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The quiescent fraction of chronic myeloid leukemic stem cells depends on BMPR1B, Stat3 and BMP4-niche signals to persist in patients in remission

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Abstract:

Chronic myelogenous leukemia arises from the transformation of hematopoietic stem cells by the BCR-ABL oncogene. Though transformed cells are predominantly BCR-ABL-dependent and sensitive to tyrosine kinase inhibitor treatment, some BMPR1B⁺ leukemic stem cells are treatment-insensitive and rely, among others, on the bone morphogenetic protein (BMP) pathway for their survival via a BMP4 autocrine loop. Here, we further studied the involvement of BMP signaling in favoring residual leukemic stem cell persistence in the bone marrow of patients having achieved remission under treatment. We demonstrate by single-cell RNA-Seq analysis that a sub-fraction of surviving BMPR1B⁺ leukemic stem cells are co-enriched in BMP signaling, quiescence and stem cell signatures, without modulation of the canonical BMP target genes, but enrichment in actors of the Jak2/Stat3 signaling pathway. Indeed, based on a new model of persisting CD34⁺CD38⁻ leukemic stem cells, we show that BMPR1B⁺ cells display co-activated Smad1/5/8 and Stat3 pathways. Interestingly, we reveal that only the BMPR1B⁺ cells adhering to stromal cells display a quiescent status. Surprisingly, this quiescence is induced by treatment, while non-adherent BMPR1B⁺ cells treated with tyrosine kinase inhibitors continued to proliferate. The subsequent targeting of BMPR1B and Jak2 pathways decreased quiescent leukemic stem cells by promoting their cell cycle re-entry and differentiation. Moreover, while Jak2-inhibitors alone increased BMP4 production by mesenchymal cells, the addition of the newly described BMPR1B inhibitor (E6201) impaired BMP4-mediated production by stromal cells. Altogether, our data demonstrate that targeting both BMPR1B and Jak2/Stat3 efficiently impacts persisting and dormant leukemic stem cells hidden in their bone marrow microenvironment.

Introduction

Chronic myelogenous leukemia (CML) represents a unique model of neoplasia driven via stem cell (SC) transformation, which is triggered by the BCR-ABL oncogene. Without treatment, this disease progresses to an inexorable fatal blast crisis. Aside from allogeneic stem cell transplantation, until 2001, interferon- α (IFN- α) was extensively used for the treatment of chronic phase (CP) CML and induced stable remission in some patients¹, which was correlated with long-term survival², but was also responsible for numerous side effects leading to drug dose reduction and lack of response. Tyrosine kinase inhibitors (TKIs), such as Imatinib (IM), are targeted therapies that specifically inhibit BCR-ABL tyrosine kinase activity. TKIs revolutionized the management of CML patients and currently represent the first line treatment.

However, TKIs fail to eliminate leukemic stem cells (LSCs), which persist in patients displaying complete cytogenetic remission (CCyR)^{3,4}, as evidenced by the indefinite detection of residual BCR-ABL⁺ cells in their blood. These cells are most likely responsible for the high rate of relapse after treatment discontinuation, even in patients with a durable undetectable status.⁵ Indeed, LSCs are highly heterogeneous, and single-cell transcriptomic analyses linked specific subpopulations to a primitive quiescent phenotype that persists throughout therapy, conferring cells upon treatment cessation with the ability to proliferate extensively, regenerate or acquire resistance.^{6,7}

As LSCs are insensitive to TKIs this strongly suggests that their survival is independent of BCR-ABL activity, relying on other factors including interactions with the bone marrow microenvironment.⁸⁻¹² Among the cytokines produced by the bone marrow stroma, Bone Morphogenetic Proteins (BMPs) are a group of growth factors involved in many processes, including stem cell regulation. During embryogenesis, BMP4 is crucial for mesodermal cell commitment to the hematopoietic lineage.¹³ BMP4-deficient mice display a reduced number of hematopoietic stem cells (HSCs), while the engraftment of wild-type HSCs into BMP4-deficient mice impairs the repopulating activity of these cells.¹⁴ Additionally, BMP4 regulates the maintenance of human primitive cord blood progenitors.¹⁵ In adults, BMPs regulate

distinct characteristics of HSCs and parameters of their niche.¹⁶ For instance, BMP4 induces CD34⁺ progenitor differentiation into megakaryocytes¹⁷, whereas BMP2 favors erythropoietic commitment.¹⁸ The BMP pathway that co-regulates the fate and proliferation of HSCs and interactions with their niche has been reported in several types of leukemia,¹⁹ such as Acute Myeloid Leukemia-AML,^{20,21} and is dysregulated in CML.²²⁻²⁴ Indeed, CP-diagnosed CML patients show higher concentrations of soluble BMP2 and BMP4 in their bone marrow (BM) compared to normal BM donors. Furthermore, the BMP receptor type-1B (BMPR1B) is overexpressed in CP-diagnosed CD34⁺ CML cells. Soluble BMPs, secreted into the tumor niche maintain a fraction of BMPR1B⁺ LSCs and amplify leukemic progenitors, contributing to disease progression.²² These alterations sustain a permanent pool of LSCs and progenitors expressing high levels of BMPR1B receptor, which evolve upon treatment²⁵ and progressively implement a BMP4 autocrine loop, leading to TKI-resistance.^{26,27}

Having unraveled a majority of the interactions/mechanisms underlying the BMP-associated survival of BMPR1B⁺ LSCs in the bone marrow niche, we herein further investigated the spatio-temporal setting of persistence mechanisms in CML TKI-responsive patients after remission, which remains a major therapeutic challenge to achieve complete recovery through eradication of BMPR1B⁺ LSCs. We identify BMP4/BMPR1B as a major bi-directional signal driving microenvironment-dependent LSC persistence alongside the Jak2-Stat3 pathway. Our data further show that targeting both BMP and Jak2/Stat3 signaling efficiently promotes LSC cell cycle re-entry and differentiation enabling their efficient eradication.

Methods

Cells. Bone marrow samples were obtained either from healthy BM donors for allogeneic transplant or from peripheral blood (PB), or from the BM of CP-diagnosed CML patients prior to or post TKI-treatment (from patients displaying cytogenetic remission CCyR). All donors provided written informed consent in accordance with the Declaration of Helsinki. Studies were approved by local ethics committee bylaws. Definitions of response respected the last European Leukemia Net Recommendations.²⁸ Mesenchymal Stem Cells (MSCs) were

isolated from BM samples and cultured as described.^{26,29} TF1 cell lines were obtained and authenticated as described.^{22,30} TF1-BA, TF1-BAP cells were cultured in RPMI1640 containing 10% fetal calf serum (FCS), with or without 2 μ M Imatinib (IM). HS5 and HS27A cell lines were obtained from ATCC and cultured in DMEM and RPMI1640 containing 10% FCS.

RNA analysis

The quantitative reverse transcription PCR protocol, reagents, and primers are depicted in²². Single-cell RNA sequencing and Gene-set enrichment analyses were performed as previously described.⁷ The BMP signature was designed as presented in the *Online Supplementary Table 1*. Briefly, we took 245 cells from group A and B derived from 16 patients, 11 and 5 of whom were good and poor responders, respectively⁷. All cells analyzed in single-cell RNA sequencing were BCR-ABL+ cells as verified by qPCR analysis. High-Throughput Microfluidic-based RT-qPCR was performed following the Fluidigm protocols (PN 68000088 K1) as described³¹.

