Hb and HCT levels with a modest increase in WBC levels while mice recipients of Mpl^{W515L} expressing Kit cells are more prone to develop leukemia (elevated WBCs) besides inducing the phenotypic characteristics of MPN, elevated RBCs, Hb, and HCT (Fig. 2C, D and Supplementary Fig. 4A–D). Unexpectedly, we observed *Dusp1* deficiency abolished $Jak2^{V617F}$ -induced MPN, while MPL^{W515L} -induced leukemogenesis was unaffected (Fig. 2E–H and Supplementary Fig. 4E–H). Strikingly, $Jak2^{V617F}$ expressing cells lacking *Dusp1* were gradually removed from the bone marrow compared to control (*Dusp1* deficient c- Kit^+ cells expressing vector), suggesting that *Dusp1* deletion is synthetic lethal to $Jak2^{V617F}$ expression (Fig. 2E, H). To ensure that $Jak2^{V617F}$ expressing cells were completely eradicated and mice were cured of the disease, secondary transplantation was performed. Secondary recipients of wild-type primary BM cells expressing $Jak2^{V617F}$ showed normal engraftment and disease development (increased RBCs, Hb, and HCT) while mice recipients of $Jak2^{V617F}$ primary BM cells lacking *Dusp1* exhibited disease-free survival without any trace of $Jak2^{V617F}$ expressing cells determined by quantitation of GFP-positive cells (data not shown). Altogether, these data provide evidence that deletion of *Dusp1* is synthetic lethality to $Jak2^{V617F}$ induced MPN.

Next, we examined the *Dusp1* dependency using a $Jak2^{V617F/+}$ knock-in mouse model to rule out retroviral-mediated bias in cellular transformation. The $Dusp1^{-/-}/RosaCre^{ER}$ mice were bred with $Jak2^{floxed/+}$ mice [30] to generate $Dusp1^{-/-}/Jak2^{floxed/+}/RosaCre^{ER}$. Bone marrow cells were harvested from the $Dusp1^{-/-}/RosaCre^{ER}.Jak2^{floxed/+}/RosaCre^{ER}$, and $Dusp1^{-/-}/Jak2^{floxed/+}/RosaCre^{ER}$ mice, treated overnight with 4-OHT to generate $Jak2^{V617F/+}$. One million $Jak2^{V617F/+}$ (CD45.2) recombined cells were mixed with equal amount of normal BM cells from the *BoyJ* (CD45.1) mice and transplanted in lethally irradiated *BoyJ* recipients (Fig. 3A). Mice recipients of $Jak2^{V617F/+}$ cells developed all features of MPN as described earlier [30] (Fig. 3). In contrast, mice transplanted with $Jak2^{V617F/+}/Dusp1^{-/-}$ cells did not develop the disease. Instead, these cells were gradually depleted and cleared from the bone marrow within thirty weeks, determined by the levels of CD45.2 (Fig. 3B). As reported earlier, the mice transplanted with $Dusp1^{-/-}/RosaCre^{ER}$ exhibited persistent engraftment like wild-type cells¹⁵. Altogether these results provide clear evidence that *Dusp1* confers dependence to $Jak2^{V617F}$ induced MPNs, and its deletion is synthetic lethal.

Chemical inhibition of DUSP1 by BCI suppresses the MPN

To determine the potential of *Dusp1* targeting towards clinical development, a small molecule DUSP1 inhibitor (BCI) [21] was evaluated alone and in combination with ruxolitinib in a competitive transplant model. One million primary bone marrow cells from the $Jak2^{V617F/+}/RosaCre^{ER}$ (CD45.2) mice or $Jak2^{V617F/+}/Dusp1^{-/-}/RosaCre^{ER}$ (CD45.2) with one million normal bone marrow cells from the *BoyJ* mice (CD45.1) were transplanted in irradiated *BoyJ* mice (Fig. 4A). Mice recipients of $Jak2^{V617F/+}/RosaCre^{ER}$ BM cells

treated with ruxolitinib (100 mg/kg daily) unexpectedly displayed increased *Jak2^{V617F}* allele burden (CD45.2) with progressive MPN phenotype compared to the wild-type cells, CD45.1 (Fig. 3B–F). Thus, recapitulating the in vitro observation of ruxolitinib resistance in MPN cells (Fig. 1C). Even *Jak2^{V617F}* cells lacking *Dusp1* showed prolonged persistence with ruxolitinib treatment than the vehicle-treated control (Fig. 3B–F). Conversely, mice treated with BCI alone exhibited modest reduction in *Jak2^{V617F}* allele burden while suppressing the WBC and HCT levels (Fig. 4B–E). Unfortunately, all treated mice displayed progressive disease. Even the combination treatment, BCI+Ruxolitinib, failed to exert clonal selectivity and all treated mice exhibited progressive disease (Fig. 4B–E). Given that BCI also inhibits DUSP6 besides inhibiting *Dusp1*, that causes phospho-Erk1/2 rebound, it is likely possible that a combination of BCI with MEK/ERK inhibitors may effectively deplete the JAK2 mutant cells. To test this, mice were treated with BCI+Trametinib combination showed more significant suppression of WBCs, RBCs, and HCT levels compared to BCI alone or BCI+ Ruxolitinib treatment. However, clonal selectivity and antileukemic effect of BCI+Trametinib treatment was not different than the BCI or BCI+ Ruxolitinib. Nonetheless, despite enhanced myelosuppressive response of BCI+Trametinib combination, all treated mice displayed progressive disease. Altogether, these data suggest that chemical inhibition of DUSP1 by BCI is not effective as noted with genetic deletion.

*Enhanced pERK and BCL2 expression due to BCI treatment abrogate *Jak2^{V617F}* clonal selectivity.* Next, we sought to understand how *Dusp1* deletion confers lethality to *Jak2^{V617F}* expressing cells and why its chemical inhibition is ineffective. Because BCI inhibits both *Dusp1* and *Dusp6*, we hypothesized that inhibition of *Dusp6* abolished *Dusp1* dependency in *Jak2^{V617F}* cells. We reasoned that a comprehensive analysis of *Dusp1* and *Dusp6* substrates from the MAPK pathway in cells lacking *Dusp1^{-/-}*, *Dusp6^{-/-}* and *Dusp1^{-/-}/*Dusp6^{-/-}** (genetic equivalence of BCI treatment) and cells treated with BCI will illuminate the underlying mechanisms driving synthetic lethality and why BCI treatment is ineffective in targeting *Jak2^{V617F}* clones (Fig. 5A, C). Bone marrow derived Kit⁺ cells from the wild-type, *Dusp1^{-/-}*, *Dusp6^{-/-}* and *Dusp1^{-/-}/*Dusp6^{-/-}** mice were transduced with either pMSCV-ires-GFP (pMIG) or pMSCV-*Jak2^{V617F}*-ires-GFP retroviruses followed with cell sorting of GFP expressing cells. Total cell extracts from these GFP-positive cells were analyzed for MAPK signaling pathway components by western blotting. As reported earlier [21], deletion of *Dusp1* resulted in higher phospho-p38 with modest increase in phospho-JNK while phospho-ERK levels were not changed compared to wild-type vector transduced control cells (Fig. 5A, B). Expression of *Jak2^{V617F}* in wild-type cells displayed increased pERK levels and significantly suppressed the phospho-p38 while pJnk levels were not significantly affected. In contrast, *Jak2^{V617F}* expression in *Dusp1* deficient cells exhibited greatly enhanced p-p38 with a modest increment in pJnk levels, while pErk levels remained the same (Fig. 5B). In contrast, the deficiency of *Dusp6* in the context of *Jak2^{V617F}* expression exhibited strong suppression of phospho-p38 levels and modest reduction in pErk1/2 levels while pJnk levels remained the same compared to wild-type cells expressing pMIG or *Jak2^{V617F}* (Fig. 5B). Interestingly, expression of *Jak2^{V617F}* in double knock-out (*Dusp1^{-/-}/*Dusp6^{-/-}**) cells displayed significantly enhanced pErk levels compared to wild-type and *Dusp1^{-/-}* while pJnk levels were unaltered compared to *Dusp1* deficient cells. Perhaps more importantly, phospho-p38 levels are significantly suppressed compared to *Jak2^{V617F}*

expressing *Dusp1*^{-/-} cells. Together these data suggest that concomitant genetic inhibition of both *Dusp1* and *Dusp6* enhances Erk activation while suppressing the p38 activity in the context of *Jak2*^{V617F} expression (Fig. 5B). In contrast, *Dusp1*^{-/-}/*Dusp6*^{-/-} expressing vector exhibited increased expression of both phospho-p38 and pErk1/2. Nonetheless, activation of Erk1/2 was lower in the normal cells compared to *Dusp1*^{-/-}/*Dusp6*^{-/-} expressing *Jak2*^{V617F} (Fig. 5B). Given that both p38 and Erk regulate the intrinsic apoptotic activity by modulating the turnover of p53 and Bcl2 family members [25, 28, 31–34], their expression and phosphorylation were evaluated by immunoblotting. Expression of *Jak2*^{V617F} induced the expression of Bcl-xl, Bax and Mcl1. We observed increased expression of proapoptotic protein Bax in *Jak2*^{V617F}/*Dusp1*^{-/-} cells while double knock-out cells exhibited lower Bax expression compared to *Dusp1*^{-/-} alone. Importantly, we noted increased expression of Bcl2 and Bcl-xl in *Jak2*^{V617F}/*Dusp1*^{-/-}/*Dusp6*^{-/-} cells and (Fig. 5B), which suggests simultaneous inhibition of both *Dusp1* and *Dusp6* would drive higher apoptotic threshold by inducing the expression of Bcl2 and Bcl-xl expression. While we noted induce expression of Mcl1 by *Jak2*^{V617F} signaling, its expression was not affected by deletion of either *Dusp1* or *Dusp6*. Strikingly, we observed a complete suppression of p53 phosphorylation at serine 15 (S15) in wild-type cells expressing *Jak2*^{V617F} (Fig. 5B). Since p38 directly phosphorylates p53 at the S15, which prevents its interaction with MDM2 and degradation. Consequently, wild-type cells expressing *Jak2*^{V617F} suppressed p38 activity by *Dusp1* that resulted in enhanced p53 degradation due to reduced phosphorylation at serine 15 (Fig. 5B). In contrast, *DUSP1* deficiency in the context of *Jak2*^{V617F} expression displayed increased p38 activity and enhanced p53 phosphorylation that induced its expression, which led to selective demise of *Jak2*^{V617F} clones (Fig. 5B).