Flow cytometry

Extracellular staining of cells was performed using PE-Cy5-conjugated anti-CD34, APC-conjugated anti-CD38, PE-conjugated anti-CD71 (BD Biosciences) or PE-conjugated anti-GPA (Invitrogen). For intracellular staining, cells were fixed with 4% paraformaldehyde, washed and permeabilized with 80% ethanol before adding PE-conjugated anti-P-Smad1/5/8 (Cell signaling) or AF647-conjugated anti-P-Stat3 (Biolegend), or PE-conjugated anti-Ki67 (BD Biosciences).

BMP quantification

BM plasma or HS5/HS27A supernatants were pre-cleared of cellular debris by rapid centrifugation and processed for BMP4 Elisa quantification (R&D systems) as previously described.²²

Western Blotting

Western blot was performed using TF1-BA and TF1-BAP cells treated with Imatinib (2 μ M), AG490 (25 μ M) or E6201 (100 nM) during 24 h, proteins were then extracted using RIPA

buffer and 40 µg of proteins were loaded. Membranes were incubated with monoclonal-antibodies against P-ERK (#4370), ERK (#4695), P-STAT3 (#9145), STAT3 (#9139), Caspase 3 (#). P-ABL (#2865), P-CRKL (#3181), P-Smad1/5/8 (#13820), GAPDH (#8884) (Cell Signalling Technology), BCR-ABL (ThermoFisher, #MA1-153) and Smad (SantaCruz Technology, #sc6031).²¹

Functional assays

Colony forming cell (CFC) and long-term culture-initiating cell (LTC-IC) assays were performed as reported.²²

Chemical inhibitors

Cells were treated with AG490 (Sigma-Aldrich) and/or E6201 (Spirita Oncology, LLC) a BMPR1B inhibitor (also shown to inhibit MEK1/2 and FLT3 without affecting BMPR1A or JAK2³²⁻³⁴, *Online Supplementary Table 2*).

Statistical analyses

Mean comparisons were performed using the bilateral Mann-Whitney test (paired or unpaired as required) and correlations were determined with the Pearson bilateral test, using the GaphPadPrism software (San Diego, CA, US). $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), $P \leq 0.0001$ (****) were defined as statistically significant values.

Results

At remission, persistent CML leukemic cells are enriched in BMP and quiescence signaling pathways

Recent single-cell analyses revealed the heterogeneity of the stem cell (SC) compartment in the bone marrow of CML patients with distinct sub-fractions of LSCs that displayed different molecular signatures.⁷ This study demonstrated the selective persistence of a BCR-ABL SC subset (group A) already present at diagnosis and from which leukemic cells emerge at relapse upon disease progression. This LSC sub-fraction progressively accumulates at the

expense of another LSC sub-fraction (group B) in patients treated with TKI. Interestingly, conversely to group B, BCR-ABL⁺ cells from group A are highly quiescent. At the beginning of the study reported here we hypothesized that BMP signaling could be involved in the maintenance of group A LSCs in CML patients that achieved remission, due to the involvement of this pathway in TKI-resistance. To address this and gain further insight into mechanisms underlying LSC persistence, we analyzed RNAseq data obtained from *Giustacchini, et al* for BMP pathway actors. Compared to the highly proliferative group B that disappeared upon TKI treatment, cells from group A displayed an enhanced BMP signaling pathway (Figure 1A and *Online Supplementary Table 1*). Both persistent LSCs from group A and B expressed *BMPR1B* transcripts at variable levels (Figure 1B left panel). However, unlike our previous observations in resistant LSCs,²⁶ persistent LSCs were devoid of autocrine *BMP4* production, suggesting their dependence upon BMP4-producing cells of their niche (Figure 1B right panel). Interestingly, neither group exhibited a significant difference in the comparative level of classical BMP-target genes as illustrated in *Online Supplementary Figure S1A* for *ID1*, *ID2*, *RUNX1* and *RUNX2* gene expression. In addition, single cell analyses of BMPR1B⁺ cells from each group revealed that LSCs of group A scarcely expressed *Id1* or *Runx1* (8% of group A cells)(*Online Supplementary Figure S1B left panel*) unlike cells of group B that frequently (50% of group B cells) co-expressed these genes with *BMPR1B* (*Online Supplementary Figure S1B right panel*).

Altogether our data indicate that the selective persistence of a quiescent BCR-ABL⁺ LSC subset (group A) upon treatment is correlated in these cells with a significant enrichment in the BMP signaling molecular signature, unrelated to the expression of BMP target genes.

Persisting quiescent leukemic stem cells are characterized by BMP and Stat3 co-activation

We then investigated whether BMPR1B⁺ cells could activate alternative non-canonical pathways in LSCs of groups A and B to enable them to persist in the bone marrow microenvironment despite TKI-treatment. Based on these single cell RNAseq data, we

identified a specific correlation between *BMPR1B* and *STAT3* pathways exclusively in group A (quiescent cells), though this was not correlated with the expression of *IL6ST* (gp130) (Figure 1C left panel). Conversely, *BMPR1B* expression was associated with kinases like *Tyk2* and *MAP3K8* in the proliferative group B (Figure 1C right panel). No correlation between *BMPR1B* and *Stat5* signaling was identified in either group (*Online Supplementary Figure S1C*).

We next evaluated the functional link between these two pathways. However, it was not technically possible to access viable *BMPR1B*⁺ quiescent LSCs subpopulation by cell sorting BM cells from patients in remission due to their extremely low frequency and the incapacity to distinguish BCR-ABL cells from dominant non-BCR-ABL stem cells. Therefore, owing to the scarcity of the sorted *BMPR1B*⁺-LSC sub-fraction from the BM of CML patients in remission, we developed a novel CML model of TKI-persistent immature cells. We used an established TF1-BCR-ABL (TF1-BA) cell line²² selected to persist upon chronic Imatinib-IM (2 μ M of TKI Imatinib-IM) exposure, designated as TF1-BAP (TF1-BCR-ABL persistent) (Figure 2A). Persistence of TF1-BAP cells was accompanied by a slight higher level of *BMPR1B* as observed by immunofluorescence, Western blot and qPCR (Figure 2B-C and *Online Supplementary Figure 2A*). Similarly to RNAseq data from primary bone marrow cells of patients in remission (Figure 1B), TF1-BAP cells do not implement an autocrine BMP4 production but inversely seemed to repress its production (*Online Supplementary Figure S2B*), corroborating our previous report in CD34⁺ CML cells from patients that achieved CCyR.²⁶

The TF1 human cell line displayed immature properties similar to human hematopoietic stem and progenitor cells, including the immature CD34⁺CD38⁻ (stem) and CD34⁺CD38⁺ (progenitor) phenotypes, and their ability to generate colony-forming progenitor cells (CFCs) in a semi-solid medium (illustrated in Figure 2A and *Supplementary Figure S3A*) from both erythroid and myeloid lineages.³⁵ Chronic treatment of TF1-BA cells with TKI led to a severe reduction of the CD34⁺CD38⁺ sub-fraction in ensuing TF1-BAP cells, which were thus characterized by a CD34⁺CD38⁻ phenotype (Figure 2D). We then evaluated the CFC output

(Figure 2E and *Supplementary Figure S3A*) or LTC-IC potential (Figure 2F and *Supplementary Figure S3B*) following TKI-persistence. We observed a drastic decrease in the ability of TF1-BAP cells to form CFCs compared to TF1-BA (Figure 2E), suggesting a loss of progenitor activity. Moreover, TF1-BAP cells display increased LTC-IC activity (Figure 2F) according to a higher CD34⁺CD38⁻ phenotype (Figure 2D). However, the W5-CFC colonies, representative of BCR-ABL TF1 immature cells derived LTC-IC, were difficult to observe similarly to what we frequently experienced with primary CML cells. We also detected a higher expression level of stemness-associated transcription factors (NANOG, MYC and FoxO1 and FoxO3a)³⁶⁻³⁸ as well as an increase in genes involved in LSC survival (CD123, TPOR, HIF2a, ALOX5, TWIST-1, BCL2 and BCL-XL)^{8,36,39-41} in TF1-BAP compared to TF1-BA cells (*Online Supplementary Figure S2A*). Altogether, these data confirmed the leukemic stem-like status of TF1-BAP cells. We also show that despite increased BCR-ABL protein levels, TF1-BAP display similar activation of P-CRKL (Figure 2G), and TKI equally impairs P-BCR-ABL in TF1-BA and TF1-BAP cells (while having no effect on P-Smad1/5/8)(*Online Supplementary Figure S4A*), indicating that there is no major involvement of the BCR-ABL kinase activity in the persistent phenotype. This set of data then further sustain what has been previously described in primary CML-LSC⁴². Lastly, we measured by intracellular flow cytometry the level of activation of BMP and Stat3 signaling, and revealed an increase in P-Smad1/5/8 and double positive P-Smad1/5/8-P-Stat3 levels in TF1-BAP cells compared to IM-sensitive TF1-BA cells (Figure 2H). However, no difference was observed in total P-Stat3 levels between the TF1-BA and TF1-BAP, as evidenced from Western blot and flow cytometry analyses (Figure 2H and *Online Supplementary Figure S4B*), suggesting that in TKI-persistent cells a specific co-activation of Smad/Stat3 occurs. Altogether these data indicate that the TF1-BAP cell line is a representative model of the primary CML persistent LSC group A characterized by a BMPR1B⁺ expression, stem-like status, the absence of autonomous BMP4 production and the co-activation of P-Smad1/5/8-P-Stat3 signals.

BMP4/BMPR1B contribute to maintaining leukemic cells in a non-differentiated state by regulating Stat3 signaling

Next, we evaluated the importance of BMP and Stat3 signaling in protecting BCR-ABL⁺ cells from TKI-induced cell death. We used the synthetic small molecule inhibitor, E6201, which has a strong affinity for BMPR1B (*Online Supplementary Table 2*). Having shown the relevance of the TF1-BAP persistent CML cell model, with increased BMPR1B expression and P-Smad1/5/8-P-Stat3 activity, we also evaluated the impact of a Jak2 inhibitor (AG490) used alone or in combination with E6201. We confirmed in TF1-BAP that E6201 inhibited P-Smad1/5/8 (Figure 3A), while AG490 decreased P-Stat3, as expected (*Online Supplementary Figure S5A*). Neither E6201 nor AG490 affected the kinase activity of BCR-ABL as revealed by the absence of a significant effect on the main BCR-ABL and ERK targets, P-Ckr1 and P-ERK, respectively (*Online Supplementary Figure S5B*). In addition, no significant change in apoptosis was observed by monitoring caspase-3 cleavage (*Online Supplementary Figure S5C*). Interestingly, E6201 alone also significantly decreased P-Stat3 levels (Figure 3B). Moreover, unlike AG490 that had no impact on the level of P-Smad1/5/8-P-Stat3 signal detected by flow cytometry, E6201 alone or in combination with AG490 decreased the cell subset presenting co-activation of P-Smad1/5/8 and P-Stat3 (Figure 3C and *Online Supplementary Figure S5D*).

At the functional level, we observed that either AG490 or E6201, or their combination, were sufficient to decrease the proliferation of persistent TF1-BAP cells (Figure 3D). The effect of both inhibitors was not modulated by the presence or absence of TKI, which by itself had no effect on TF1-BAP cell proliferation, as expected (Figure 3D). Treatment of TF1-BAP with AG490 or E6201, alone or in combination significantly increased the progenitor outcome as evidenced by CFC assays (Figure 3E). We then measured by flow cytometry the appearance of cell surface markers indicative of early progenitor expansion (CD38 and CD71) and differentiation (GPA) into a non-myeloid lineage to evaluate whether this increase in CFC and proliferation arrest could be due to the engagement of the pluripotent immature leukemic cell towards a more differentiated stage. Results indicate that inhibition of the JAK2/Stat3

and BMP signaling induced TF1-BAP cells to differentiate into a more committed progenitor status, as indicated by the increased number of CD38⁺ (3.26 fold) or CD71⁺ cells (1.72 fold) and a trend to increase GPA cell membrane expression (1.42 fold) (Figure 3F).

Collectively, these results suggest that simultaneous inhibition of BMP and JAK2 signaling could induce a decrease in persistent LSCs through the induction of their differentiation towards progenitors.

Targeting of BMP4/BMPR1B impairs CML persistent cells mediated by their niche

As we have previously shown that BMP2 and BMP4 secreted by the niche are drivers of LSC survival and expansion at diagnosis and following the development of resistance^{22,26}, we analyzed whether patients having achieved CCyR displayed normal levels of these molecules following treatment. The level of both BMP2 and BMP4 was significantly reduced in CCyR patients compared to values obtained at diagnosis (*Online Supplementary Figures S6A-B*). However, a higher concentration (2.7 fold) of BMP2 and BMP4 persists in the bone marrow plasma of patients in remission compared to healthy donors (Figure 4A), indicating that despite eradication of a majority of leukemic cells, soluble BMPs remain abnormally elevated in the bone marrow niche. Our former findings also unveiled that BMP4, which contributes to LSC survival under TKI treatment, is provided by the leukemic niche^{22,26}. Consequently, we evaluated whether combinations of BMP and/or Jak2 inhibitors (E6201 and AG490, respectively) impacted its production by bone marrow mesenchymal stem cells (MSCs). Surprisingly, AG490 alone increased BMP4 expression in both CML (Figure 4B) and healthy MSCs (*Online Supplementary Figure S6C*), while TKI (Imatinib-IM) and E6021 had no effect individually. BMP4 production induced by AG490 treatment was prevented by adding E6201.

In order to evaluate if soluble BMPs contribute to LSC persistence, we measured the impact of BMP4 treatment on Smad and Stat3 signaling. TF1-BA and TF1-BAP cells treated with exogenous BMP4 further increased their P-Smad1/5/8 and double positive P-Smad1/5/8-P-Stat3 levels (Figure 4C), while this treatment had no effect on cells displaying only P-Stat3

activation (*Online Supplementary Figure S6D*).

To evaluate the functional importance of BMP4 production by MSCs, we then performed co-culture experiments of TF1-BA or TF1-BAP and HS27A cells (a model of BMP4-producing MSCs)²². We first observed that despite the fact that TF1-BA cells in suspension were sensitive to IM, the addition of HS27A cells increased their persistence following 6 days of TKI treatment (Figure 4D). Drug withdraw led TF1-BA cells to re-establishing a high level of proliferation after 3 (Figure 4E, left panel) or 6 (Figure 4E, right panel) days of co-culture with HS27A in the presence of TKI. In addition, release from the niche leads to clonogenic expansion in favor of TKI-treatment arrest (Figure 4F).