To examine why chemical inhibition of *DUSP1* by BCI was ineffective in targeting the *Jak2*^{V617F} expressing cells, MAPK signaling and levels of p53 and Bcl2 were evaluated in cells treated with BCI alone and with MEK/ERK inhibitors (Fig. 5C). BCI treatment alone exhibited increased phospho-p38 in both vector and *Jak2*^{V617F} expressing cells compared to vehicle control (Fig. 5D). As expected, it also significantly increased pErk levels in *Jak2*^{V617F} expressing cells likely due to the inhibition of *Dusp6* (Fig. 5D). Surprisingly, BCI treatment alone significantly induced Bcl2, Bcl-xl, and Mcl1 levels (Fig. 5D, E). These data imply that sustained Erk activity in *Jak2*^{V617F} expressing cells due to *Dusp1* and *Dusp6* inhibition induces antiapoptotic Bcl2 proteins and disrupted the selective activation of p38 mediated p53 stabilization. This resulted in a loss of therapeutic window and clonal selectivity, as an equal amount of p53 protein was observed in both vector and *Jak2*^{V617F}-expressing cells (Fig. 5D). We sought to test whether inhibition of Mek/Erk with trametinib would restore the stabilization of p53. Cells treated with trametinib (Mek inhibitor) alone, nonetheless, exhibited modest increase in phospho-p38 and p53 levels in *Jak2*^{V617F} expressing cells compared to vector control, but also displayed modest induction in Bcl2 and Mcl1 levels (Fig. 5D, E). Paradoxically, treatment with Trametinib+BCI combination resulted in pErk rebound (Fig. 5D). Consequently, it failed to achieve selective stabilization of p53 in *Jak2*^{V617F} expressing cells. Similarly, Erk inhibitor in combination with BCI was ineffective in suppressing the pErk and selective stabilization of p53 in *Jak2*^{V617F} expressing cells (data not shown). Altogether these data imply that *Dusp6* functions as tumor suppressor in MPN. However, a recent study using BCI, which inhibits both *Dusp1* and *Dusp6*

concluded that *Dusp6* drives leukemogenesis and resistance to *Jak2* inhibition in MPN [35]. To rule out this discrepancy, MPN development were assessed using bone marrow cells from *Dusp6* deficient mice. Bone marrow derived c-Kit⁺ cells from the wild-type and *Dusp6* deficient mice were transduced with retroviruses pMSCV-Ires-GFP, *Jak2*^{V617F}-Ires-GFP and *MPL*^{W515L}-ires-GFP. Eighty to one-hundred thousand FACS-sorted GFP expressing cells were transplanted in the recipient mice. As noted earlier, mice transplanted with wild-type c-Kit⁺ cells expressing *Jak2*^{V617F} and *MPL*^{W515L} developed MPN (Fig. 5F). Notably, mice recipients of *Jak2*^{V617F} and *MPL*^{W515L} cells lacking *Dusp6* exhibited more aggressive MPN phenotype, significantly increased WBC, RBC and HCT (Fig. 5G–I). These data provide a direct and clear evidence that DUSP6, unlike DUSP1, functions as a tumor suppressor in MPN.

To ensure that the inhibition of DUSP6 abrogated BCI treatment, mice were transplanted with *Jak2*^{V617F} expressing c-Kit⁺ cells lacking both *Dusp1* and *Dusp6*. As expected, mice transplanted with *Dusp1*^{-/-}/*Dusp6*^{-/-} cells expressing *Jak2*^{V617F} exhibited progressive disease (Fig. 5J). Thus, confirming that the inhibition of DUSP6 abrogates DUSP1 dependency in *Jak2*^{V617F} induced MPN. Together, these data prove that the off-target inhibition of DUSP6 by BCI causes pErk rebound that resulted in higher expression of Bcl2 and Bcl-xl expression and non-selective p53 stabilization.

Ectopic expression of *Dusp6* and BCI treatment selectively eradicated the *Jak2*^{V617F} cells

To further confirm that the inhibition of DUSP6 abolishes *Jak2*^{V617F} clonal selectivity, we transplanted *Jak2*^{V617F/+} *RosaCreER* c-Kit⁺ cells expressing pMSCV-Ires-cherry (vector) or pMSCV-*Dusp6*-cherry in C57Bl/6 recipient mice (Fig. 6A). Because *Dusp6* overexpression will inhibit pERK levels, as expected, mice recipients of *Jak2*^{V617F/+} cells overexpressing *Dusp6* displayed slower disease progression with a reduced leukemic burden (Fig. 6B). Strikingly, BCI treatment of mice transplanted with *Dusp6* overexpressing *Jak2*^{V617F/+} cells not only normalized the WBC, RBC, HCT, and platelets levels but also selectively eradicated the *Jak2*^{V617F/+} cells (Fig. 6C-F). To confirm that the JAK2 mutant cells are fully eradicated, secondary transplantation was performed. Secondary bone marrow recipients of BCI-treated *Jak2*^{V617F/+} +*Dusp6* overexpressing cells did not show any presence of cherry-positive cells determined by FACS while mice recipients of vehicle-treated mice *Jak2*^{V617F/+} +*Dusp6* overexpression, exhibited robust engraftment (Fig. 6G). Thus, confirming that *Dusp6* overexpression and BCI treatment selectively eradicated the *Jak2*^{V617F/+} cells. Altogether these data provide evidence that *Dusp6* functions as a tumor suppressor in the context of *Jak2*^{V617F/+}, and its off-target inhibition by BCI abolishes DUSP1 dependency in *Jak2*^{V617F/+} driven MPN.

DISCUSSION

Unlike the clinical efficacy of ABL inhibitors in CML, treatment outcomes to JAK2 inhibitors are limited to reducing the spleen size and alleviating the constitutional symptoms [36, 37]. Importantly, *Jak* inhibitors failed to induce cellular or molecular remission as they lacked clonal selectivity [38]. Besides, resistance, disease relapse, and treatment-related toxicities resulting in treatment discontinuation within months are common [39]. The

persistence of *Jak2*^{V617F} clones is mainly responsible for treatment failure. Therefore, therapeutic approaches are needed to eradicate the JAK2 mutant clones for durable or curative response.

Efficacy of TKI treatment is mediated by oncogene addiction [13, 40, 41]. However, genetic, cellular, and environmental contexts alter the oncogene dependence that results in treatment failure. For instance, cancer stem cells from both leukemia and solid tumors are intrinsically resistant to TKI treatment where growth-factor signaling abrogates oncogene dependence that causes intrinsic TKI resistance [16–18, 21]. MPNs are considered as oncoinflammatory disorders where malignant clones are the main source and target of cytokine storm acting both locally and systemically [42]. Significantly elevated levels of almost fifty different cytokines have been reported in MPN patients by different groups [42, 43]. Among these are hematopoietic cytokines, such as IL3, TPO, GM-CSF, HGF, and TGFβ that are implicated in abrogating TKI response were noted [21]. As a proof, we provide evidence that the addition of IL3 is sufficient to abrogate TKI response in BaF3 cells. Similarly, hematopoietic cytokines in combination significantly increased the IC50 of ruxolitinib in human and primary mouse MPN cells (Fig. 1C), which also implied that continued ruxolitinib treatment not only cause persistence rather it will exert *Jak2*^{V617F} clonal dominance. As envisioned from the in vitro data, ruxolitinib treatment exhibited clonal dominance of *Jak2*^{V617F} mutant clones in competitive transplants (Fig. 3). Comparative whole genome gene expression analysis of *Jak2*^{V617F} and *Mpl*^{W515L} in primary cells revealed that both oncogenes, besides commonly regulating a significant number of genes, also modulate the expression of a distinct set of genes. As reported earlier [23], we observed increased expression of key signaling intermediates driving JAK-STAT pathway by both oncogenes. In addition, we noted increased expression of, *Csf1r*, *Csf3r* and *HRas* implicated in driving JAK2^{V617F}-negative MPN which is shown to be addicted to enhanced MAPK signaling [44, 45]. Collectively, these data support that oncogenic JAK2^{V617F} and MPL^{W515L} signaling beside activating JAK-STAT pathway also modulate MAPK signaling. Consequently, MAPK pathway inhibitors targeting BRAF, MEK and ERK alone or in combination with ruxolitinib have shown better therapeutic response than ruxolitinib alone [9, 11]. However, both MEK and ERK inhibitors in combination with ruxolitinib, nonetheless, exerted improved efficacy in ameliorating the disease phenotype but lacked clonal selectivity [9, 11, 45].