In order to evaluate the effect of BMP4 provided by the stroma on long-term persistent LSCs, we separately analyzed adherent and non-adherent TF1-BAP cell fractions following 3 days of HS27A co-culture (Figure 5A). Flow cytometry analysis provided Ki67 expression (proliferation marker) of hematopoietic cells exclusively by gating GFP-expressing TF1-BAP cells. Figure 5A clearly shows a decrease in Ki67⁺ cells in adherent TKI-treated TF1-BAP cells, while the non-adherent fraction expressed similar Ki67 levels as untreated cells (normalized to 1). This effect on proliferation appeared to be dependent on the activation of BMP and Jak2 signaling, as it was reversed in the presence of both AG490 and E6201 (Figure 5B). The importance of BMP4 to mediate TKI-induced quiescence was confirmed by the absence of any significant effect when TF1-BAP cells were co-cultured with HS5 MSCs (isolated from the bone marrow of the same patient as HS27A) that express lower levels of BMP4 (*Online Supplementary Figure S7A*), on which TKI did not induce a decrease in Ki67⁺ cells (*Online Supplementary Figure S7B*). Lastly, the addition of anti-BMP4 blocking antibody further demonstrate that the Ki67 decrease by stroma coculture under TKI treatment is impaired by inhibiting the BMP4 signal (Figure 5C), and can potentiate the effect of AG490 and E6201.

Overall these data suggest that BMP4 controls Stat3 activation through Smad1/5/8 in persistent LSCs to favor the maintenance of low cycling cells close to BMP4-expressing stromal cells.

Discussion

In CML, the oncogene BCR-ABL alters numerous signaling pathways that control HSCs, such as the BMP pathway that sustains the survival of BMPR1B+ LSCs and progenitors²². Under continuous pressure of the BCR-ABL-specific TKI-treatment, LSCs evolved and established a BMP4 autocrine loop, which contributes to treatment escape.²⁶ Furthermore, a sub-fraction of LSCs survives in both TKI-sensitive or -resistant CML patients.⁷ Here, we revealed that, in the bone marrow of CML patients in remission, residual LSCs that displayed a quiescent molecular signature are also enriched in BMP signaling. Moreover, we show that a sub-fraction of quiescent cells present a correlation between BMPR1B expression and the Stat3 pathway, but not with Stat5. Both BMPR1B,²⁶ and Stat3⁴³ have been implicated in CML LSC resistance/persistence to TKI treatment independently of the BCR-ABL oncogene.^{26,44} Targeting Jak2 and/or Stat3 alone or in combination with other pathways has been proposed to constitute an efficient way to target LSCs and counteract their TKI-resistance.⁴⁵⁻⁴⁷ Here, using primary cells and a novel model of CD34⁺CD38⁻ TKI-persistent LSCs, we demonstrate that BMPR1B expression is linked to P-Smad1/5/8 and P-Stat3 activation, and that concomitant targeting of these pathways impairs cell proliferation in suspension. This raised the hypothesis that the BMP pathway is responsible for the Stat3 activation in a small subset of persisting LSCs. Indeed, we showed that BMP4 controls the concomitant activation of Smad1/5/8 and Stat3 in TKI persistent CD34⁺ cells, identifying the soluble factor produced by stromal cells and involved in the JAK/STAT3 activation as suspected by Kuepper *et al.*⁴³ This is in accordance with results obtained in embryonic SCs, showing that BMP controls Stat3 expression to sustain self-renewal and prevents cell differentiation through the induction of Id gene expression.⁴⁸ At a molecular level, it is interesting to note that transcription factor binding site analysis revealed that Smad1 and Stat3 often co-occupy similar loci⁴⁹ and that Jak2 signaling regulates CML LSCs^{8,45}. Moreover, a direct link between the BMP and Jak/Stat pathway has been established in the

neural system through the identification of a molecular complex between Smad1 and Stat3.⁵⁰

We have previously demonstrated that BMP4 regulates human normal megakaryopoiesis, which is prevented in the presence of the Jak2 inhibitor, AG490, but not of MEK1/2 inhibitors.¹⁷ In addition, we have shown that TPO-induced megakaryopoiesis was partly mediated by the induction of a BMP4/BMPR1 autocrine loop.¹⁷ In the context of stem cell dormancy, TPO has been identified in an *in vivo* mouse model as a master cytokine controlling HSC quiescence in the bone marrow niche through the Jak2 pathway.⁵¹ Moreover, BMP4 was shown to induce SC quiescence in different tissues such as the eyes,⁵² neural⁵³⁻⁵⁵ and skin.⁵⁶ In keratinocytes, BMP4 induces SC dormancy by controlling DNA hypomethylation.⁵⁶ In the neural system, BMP4 decreases cell proliferation through P27Kip1 regulation⁵³ and prevents neural SC differentiation.⁵⁵ Altogether, these observations suggest that BMP4 could directly control CML LSC quiescence through its modulation of the JAK/Stat3 pathway in a non-canonical signal, dependent upon BMPR1B kinase activity. We now provide evidence that residual bone marrow BMPR1B⁺ LSCs include (at least) two distinct subsets of leukemic persisting cells. *In vitro* data obtained with the TF1-BAP model of persisting immature BCR-ABL⁺ cells indicate that cell adhesion to stromal BMP4-producing MSCs results in a drastically decreased proliferation in the presence of TKI, mediated by BMPR1B and Jak2 signaling. Conversely, non-adherent BMPR1B⁺ TF1-BAP-cells do not display a decrease in Ki67 upon TKI treatment. These data suggest that LSCs could be maintained into a quiescent stage through a synergistic mechanism between the BMP signaling and adhesion to bone marrow stromal cells. Indeed, in the drosophila model, it has been demonstrated that integrin signaling enhances BMP pathway activation through interactions with the BMP receptors, independently of the FAK-signaling.⁵⁷ In addition, the authors showed that integrin themselves are a target of BMP signaling. Moreover, extracellular matrix molecules such as integrin promote Stat3-dependent mitochondrial functions and cell survival through integrin-FAK signaling.⁵⁸ Murine BMP4 regulates the expression of integrin alpha4 in HSC through a Smad-independent non-canonical signal.¹⁶ BMP4 induces HSC adhesion to stromal cells resulting in an increase in stem cell

maintenance and expansion.¹⁷ Altogether, these data suggest that integrin could contribute to maintaining and amplifying the BMP4 signal to promote adherent LSCs persistence. Since LSCs of group A appeared to constitute a reservoir from which leukemic resistant or progressing cells could re-emerge,⁷ we hypothesized that these characteristics may enable the targeting of those persistent and quiescent LSCs in their niche. Indeed, we then demonstrated that the combination of AG490-E6201 is optimal to prevent BMP4-induced MSC protection, reinforcing the importance of combining BMPR1B inhibition with Jak2 targeting. ,

Altogether, our results raise the hypothesis that close proximity of a subset of LSCs to BMP4-producing MSCs of the leukemic niche, increases the binding rate of BMP4 to its receptor BMPR1B to promote cell cycle exit in cooperation with integrin signaling and amplified by TKI exposure (Figure 6). This subpopulation of dormant LSCs then re-enter the cell cycle upon TKI treatment cessation to slowly expand, progress⁷ and mediate patient relapse after several months as observed in 60% of the cases reported in the STIM clinical trial of treatment arrest.⁵⁹ In conclusion, we identified BMP4/BMPR1B as a major bi-directional signal driving microenvironment-dependent LSC persistence alongside the Jak2-Stat3 pathway. Our data may prompt new therapeutic strategies to efficiently target LSCs and their microenvironment and prevent disease persistence during the treatment of CML and other leukemic malignancies.

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Authorship Contributions

S.J, K.A, S.T, E.G, KG, and S.L. performed experiments and data analysis. T.V developed the LSC persistent model. F-E.N. provided CML patients and donor samples and related clinical follow-up. A.J.M. and S.T. designed protocols and data analysis for single cell RNA-Seq experiments. A.G., S.G-G. and O.G. designed protocols and data analysis for Fluidigm RNA quantification. S.L. and V.M.-S. designed the study, analyzed data and wrote the manuscript. All authors approved the final manuscript.

The authors declare no competing financial interests.