Pharmacological targeting of MEK/ERK and JAK2 either alone or in combination exert cytostatic response. Therefore, treatment outcomes to cytostatic drugs are short-lived as most patients develop resistance within short duration. The reasons for cytostatic response exerted by MER/ERK or JAK2 inhibitors is not fully understood. Enhanced MAPK-ERK activity is one of the central features in many cancers implicated in driving proliferation and survival by modulating the apoptotic threshold mainly by stabilizing and/or activating the antiapoptotic proteins, such as BCL2 family members and repressing the proapoptotic proteins, such as BIM [46–48]. Paradoxically, sustained ERK activation also induces a robust apoptotic response, which will be counter-selective for cellular transformation [25, 27, 49, 50]. To counter this, most cancers induce MAPK negative regulators to fine-tune the MAPK signaling output to suppress ERK-induced apoptotic signaling while selectively promoting survival and proliferation [51, 52]. We observed a significant induction of MAPK signaling genes in both JAK2^{V617F} and MPL^{W515L} cells

where JAK2^{V617F} signaling exhibited a more robust induction of MAPK network genes compared to Mpl^{W515L} (Supplementary Fig. 2A, 18 genes are not induced in Mpl^{W515L} cells from the MAPK network; Supplementary Fig. 3A, 7 genes are under-expressed in Mpl^{W515L} cells from the ERK1/2 network). Consequently, both JAK2^{V617F} and MPL^{W515L} cells exhibited induced expression of several MAPK negative regulators, including DUSP family members (Supplementary Fig. 1H, I). Interestingly, a recent study implicated the role of DUSP1 in protecting the *Jak2*^{V617F} MPN cells from cellular stress caused by the inflammatory cytokines [53]. Importantly, we observed induced expression MAPK negative regulators (*Sfrp1*, *Sfrp5*, *Mapk8ip1* and *Nppa*) in *Jak2*^{V617F} cells while their expressions are significantly suppressed in MPL^{W515L} cells which may be a reason for conferring the dependence of JAK2^{V617F} to DUSP1. SFRP proteins negatively regulate Wnt/β-catenine signaling by preventing the Wnt binding to its receptors, Fzds. There is extensive crosstalk between MAPK and WNT/β-catenine signaling [54]. Depending on the cellular context, a positive interaction between MAPK and WNT/β-catenine drives cellular transformation, while a negative interaction promotes anti-tumor response [54]. For instance, overexpression of SFRP1 in colon cancer cells significantly decreased the protein levels of WNT, β-catenin and apoptosis-related proteins, including MMP2, MMP9, Twist, CDK1, TGF, and BCL2 [55]. Likewise, reduced expression of scaffold protein MAPL8ip1 would result in reduced JNK activation that will alter apoptotic threshold due to reduced AP1 activation [21]. Cumulatively induced expression of these negative regulators in *Jak2*^{V617F} cells makes it more vulnerable to MAPK negative feedback perturbations. Consequently, the deletion of *Dusp1* resulted in the selective demise of *Jak2*^{V617F} but not the *Mpl*^{W515L} cells. Future studies will uncover the molecular basis of altered dependence on MAPK negative feedback in JAK2^{V617F} and MPL^{W515L}. Perhaps suppressing β-catenin by overexpressing the *Sfrp1* in combination with DUSP1 depletion may help in eradicating the *MPL*^{W515L} cells.

Chemical inhibition of DUSP1 by BCI failed to recapitulate DUSP1 deletion-induced synthetic lethality to JAK2^{V617F}. Because BCI inhibits both DUSP1 and DUSP6 [21], we hypothesized that simultaneous inhibition of DUSP6 induces ERK1/2 activity, neutralizing the effect of DUSP1 inhibition. As expected, BCI treatment or genetic deletion of *Dusp1*^{-/-}/*Dusp6*^{-/-} in the context of *Jak2*^{V617F} expression significantly increased ERK1/2 activity (Fig. 5). In contrast, cells lacking *Dusp1* only exhibited strong p38 activity compared to *Jak2*^{V617F} expressing wild-type and *Dusp6* deficient cells. Even cells lacking both *Dusp1* and *Dusp6* exhibited suppressed p38 activity in the context of *Jak2*^{V617F} expression. This suggests a complex regulatory network between *Dusp1* and *Dusp6* modulating the ERK1/2 and p38 activity in normal and oncogenic contexts. Mechanistically, inhibition of DUSP1 activated p38 that phosphorylates p53 at S15, which blocks its MDM2 mediated degradation. Consequently, higher p53 expression selectively induced apoptosis in JAK2^{V617F} expressing cells as vector expressing *Dusp1* deficient cells did not show induced p53 expression. To ascertain the role of p38 in p53 stabilization, p38 inhibition and overexpression studies were performed (Supplementary Fig. 5A). As expected, p38 inhibition increased the CFU numbers in wild-type cells while expression of JAK2^{V617F} significantly increased the CFU number and did not show any response to p38 inhibition as p38 is already inhibited into these cells. In contrast, inhibition of p38 in *Jak2* expressing cells lacking *Dusp1* exhibited increased CFU while its inhibition in control cells had no

effect. Interestingly, ectopic expression of MAPK14 (p38- α) suppressed the CFU numbers in *Jak2^{V617F}* expressing wild-type cells while its overexpression did not show any change in CFU numbers in control and *Dusp1* deficient *JAK2^{V617F}* expressing cells (Supplementary Fig. 5B). These results provide direct evidence that downregulation of p53 under *JAK2^{V617F}* signaling is caused by *Dusp1* mediated p-p38 blockade.

The enhanced pERK1/2 activity due to BCI treatment suggested that perhaps a combination of BCI with MEK or ERK inhibitor may likely restore clonal selectivity. Unfortunately, treatment with BCI or trametinib alone significantly induced the expression of BCL2 and lost selective stabilization of p53 likely due to pERK rebound in both normal and *Jak2^{V617F}* expressing cells (Fig. 5D). This prompted us to test whether a combination of BCI with MDM2 inhibitor (idasanutlin) and BCL2 inhibitor (venetoclax) might be effective in depleting the *Jak2^{V617F}* clones. In vitro evaluations showed significant suppression of *Jak2^{V617F}* cells by BCI+Idasanutlin+venetoclax combination (Supplementary Fig. 6A). Mice treated with idasanutlin+venetoclax or BCI+idasanutlin+venetoclax showed significant suppression compared to vehicle-treated mice but all treated mice displayed progressive disease (Supplementary Fig. 6B). Unfortunately, venetoclax+ idasanutlin treatment exerted significant toxicity (lethargy and scruffy coat) that resulted in treatment discontinuation and prevented us from further evaluation. Our data suggest that the inefficacy of BCI treatment was due to DUSP6 inhibition. *Dusp6* has been shown to have tumor suppressor as well as oncogenic role in different tumors [56–58]. A recent report during the preparation of this manuscript implicated DUSP6 in leukemic transformation of MF to secondary AML (sAML) and conferring resistance to JAK2 inhibitors [59]. They performed both shRNA-mediated knockdown and chemical inhibition for validation studies. Both shRNA-mediated knockdown and a higher concentration of BCI are prone to have off-target inhibitory effect. Using DUSP6 knock-out mice, we clearly demonstrate that the loss of DUSP6 had no effect in MPNs induced by *JAK2^{V617F}* or *MPL^{W515L}* rather its absence causes aggressive disease progression compared to wild-type BM donors, which supports its role as a tumor suppressor. Besides, using mice lacking both *Dusp1* and *Dusp6* mimicking the BCI treatment, we provide evidence that inhibition of DUSP6 either chemically or genetically abolishes DUSP1 dependency in *Jak2^{V617F}* induced MPN. Moreover, ectopic overexpression of *Dusp6* in *Jak2^{V617F}* cells restored BCI sensitivity. Mice transplanted with *Jak2^{V617F/+}* cells overexpressing *Dusp6* exhibited disease suppression likely due to inhibition of pERK1/2. Strikingly, BCI treatment of mice transplanted with *Dusp6* overexpressing *Jak2^{V617F}* cells cured the mice of disease. These data provide evidence that the inefficacy of BCI was due to pERK rebound because of *Dusp6* inhibition. Importantly, our study demonstrates that the dynamic balance between Erk and p38 activation determines whether the cell survives or undergoes apoptosis. Mapk-negative regulator, DUSP1, regulates this balance to support the *JAK2^{V617F}*-driven neoplasm.

In conclusion, we show that *JAK2^{V617F}* signaling induces DUSP1 to fine-tune MAPK signaling output that suppresses p53 expression by enhancing its degradation. Consequently, DUSP1 confers dependence to *JAK2^{V617F}* driven malignancies. Deletion of *Dusp1* selectively induces p38 activity that promotes p53 phosphorylation and stabilization, which results in the selective eradication of *Jak2^{V617F}* cells. In contrast, ERK1/2 negative regulator, DUSP6, functions as a tumor suppressor as its inhibition abrogated DUSP1 dependence,

likely due to negative crosstalk between ERK and p38 [60]. Altogether these data provide evidence that the inhibitors selectively targeting DUSP1 would exert a durable or curative response to JAK2^{V617F} driven MPN. Finally, our study exposes a new Achilles' heel to malignancies fueled by enhanced MAPK signaling and support for selective targeting of MAPK negative regulators for effective treatment outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Benjamin Ebert and Ann Mullaly for providing the *Jak2*^{V617F/+} mice and pMSCV-CALR^{wt} and pMSCV-CALR^{mut} retroviral constructs. This study was supported by grants to Mohammad Azam (M Azam) from the National Cancer Institutes at NIH (RO1CA211594) and (RO1CA250516) and to RL by MSKCC Support Grant/Core Grant P30 CA008748. MA is a recipient of the bridge award from the American Society of Hematology (ASH).

FUNDING

This study was supported by grants to MA from the National Cancer Institutes at NIH (RO1CA211594) and (RO1CA250516) and to R.L.L. by MSKCC Support Grant/Core Grant P30 CA008748.

DATA AVAILABILITY

RNA-seq data are publicly available at GSE229318. For materials and other resources, please contact the corresponding author; Mohammad.Azam@cchmc.org.

REFERENCES

1. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*. 2005;7:387–97. [PubMed: 15837627]
2. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434:1144–8. [PubMed: 15793561]
3. Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med*. 2006;3:e270. [PubMed: 16834459]
4. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 2013;369:2379–90. [PubMed: 24325356]
5. Fisher DAC, Fowles JS, Zhou A, Oh ST. Inflammatory pathophysiology as a contributor to myeloproliferative neoplasms. *Front Immunol*. 2021;12:683401. [PubMed: 34140953]
6. Wolf A, Eulenfeld R, Gäbler K, Rolvering C, Haan S, Behrmann I, et al. JAK2-V617F-induced MAPK activity is regulated by PI3K and acts synergistically with PI3K on the proliferation of JAK2-V617F-positive cells. *JAKSTAT*. 2013;2:e24574. [PubMed: 24069558]
7. Bartalucci N, Guglielmelli P, Vannucchi AM. Rationale for targeting the PI3K/Akt/mTOR pathway in myeloproliferative neoplasms. *Clin Lymphoma Myeloma Leuk*. 2013;13:S307–9. [PubMed: 24290217]
8. Gerds AT, Bartalucci N, Assad A, Yacoub A. Targeting the PI3K pathway in myeloproliferative neoplasms. *Expert Rev Anticancer Ther*. 2022;22:835–43. [PubMed: 35763287]

9. Stivala S, Codilupi T, Brkic S, Baerenwaldt A, Ghosh N, Hao-Shen H, et al. Targeting compensatory MEK/ERK activation increases JAK inhibitor efficacy in myeloproliferative neoplasms. *J Clin Investig.* 2019;129:1596–611. [PubMed: 30730307]
10. Jayavelu AK, Schnoder TM, Perner F, Herzog C, Meiler A, Krishnamoorthy G, et al. Splicing factor YBX1 mediates persistence of JAK2-mutated neoplasms. *Nature.* 2020;588:157–63. [PubMed: 33239784]
11. Brkic S, Stivala S, Santopolo A, Szybinski J, Jungius S, Passweg JR, et al. Dual targeting of JAK2 and ERK interferes with the myeloproliferative neoplasm clone and enhances therapeutic efficacy. *Leukemia.* 2021;35:2875–84. [PubMed: 34480104]
12. Sawyers CL. Shifting paradigms: the seeds of oncogene addiction. *Nat Med.* 2009;15:1158–61. [PubMed: 19812578]
13. Weinstein IB. Cancer. Addiction to oncogenes—the Achilles heel of cancer. *Science.* 2002;297:63–4. [PubMed: 12098689]
14. Pagliarini R, Shao W, Sellers WR. Oncogene addiction: pathways of therapeutic response, resistance, and road maps toward a cure. *EMBO Rep.* 2015;16:280–96. [PubMed: 25680965]
15. O'Hare T, Zabriskie MS, Eiring AM, Deininger MW. Pushing the limits of targeted therapy in chronic myeloid leukaemia. *Nat Rev Cancer.* 2012;12:513–26. [PubMed: 22825216]
16. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Investig.* 2011;121:396–409. [PubMed: 21157039]
17. Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature.* 2012;487:505–9. [PubMed: 22763448]
18. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature.* 2012;487:500–4. [PubMed: 22763439]
19. Azhar M, Kincaid Z, Kesarwani M, Menke J, Schwieterman J, Ansari S, et al. Rational polypharmacological targeting of FLT3, JAK2, ABL, and ERK1 suppresses the adaptive resistance to FLT3 inhibitors in AML. *Blood Adv.* 2023;7:1460–76. [PubMed: 36044389]
20. Azhar M, Kincaid Z, Kesarwani M, Ahmed A, Wunderlich M, Latif T, et al. Momelotinib is a highly potent inhibitor of FLT3-mutant AML. *Blood Adv.* 2022;6:1186–92. [PubMed: 34768286]
21. Kesarwani M, Kincaid Z, Gomaa A, Huber E, Rohrabough S, Siddiqui Z, et al. Targeting c-FOS and DUSP1 abrogates intrinsic resistance to tyrosine-kinase inhibitor therapy in BCR-ABL-induced leukemia. *Nat Med.* 2017;23:472–82. [PubMed: 28319094]
22. Fisher DAC, Miner CA, Engle EK, Hu H, Collins TB, Zhou A, et al. Cytokine production in myelofibrosis exhibits differential responsiveness to JAK-STAT, MAP kinase, and NFkappaB signaling. *Leukemia.* 2019;33:1978–95. [PubMed: 30718771]
23. Rupal R, Al-Shahrour F, Abdel-Wahab O, Patel JP, Brunel JP, Mermel CH, et al. Integrated genomic analysis illustrates the central role of JAK-STAT pathway activation in myeloproliferative neoplasm pathogenesis. *Blood.* 2014;123:e123–33. [PubMed: 24740812]
24. Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. *Trends Biochem Sci.* 2006;31:268–75. [PubMed: 16603362]
25. Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death—apoptosis, autophagy and senescence. *FEBS J.* 2010;277:2–21. [PubMed: 19843174]
26. Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* 1995;80:179–85. [PubMed: 7834738]
27. Subramaniam S, Unsicker K. ERK and cell death: ERK1/2 in neuronal death. *FEBS J.* 2010;277:22–9. [PubMed: 19843173]
28. Martin P, Pognonec P. ERK and cell death: cadmium toxicity, sustained ERK activation and cell death. *FEBS J.* 2010;277:39–46. [PubMed: 19843171]
29. Zaleskas VM, Krause DS, Lazarides K, Patel N, Hu Y, Li S, et al. Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F. *PLoS ONE.* 2006;1:e18. [PubMed: 17183644]