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Figures legends

Figure 1. **BMPR1B-positive CML persistent cells signal through the Stat3 pathway.** (A) GSEA of group-A versus group-B *BCR-ABL*⁺ SCs at remission ($n = 122$ and $n = 123$, respectively). Gene set shown is BMP signaling pathway. (B) Beeswarm plot of *BMPR1B* (left) or *BMP4* (right) expression between group-A (light blue; $n = 122$) and group-B *BCR-ABL*⁺ SCs from patients at remission (purple; $n = 123$). Number of cells analyzed and displaying amplification for the selected genes are shown below the plot. The average gene expression level is indicated by red squares, and the boxes represent the median and quartiles of gene expression levels. (C) Heat map shows hierarchical clustering of *IL6_JAK_STAT3* signaling pathways genes in *BMPR1B*⁺ cells ($n = 8$) from group-A *BCR-ABL*⁺ SCs (left) or group-B *BCR-ABL*⁺ SCs (right).

Figure 2. **The TF1-BAP model displays similar features as CML persistent cells** (A) Strategy for generating TF1-BA and TF1-BAP cell lines. (B) Representative images of *BMPR1B* staining of TF1-BA and TF1-BAP cells. (C) Western blot analysis showing *BMPR1B* levels in TF1-BA and TF1-BAP cells; Scatter plot showing *BMPR1B* (*GAPDH* used as an internal control) $n = 5$. (D) Representative FACS plots of TF1-BA and TF1-BAP cells analyzed for their *CD34* and *CD38* content. (E) Dot plot showing CFC activity from TF1-BA and TF1-BAP cells. (F) Dot plot showing LTC-IC activity from TF1-BA and TF1-BAP cells. (G) Western blot analysis showing *BCR-Abl* and *P-CRKL* levels in TF1-BA and TF1-BAP cells; Bar graph showing *BCR-Abl* and *P-CRKL* (*GAPDH* used as an internal control) $n = 4$. (H) TF1-BA and TF1-BAP cells were analyzed for their content in *P-Smad1/5/8* and *P-Stat3*. Dot plots represent percentage of *P-Smad1/5/8* (left panel), *P-Stat3* (middle panel) or double positive *P-Smad1/5/8-P-Stat3* (right panel). (E-H) Unpaired t-test significant *P*-values are indicated in the figure, $P < 0.05$, $*P < 0.01$, $**$.

Figure 3. **Dual targeting of *BMPR1B* and *Stat3* impairs proliferation and promotes differentiation of CML persistent cells.** (A-B) TF1-BAP cells treated with E6201 (100 nM) for 24 h and analyzed for their content of *P-Smad1/5/8* (A) or *P-Stat3* (B). (C) TF1-BAP cells

treated or not with AG490 (25 μ M) or E6201 (100 nM) for 24 h and analyzed for their content of double positive P-Smad1/5/8-P-Stat3. (D-F) TF1-BAP cells were treated for 3 days with Imatinib (IM) (2 μ M), and/or AG490 (25 μ M), and/or E6201 (100 nM), and then (D) cell viability was determined by counting viable cells (Trypan blue exclusion), (E) CFC activity was assessed, or (F) cells were analyzed for their content of CD38 (left panel), CD71 (middle panel) or GPA (right panel). Unpaired (C-E) or paired (A-B, F) t-test significant *P*-values are indicated in the figure, *P*<0.05, **P*<0.01, **.

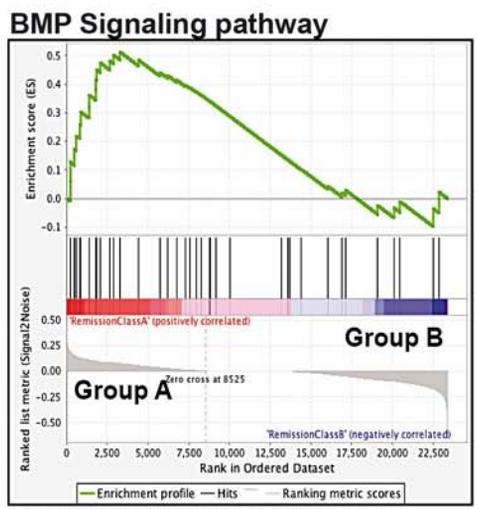
Figure 4. CML niche promotes TKI persistence of leukemic cells. (A) ELISA quantification of BMP2 and BMP4 in BM plasma obtained from healthy donors and CCyR patients. Results from individual samples are expressed in picograms per milliliter, and horizontal lines represent mean values \pm SEM of the indicated number of analyzed samples. (B) Mesenchymal stem cells from CML patients were treated with IM, and/or AG490, and/or E6201 for 3 days. BMP4-transcript expression from 3 samples is expressed in arbitrary units, and lines represent mean values \pm SEM, compared to untreated. (C) TF1-BA and TF1-BAP cells treated or not with BMP4 (20 ng/ml for 24 h) were analyzed for their content in P-Smad1/5/8 (left panel), or double positive P-Smad1/5/8-P-STAT3 (right panel). (D) TF1-BA cells were cultured in suspension or seeded on top of a HS27A layer and then treated for 6 days with Imatinib (IM) (1.5 μ M), before viable cell number was analyzed. Scatter plot showing fold to input (Day 6 / Day 0), with dashed line representing no proliferation compared to input; n = 7. (E-F) TF1-BA cells were seeded on top of a HS27A layer for 3 (E, left panel) or 6 days (E-F) with Imatinib (IM) (1.5 μ M), before adherent TF1-BA were harvested and further divided into suspended cells without IM or seeded on top of a HS27A layer with IM. After 3 days, cell numbers were analyzed (E) or CFC assays were performed (F). Scatter plot showing fold to input (E) (Day 6 / Day 3 (left panel), Day 9 / Day 6(right panel)), or CFC activity (F). Mann-Whitney test significant *P*-values are indicated in the figure, *P*<0.05, **P*<0.01, **

Figure 5. **Targeting of CML niche impacts the quiescence of persisting cells.** (A-C) TF1-BAP cells were seeded on top of a HS27A layer and then treated for 3 days with Imatinib (IM) (2 μ M) (A), with or without AG490 (25 μ M) + E6201 (100 nM) (B-C), and addition of anti-BMP4 (C). Then TF1-BAP (GFP tagged) cells adherent and non-adherent fractions were harvested and analyzed separately for their content of Ki67-PE. Unpaired t-test significant *P*-values are indicated in the figure, *P*<0.05, **P*<0.01, **, *P*<0.001, ***.

Figure 6. **Proposed model to illustrate the regulation of quiescence stage of persisting CML leukemic stem cells by their microenvironment.** (Left panel) Leukemic Stem Cells dormancy of cells that interact with stromal cells of the niche is partly driven by BMP4 binding to its BMPR1B receptor overexpressed in those cells. This leads to phosphorylation of the Smad signaling (P-Smad 1/5/8) canonical pathway. It cooperates with the activation of Jak2/Stat3 signal through P-Stat3 induced directly by BMPR1B and/or by other cytokines binding to their receptors. Altogether, it results in repressing proliferation while inducing quiescence genes expressions. (Left panel) Using a chemical inhibitor toward BMPR1B (E6201) in combination with a Jak2 inhibitor (AG490) the targeting of the LSC could revert it to induce proliferation and repress their quiescence. In addition, E6021 is preventing BMP4-induced production by stromal cells upon AG490 exposure, allowing a simultaneous dual targeting of the LSC and their leukemic protecting microenvironment.