30. Mullally A, Lane SW, Ball B, Megerdichian C, Okabe R, Al-Shahrour F, et al. Physiological Jak2V617F expression causes a lethal myeloproliferative neoplasm with differential effects on hematopoietic stem and progenitor cells. *Cancer Cell*. 2010;17:584–96. [PubMed: 20541703]
31. Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. *Exp Ther Med*. 2020;19:1997–2007. [PubMed: 32104259]
32. De Chiara G, Marcocci ME, Torcia M, Lucibello M, Rosini P, Bonini P, et al. Bcl-2 Phosphorylation by p38 MAPK: identification of target sites and biologic consequences. *J Biol Chem*. 2006;281:21353–61. [PubMed: 16714293]
33. Whitaker RH, Cook JG. Stress Relief Techniques: p38 MAPK determines the balance of cell cycle and apoptosis pathways. *Biomolecules*. 2021;11:1–16.
34. Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E, et al. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J*. 1999;18:6845–54. [PubMed: 10581258]
35. Kong T, Laranjeira ABA, Yang K, Fisher DAC, Yu L, Poittevin De La Fregoniere L, et al. DUSP6 mediates resistance to JAK2 inhibition and drives leukemic progression. *Nat Cancer* 2023;4:108–27. [PubMed: 36581736]
36. Verstovsek S, Kantarjian H, Mesa RA, Pardanani AD, Cortes-Franco J, Thomas DA, et al. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. *N Engl J Med*. 2010;363:1117–27. [PubMed: 20843246]
37. Pardanani A, Tefferi A, Jamieson C, Gabrail NY, Lebedinsky C, Gao G, et al. A phase 2 randomized dose-ranging study of the JAK2-selective inhibitor fedratinib (SAR302503) in patients with myelofibrosis. *Blood Cancer J*. 2015;5:e335. [PubMed: 26252788]
38. Talpaz M, Kiladjian JJ. Fedratinib, a newly approved treatment for patients with myeloproliferative neoplasm-associated myelofibrosis. *Leukemia*. 2021;35:1–17. [PubMed: 32647323]
39. Kirito K Recent progress of JAK inhibitors for hematological disorders. *Immunol Med*. 2022;45:1–12. [PubMed: 34053404]
40. Sharma SV, Settleman J. Oncogene addiction: setting the stage for molecularly targeted cancer therapy. *Genes Dev*. 2007;21:3214–31. [PubMed: 18079171]
41. Weinstein IB, Joe A. Oncogene addiction. *Cancer Res*. 2008;68:3077–80. discussion 80 [PubMed: 18451130]
42. Masselli E, Pozzi G, Gobbi G, Merighi S, Gessi S, Vitale M, et al. Cytokine profiling in myeloproliferative neoplasms: overview on phenotype correlation, outcome prediction, and role of genetic variants. *Cells*. 2020;9:1–23.
43. Wang Y, Zuo X. Cytokines frequently implicated in myeloproliferative neoplasms. *Cytokine X* 2019;1:100005. [PubMed: 33604548]
44. Maxson JE, Gotlib J, Pollyea DA, Fleischman AG, Agarwal A, Eide CA, et al. Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. *N Engl J Med*. 2013;368:1781–90. [PubMed: 23656643]
45. Rohrabough S, Kesarwani M, Kincaid Z, Huber E, Leddonne J, Siddiqui Z, et al. Enhanced MAPK signaling is essential for CSF3R-induced leukemia. *Leukemia* 2017;31:1770–8. [PubMed: 28031554]
46. Balmanno K, Cook SJ. Tumour cell survival signalling by the ERK1/2 pathway. *Cell Death Differ*. 2009;16:368–77. [PubMed: 18846109]
47. Yue J, Lopez JM. Understanding MAPK signaling pathways in apoptosis. *Int J Mol Sci*. 2020;21:1–22.
48. Clybourn C, Merino D, Nebl T, Masson F, Robati M, O'Reilly L, et al. Alternative splicing of Bim and Erk-mediated Bim(EL) phosphorylation are dispensable for hematopoietic homeostasis in vivo. *Cell Death Differ*. 2012;19:1060–8. [PubMed: 22240894]
49. Sugiura R, Satoh R, Takasaki T. ERK: a double-edged sword in cancer. ERK-dependent apoptosis as a potential therapeutic strategy for cancer. *Cells*. 2021;10:1–29.
50. Teixeira E, Daniels MA. ERK and cell death: ERK location and T cell selection. *FEBS J*. 2010;277:30–8. [PubMed: 19843172]
51. Lake D, Correa SA, Muller J. Negative feedback regulation of the ERK1/2 MAPK pathway. *Cell Mol Life Sci*. 2016;73:4397–413. [PubMed: 27342992]

52. Seternes OM, Kidger AM, Keyse SM. Dual-specificity MAP kinase phosphatases in health and disease. *Biochim Biophys Acta Mol Cell Res.* 2019;1866:124–43. [PubMed: 30401534]
53. Stetka J, Vyhlidalova P, Lanikova L, Koralkova P, Gursky J, Hlusi A, et al. Addiction to DUSP1 protects JAK2V617F-driven polycythemia vera progenitors against inflammatory stress and DNA damage, allowing chronic proliferation. *Oncogene.* 2019;38:5627–42. [PubMed: 30967632]
54. Guardavaccaro D, Clevers H. Wnt/beta-catenin and MAPK signaling: allies and enemies in different battlefields. *Sci Signal.* 2012;5:pe15. [PubMed: 22494969]
55. Wang Z, Li R, He Y, Huang S. Effects of secreted frizzled-related protein 1 on proliferation, migration, invasion, and apoptosis of colorectal cancer cells. *Cancer Cell Int.* 2018;18:48. [PubMed: 29610564]
56. Ahmad MK, Abdollah NA, Shafie NH, Yusof NM, Razak SRA. Dual-specificity phosphatase 6 (DUSP6): a review of its molecular characteristics and clinical relevance in cancer. *Cancer Biol Med.* 2018;15:14–28. [PubMed: 29545965]
57. Keyse SM. Dual-specificity MAP kinase phosphatases (MKPs) and cancer. *Cancer Metastasis Rev.* 2008;27:253–61. [PubMed: 18330678]
58. Okudela K, Yazawa T, Woo T, Sakaeda M, Ishii J, Mitsui H, et al. Down-regulation of DUSP6 expression in lung cancer: its mechanism and potential role in carcinogenesis. *Am J Pathol.* 2009;175:867–81. [PubMed: 19608870]
59. Kong T, Laranjeira ABA, Yang K, Fisher DAC, Yu L, Poittevin De La Fregonniere L, et al. DUSP6 mediates resistance to JAK2 inhibition and drives leukemic progression. *Nat Cancer.* 2022;4:108–27. [PubMed: 36581736]
60. Fey D, Croucher DR, Kolch W, Kholodenko BN. Crosstalk and signaling switches in mitogen-activated protein kinase cascades. *Front Physiol.* 2012;3:355. [PubMed: 23060802]

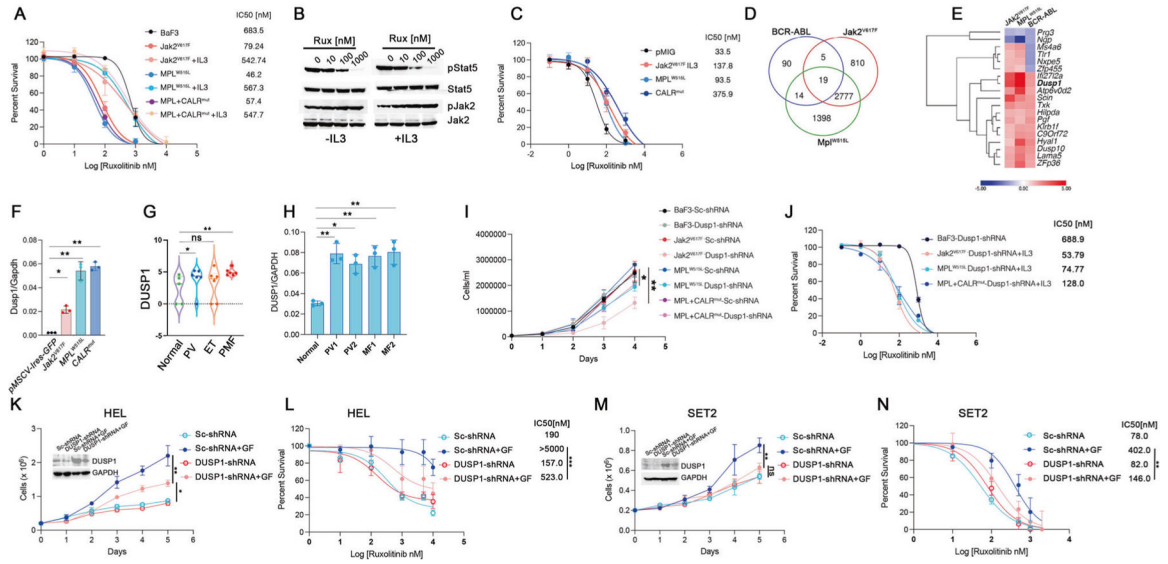


Fig. 1. Growth factor induced DUSP1 expression confers resistance to ruxolitinib.

A A dose dependent cell proliferation assay of BaF3 cells expressing MPN inducing genes (*Jak2^{V617F}*, *MPL^{W515L}*, and *CALR^{mut}*) showing resistance to ruxolitinib in the presence of growth-factor (IL3) signaling. **B** Immunoblot analysis of BaF3-*Jak2^{V617F}* cells showing complete inhibition of Jak2 (increased phospho-JAK2 protein) and phosphoStat5 by ruxolitinib in both IL3+/- conditions. **C** Bone marrow derived primary Kit⁺ cells expressing *Jak2^{V617F}*, *MPL^{W515L}*, and *CALR^{mut}* showing resistance to ruxolitinib in the presence of hematopoietic cytokines compared to control vector (pMIG). **D** Venn diagram showing the commonly expressed genes between *Jak2^{V617F}*, *MPL^{W515L}*, and *BCR-ABL* (treated with IL3 and imatinib). **E** Heat map showing the expression of 19 commonly deregulated genes in *Jak2^{V617F}*, *MPL^{W515L}*, and *BCR-ABL* cells. **F** Bar graph showing the relative expression of *Dusp1* in LSK cells (Lin⁻, Sca1⁺ and Kit⁺) expressing *Jak2^{V617F}*, *MPL^{W515L}*, and *CALR^{mut}*. **G** Violin plot showing significant induction of *DUSP1* expression in PV and PMF patients but not ET. Expression data (GSE55976) is derived from the CD34⁺ cells from the MPN patients. **H** Real-time qPCR analysis of *DUSP1* expression in primary MPN patient-derived mononuclear cells. Two PV and two MF patients were analyzed. Data are shown from two independent qPCR analysis ± SD. **I** A cell proliferation growth curve of *Dusp1* knockdown in BaF3 cells expressing *Jak2^{V617F}*, *MPL^{W515L}*, and *CALR^{mut}* showing significantly reduced proliferation compared to the parental BaF3 control. **J** Dose dependent cell proliferation assays showing *Dusp1* depletion abrogated IL3 induced resistance to ruxolitinib in *Jak2^{V617F}*, *MPL^{W515L}*, and *CALR^{mut}* expressing BaF3 cells. **K** DUSP1 knockdown by shRNA in HEL cells significantly suppressed the proliferation under growth-factor signaling (GF). Extent of DUSP1 knockdown and its expression ± GF conditions are shown in western blot (inset). Please note significant induction of DUSP1 during GF signaling. **L** Dose dependent cell proliferation assay showing *DUSP1* depletion in HEL cells significantly reduced the resistance conferred by GF signaling. **M** Depletion of *DUSP1* by shRNA-mediated knockdown in SET2 cells significantly suppressed the proliferation under growth-factor signaling (GF). Extent of DUSP1 knockdown and its expression in GF ± conditions are shown in western blot (inset). Please note significant induction of DUSP1

during GF signaling. **N** Dose dependent cell proliferation assay showing *DUSP1* depletion in SET2 cells significantly reduced the resistance conferred by GF signaling. Presented data are from two independent experiments shown as the means \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

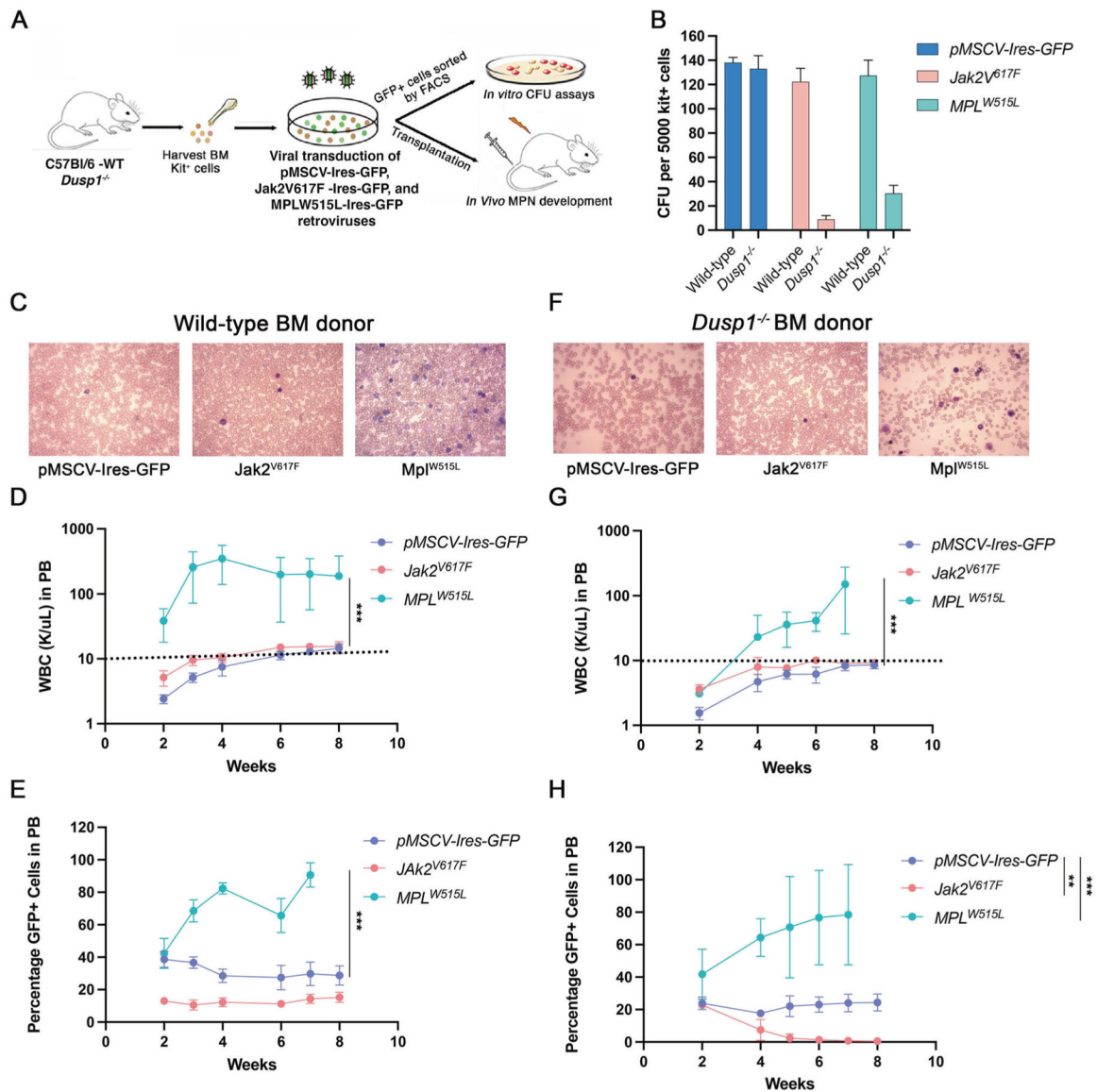


Fig. 2. Deletion of *Dusp1* is synthetic lethal to *JAK2*^{V617F} induced MPN.

A An schematic showing the experimental design for evaluating the role of DUSP1 in mouse model of MPN. **B** Percent CFUs from the C57Bl/6-WT and *Dusp1*^{-/-} Kit⁺ cells expressing *Jak2*^{V617F} and *MPL*^{W515L}. The presented data are the mean colony number from two independent experiments ± SD. **C–E** MPN development in mice transplanted with wild-type BM-derived Kit⁺ cells expressing *Jak2*^{V617F} and *MPL*^{W515L}. **C** Peripheral blood smears showing blast cells from the transplanted mice at week six. **D** WBC levels determined biweekly. **E** Graph showing GFP⁺ cells as a surrogate leukemic burden from the peripheral blood. Dotted lines represent normal WBC levels. Representative data are from the two independent transplant experiments. **F–H**. Mice transplanted with *Dusp1* deficient BM-derived Kit⁺ cells expressing *Jak2*^{V617F} and *MPL*^{W515L}. Note, normalized blood count from the mice recipients of *Jak2*^{V617F} expressing cells. **F** Peripheral blood smear from the transplanted mice at week six. **G** Graph showing the normalized and reduced WBC levels

in *Jak2^{V617F}* and *MPL^{W515L}* transplanted mice, respectively. **H** Graph showing gradual depletion of GFP⁺ cells expressing *Jak2^{V617F}* but not the *MPL^{W515L}* or pMIG transplanted Kit⁺ cells. Representative data are from two independent experiments (five mice per group) shown as the means ± SD. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

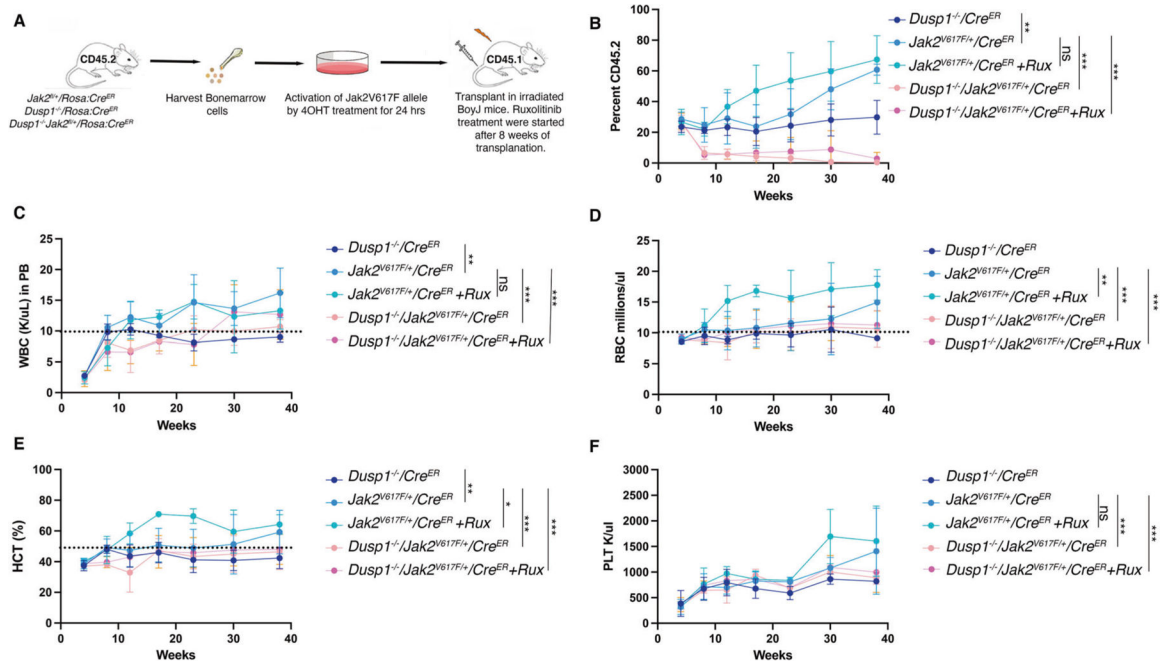


Fig. 3. *Dusp1* deletion eradicates *Jak2*^{V617F} expressing primary bone marrow cells.