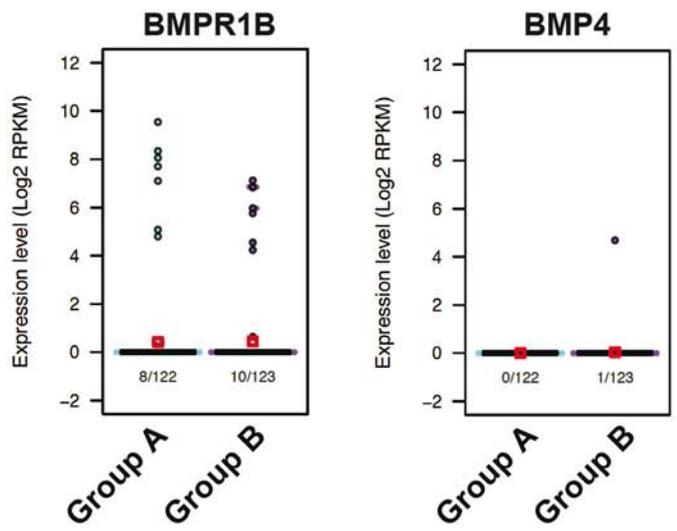
Figure 1

A



NES 1.56; *P*-value 0.002;
FDR *q*-value 0.03

B



C

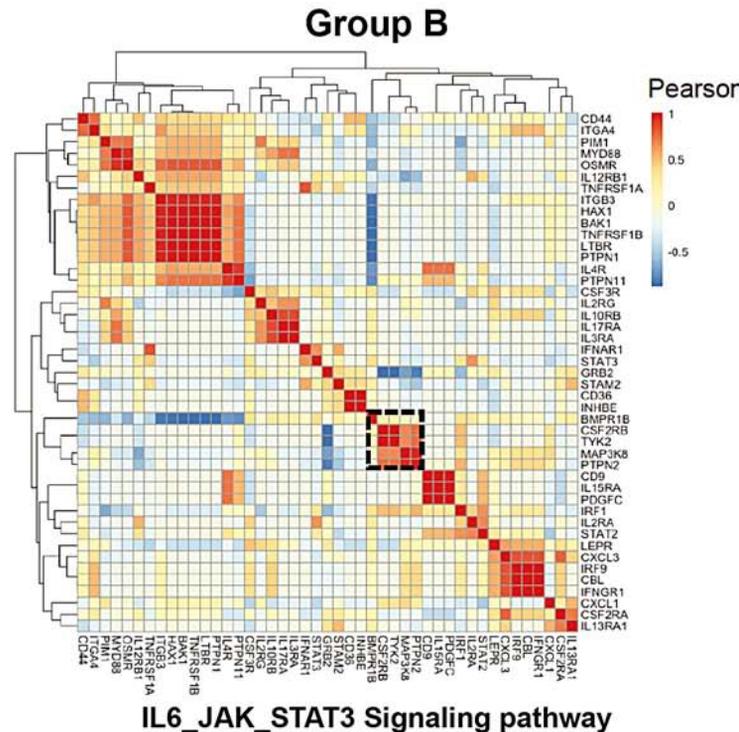
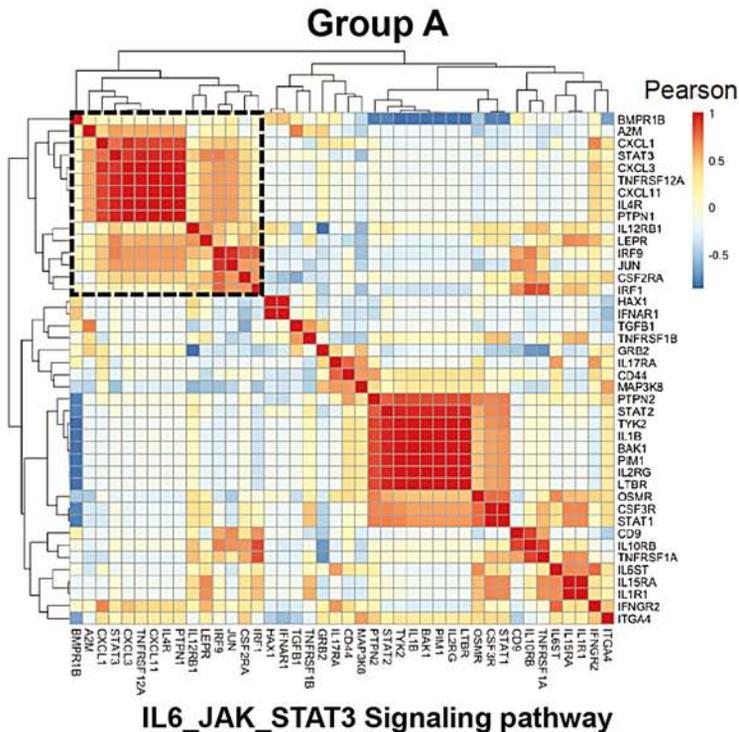