A An schema showing the experimental design for evaluating the role of *Dusp1* in *Jak2*^{V617F} transgenic mouse model. One million *Jak2*^{V617F} (CD45.2) cells mixed with normal boyJ (CD45.1) BM cells were transplanted in irradiated BoyJ mice. Engraftment and levels of *Jak2*^{V617F} cells were determined after four weeks of transplantation. Drug treatments were started after week eight and continued for forty weeks. **B** Graph showing the CD45.2 levels in transplanted mice exhibiting normal progression of wild-type *Jak2*^{V617F} and *Dusp1*^{-/-} cells while *Jak2*^{V617F}/*Dusp1*^{-/-} cells are gradually depleted and completely removed by 30th week. Note, ruxolitinib treatment in contrast causes rapid progression of *Jak2*^{V617F} cells which is likely due to higher IC50 values of *Jak2*^{V617F} for ruxolitinib compared to normal cells. Shown are the WBC (**C**), RBC (**D**), HCT (**E**), and platelets (**F**) in transplanted mice with and without treatment. Mice transplanted with *Jak2*^{V617F/+}/*Dusp1*^{-/-} cells do not show any sign of MPN. As expected, mice transplanted with *Jak2*^{V617F/+} cells developed all signs of MPN, increased RBC, WBCs and HCT. Representative data are from two independent experiments (five mice per group) shown as the means \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

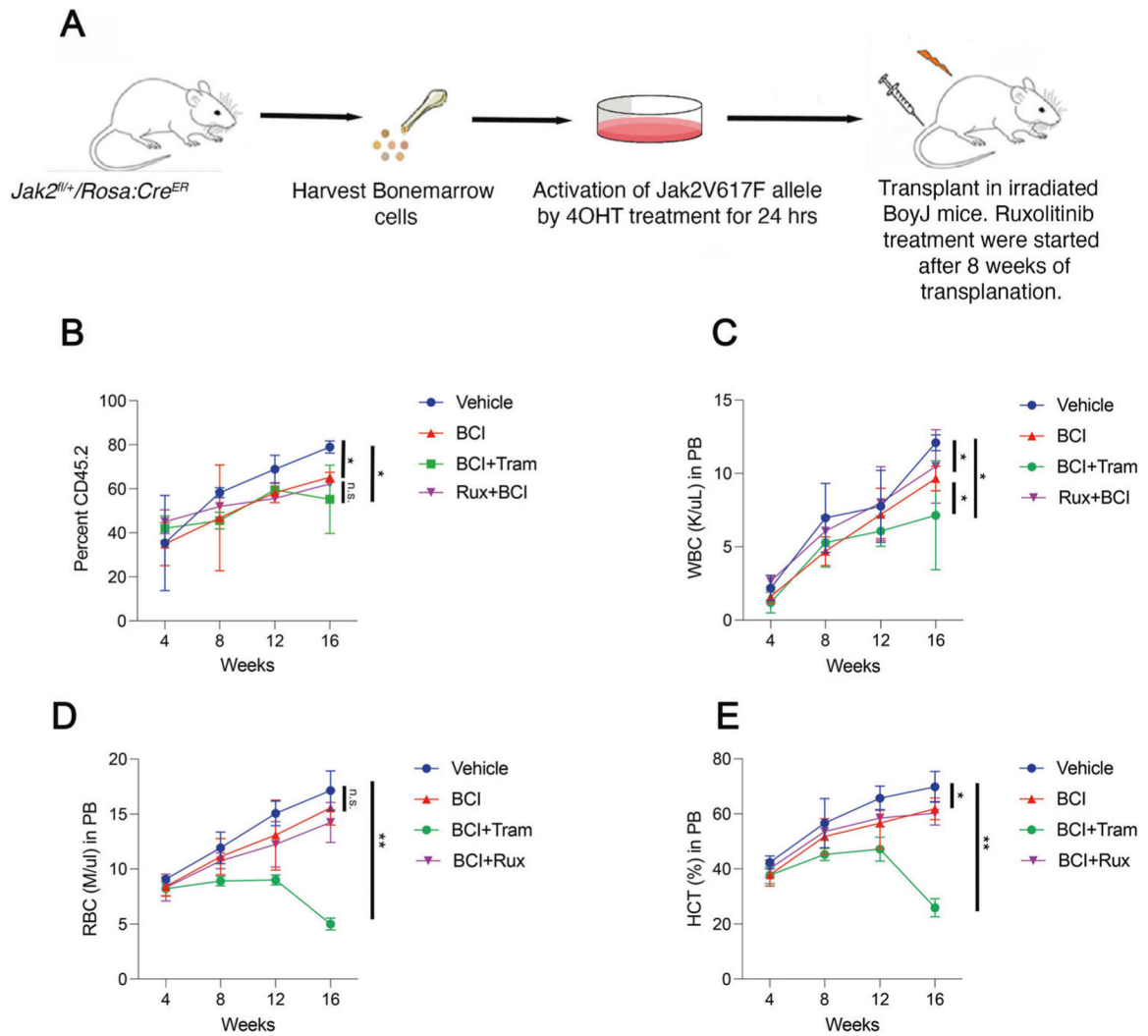


Fig. 4. Chemical inhibition of DUSP1 by BCI suppresses the MPN but lacks clonal selectivity. **A** An schema showing the evaluation of DUSP1 inhibitor (BCI) in *Jak2^{V617F/+}* induced MPN. **B** After 8 weeks of transplantation blood chimerism for CD45.2 were assessed, and mice were grouped for treatment. Graph showing the CD45.2 levels in drug treated and vehicle-treated mice. BCI treatment modestly suppressed the *Jak2^{V617F/+}* clone but all transplanted mice showing progressive disease. Even the combination of BCI with ruxolitinib or Trametinib failed to fully suppress the *Jak2^{V617F/+}* clone. **C** Graph showing WBC mice in treated and control mice. Note BCI treatment alone suppresses the WBCs but in combination with trametinib suppression is higher. Graphs are showing the RBC (**D**) and HCT (**E**) levels in treated and control mice. Treatment with BCI alone or with ruxolitinib is ineffective in suppressing the RBCs, however, in combination with trametinib it significantly suppressed the HCT and RBC levels. Representative data are from two independent experiments (three mice per group) shown as the means \pm SD. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

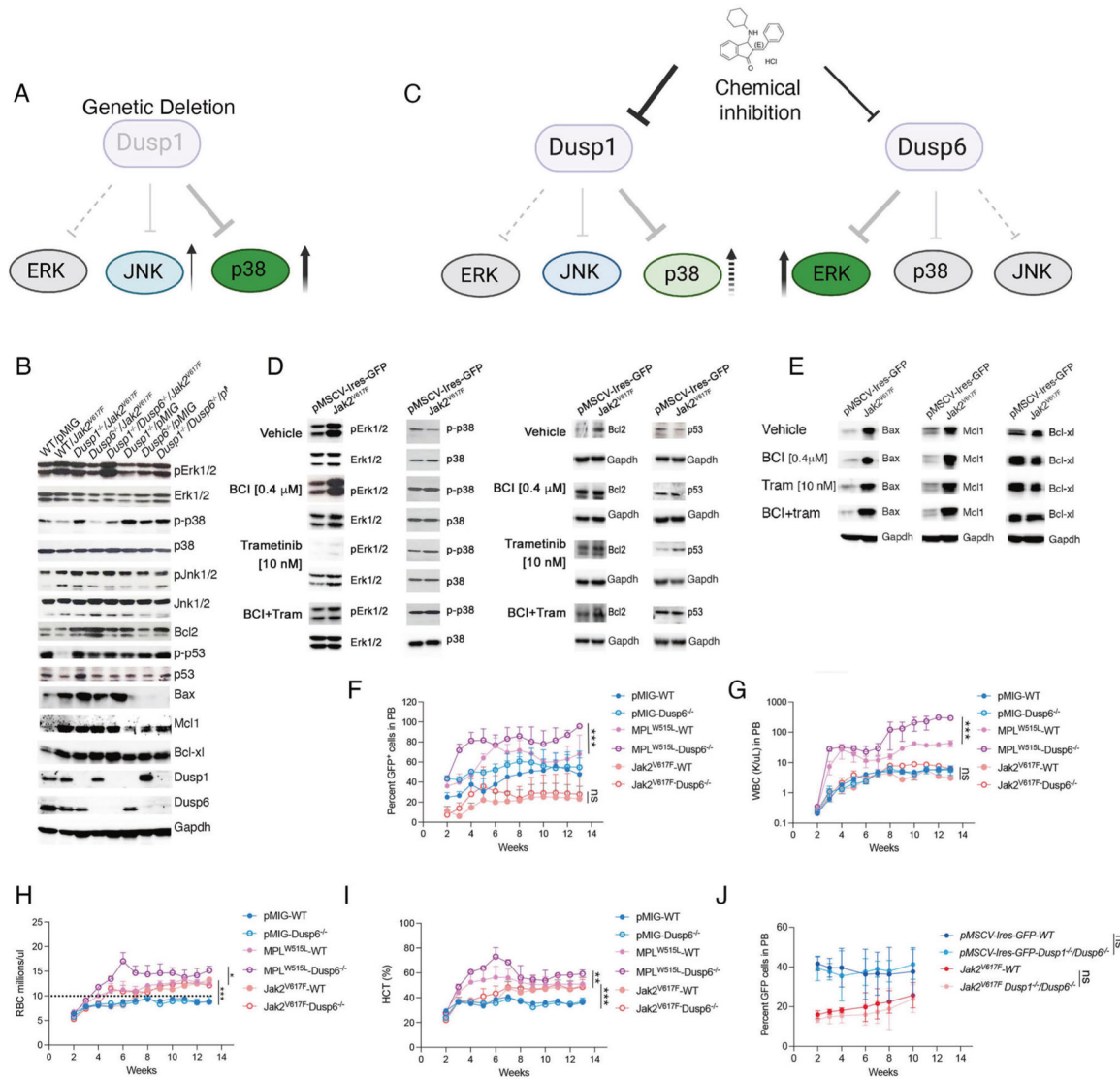


Fig. 5. Activation of pERK1/2 due to off-target inhibition of DUSP6 by BCI abrogated its antileukemic response.