Figure 2

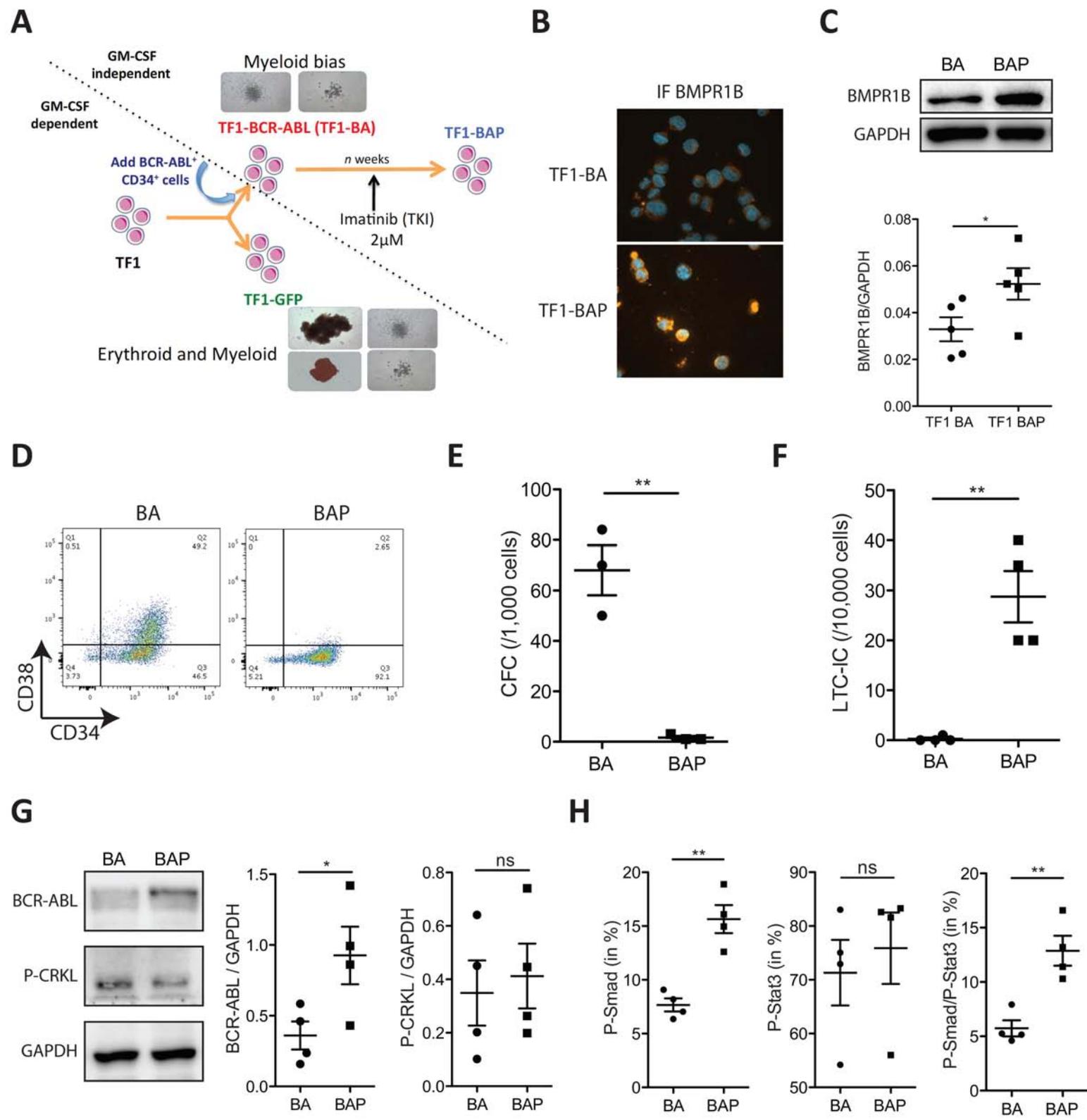


Figure 3

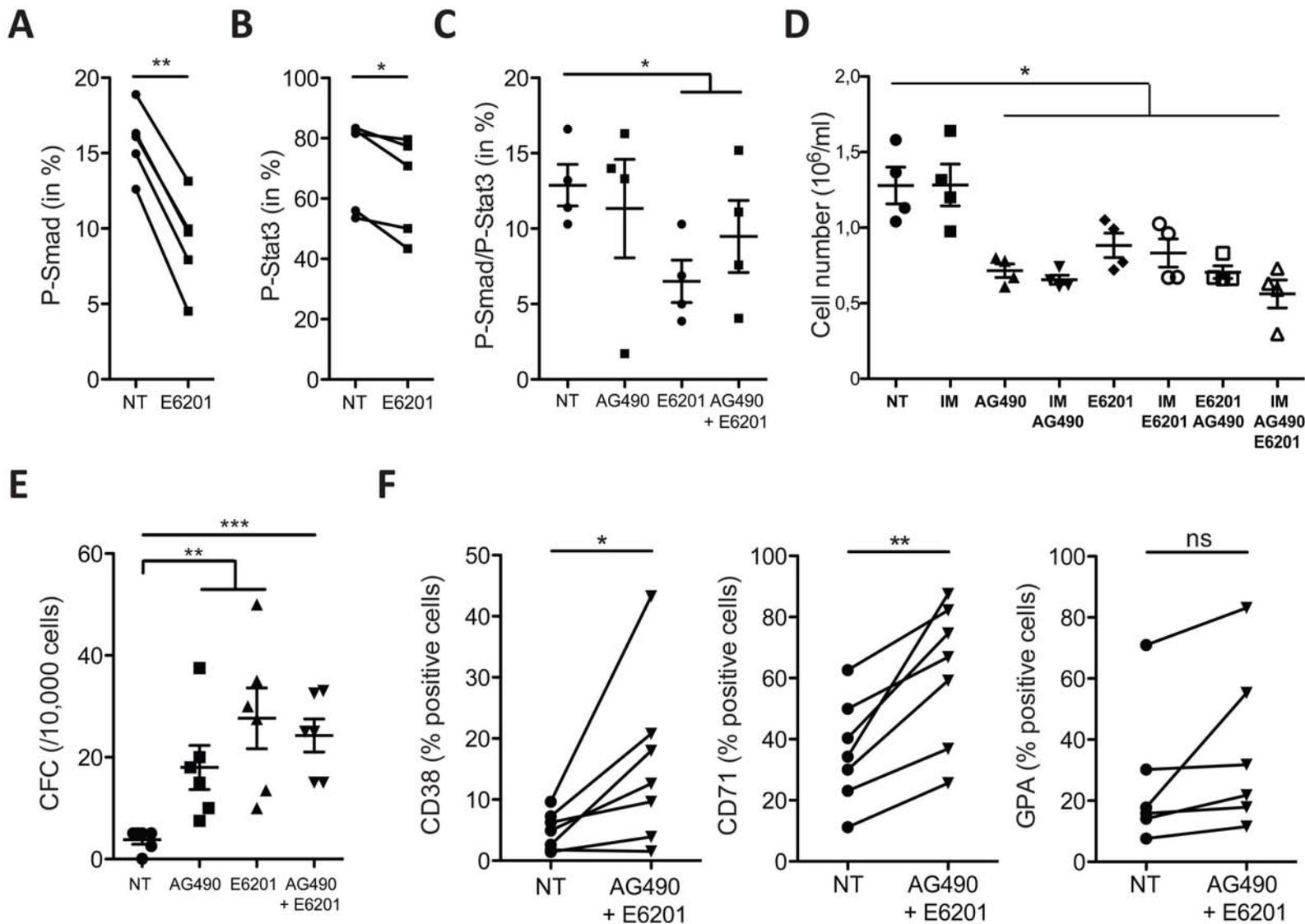


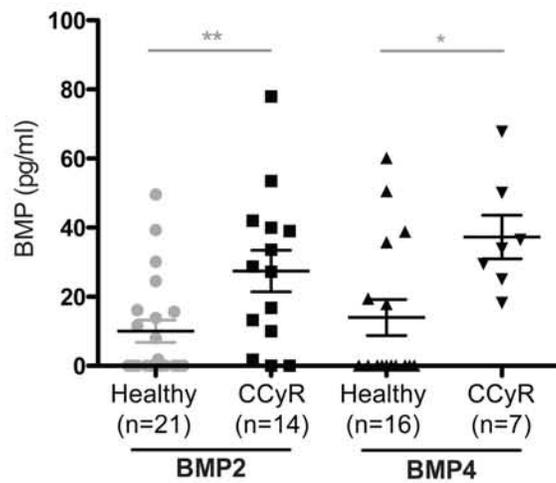
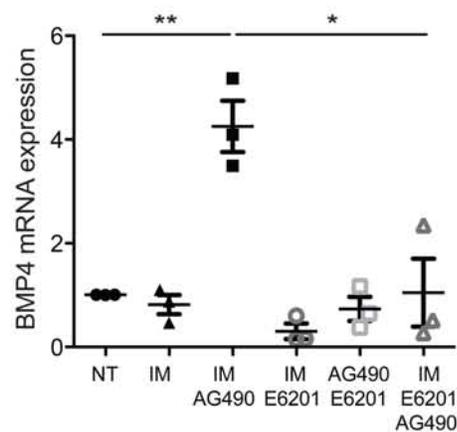
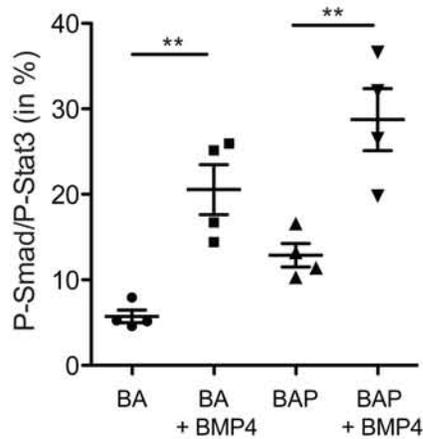
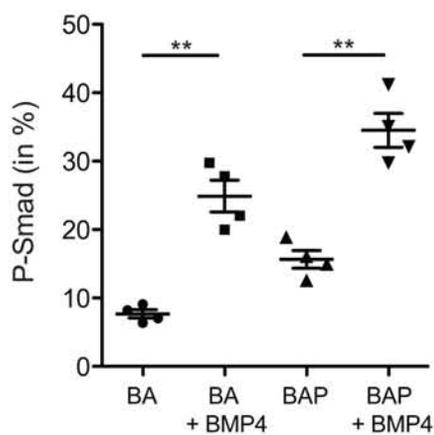
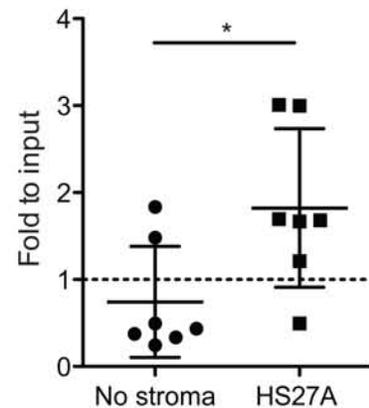
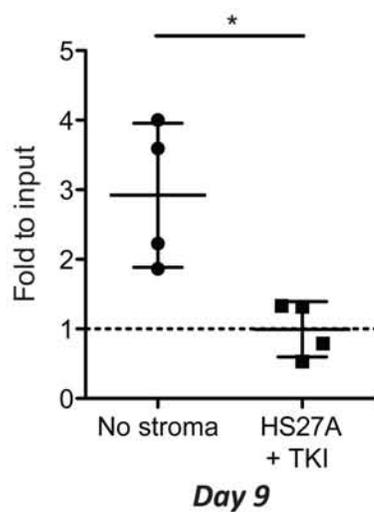
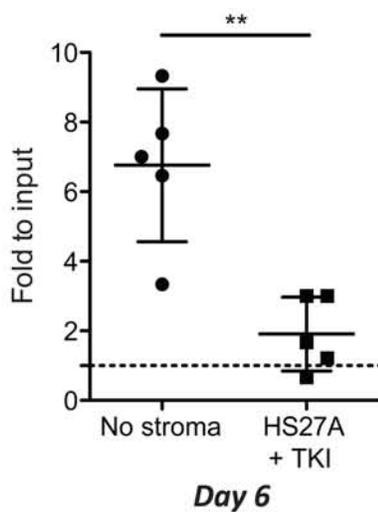
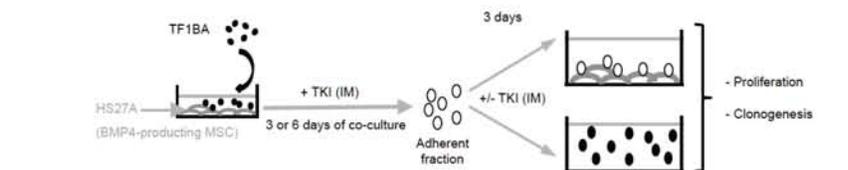
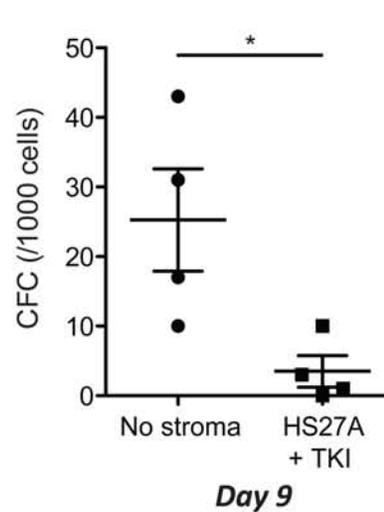
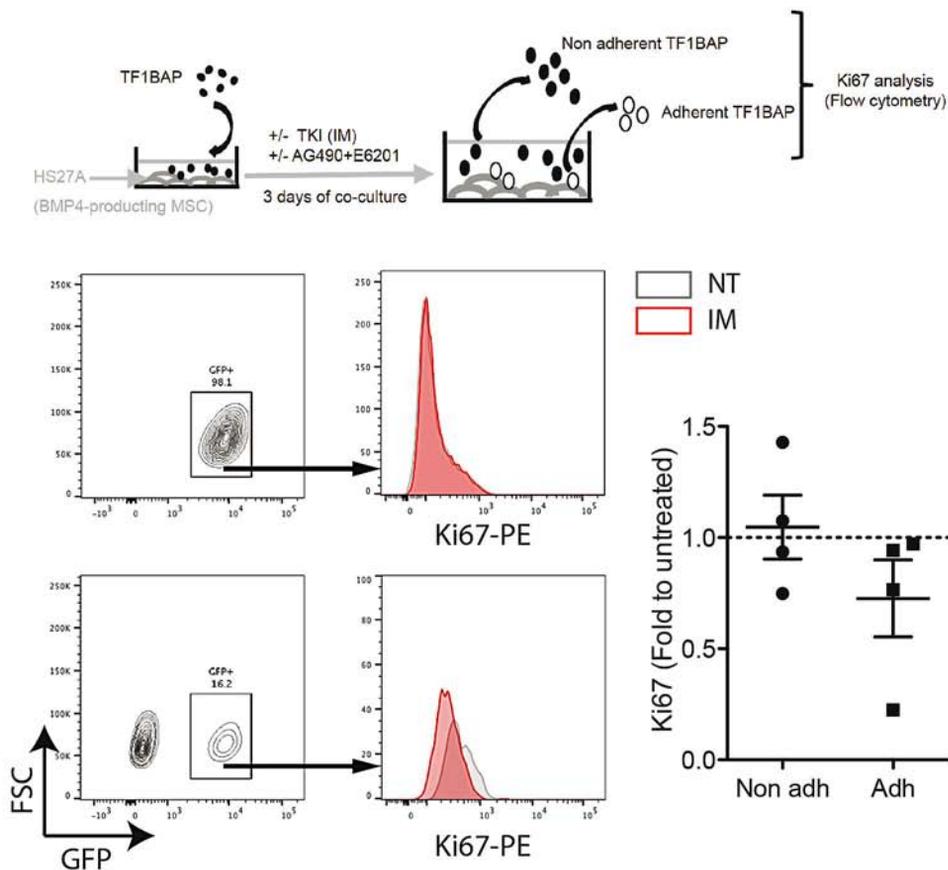
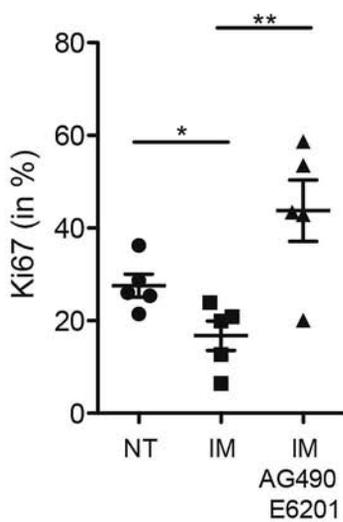
Figure 4**A****B****C****D****E****F**

Figure 5

A



B



C

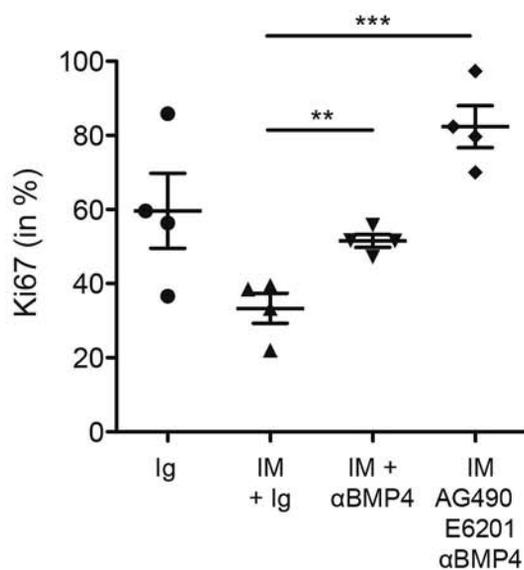