A A model depicting p38 and Jnk1/2 activation following *Dusp1* deletion. **B** Immunoblots from primary BM-derived Kit⁺ cells showing enhanced activation of Erk1/2, suppression of 38 and downregulation of phospho-p53 at S15 in WT cells expressing *Jak2^{V617F}*. *Dusp1* deletion activates 38 that selectively induces the expression of total and phospho-p53 in *Jak2^{V617F}* expressing cells compared to vector controls. **C** A model depicting chemical inhibition of DUSP1 and DUSP6 by BCI activated pErk1/2 and failed to selectively activate p38 in *Jak2^{V617F}* expressing cells. **D** Immunoblots showing the BCI treatment alone, nonetheless, activated phospho-p38 but lacked selectivity as both vector and *Jak2^{V617F}* expressing cells displayed equal phospho-p38 and p53 levels. Importantly, BCI treatment alone induced Bcl2 expression displayed significantly higher pErk1/2 levels in *Jak2^{V617F}* expressing cells compare to vector control. Treatment with trametinib alone suppressed the pErk1/2 but in combination with BCI *Jak2^{V617F}* expressing cells retained higher pErk1/2 possibly due to inhibition of Dusp6. **E** Immunoblots showing that treatment with BCI,

Trametinib and in combination induce the expression of antiapoptotic proteins, Mc11 and Bcl-xl. **F–I** MPN development in mice transplanted with BM-derived Kit⁺ cells from the wild-type and *Dusp6*^{-/-} mice expressing *Jak2*^{V617F}, *MPL*^{W515L}, and *CALR*^{mut}. **F** Graph showing progression of leukemic cells (GFP⁺ cells) in mice recipients of both wild-type and *Dusp6*^{-/-} donors. Note, *Dusp6* deficiency causes aggressive leukemia in mice transplanted with *MPL*^{W515L} while mice recipients of *Jak2*^{V617F} kit cells lacking *Dusp6* do not show any difference compared to wild-type donor. **G** Graph showing WBC levels in *Jak2*^{V617F} and *MPL*^{W515L} transplanted mice. Note significantly higher WBC levels in *MPL*^{W515L} transplanted mice lacking DUSP6. **H** Graph showing significantly higher RBC levels in both *Jak2*^{V617F} and *MPL*^{W515L} transplanted mice in the absence of DUSP6 compared to vector control. **I** Graph showing significantly increased HCT levels in both *Jak2*^{V617F} and *MPL*^{W515L} transplanted mice lacking DUSP6. **J** Graphs showing progressive disease transplanted with *Jak2*^{V617F} lacking both *Dusp1* and *Dusp6*. Representative data are from two independent experiments (three mice per group) shown as the means ± SD. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

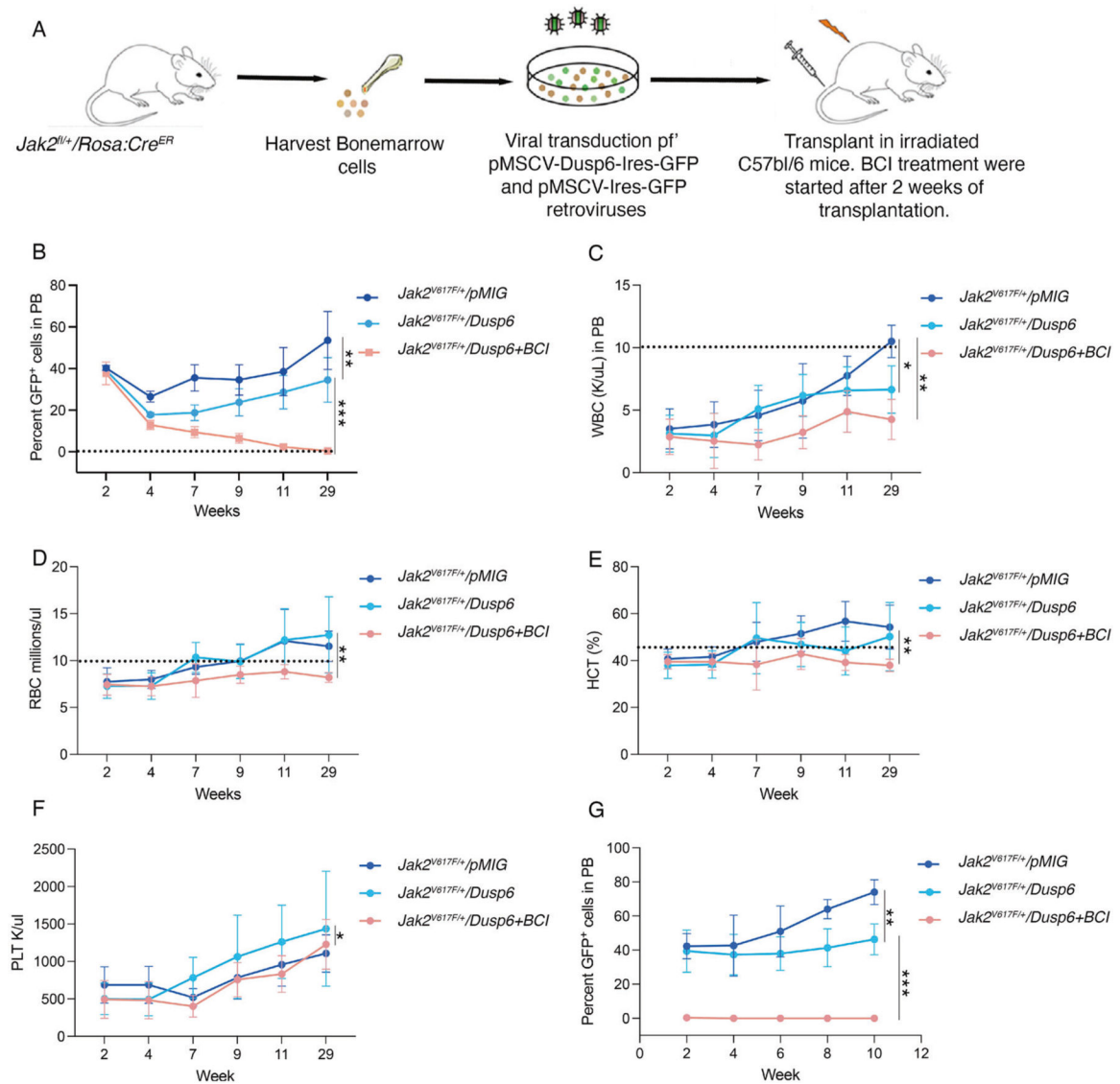


Fig. 6. *Dusp6* overexpression and BCI treatment eradicated the *Jak2^{V617F}* clone.

A An schema showing the experimental design for evaluating the role of *Dusp6* in *Jak2^{V617F}* transgenic mouse model. BM-derived Kit⁺ cells from *Jak2^{V617F}* mice were transduced with pMIG or pMSCV-*Dusp6*-Ires-*cherry*. One hundred thousand cherry-positive cells were transplanted in each lethally irradiated C57Bl/6 recipient mice. Engraftment and levels of *Jak2^{V617F}*-pMIG or *Jak2^{V617F}*+*Dusp6* cells were determined after two weeks of transplantation by examining the cherry-positive cells. BCI treatment was started after two weeks of transplantation. **B** Graph showing the cherry-positive cells in transplanted mice exhibiting normal disease progression in mice transplanted with *Jak2^{V617F}*-pMIG cells while it is significantly suppressed in mice recipients of *Jak2^{V617F}*+*Dusp6* cells. Strikingly, BCI treatment of *Jak2^{V617F}*+*Dusp6* eradicated the MPN cells by 14th week. And cured the mice as treatment discontinuation after week 14th did not result in disease relapse. Shown are the WBC (**C**), RBC (**D**), HCT (**E**), and platelets (**F**) in transplanted mice with and without BCI treatment. Note, overexpression of *Dusp6* alone suppresses the WBC levels but not

the RBCs, HCT and PLTs while BCI treatment in the context of *Dusp6* overexpression exhibited normal blood counts (dotted lines are showing the normal range). **G** Peripheral blood chimerism of cherry-positive cells in secondary recipients of primary transplants shown in **(B)**. Note slow progression of *Dusp6* overexpressing *Jak2^{V617F}* cells while BCI treatment cured the mice. Representative data are from two independent experiments (five mice per group) shown as the means \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript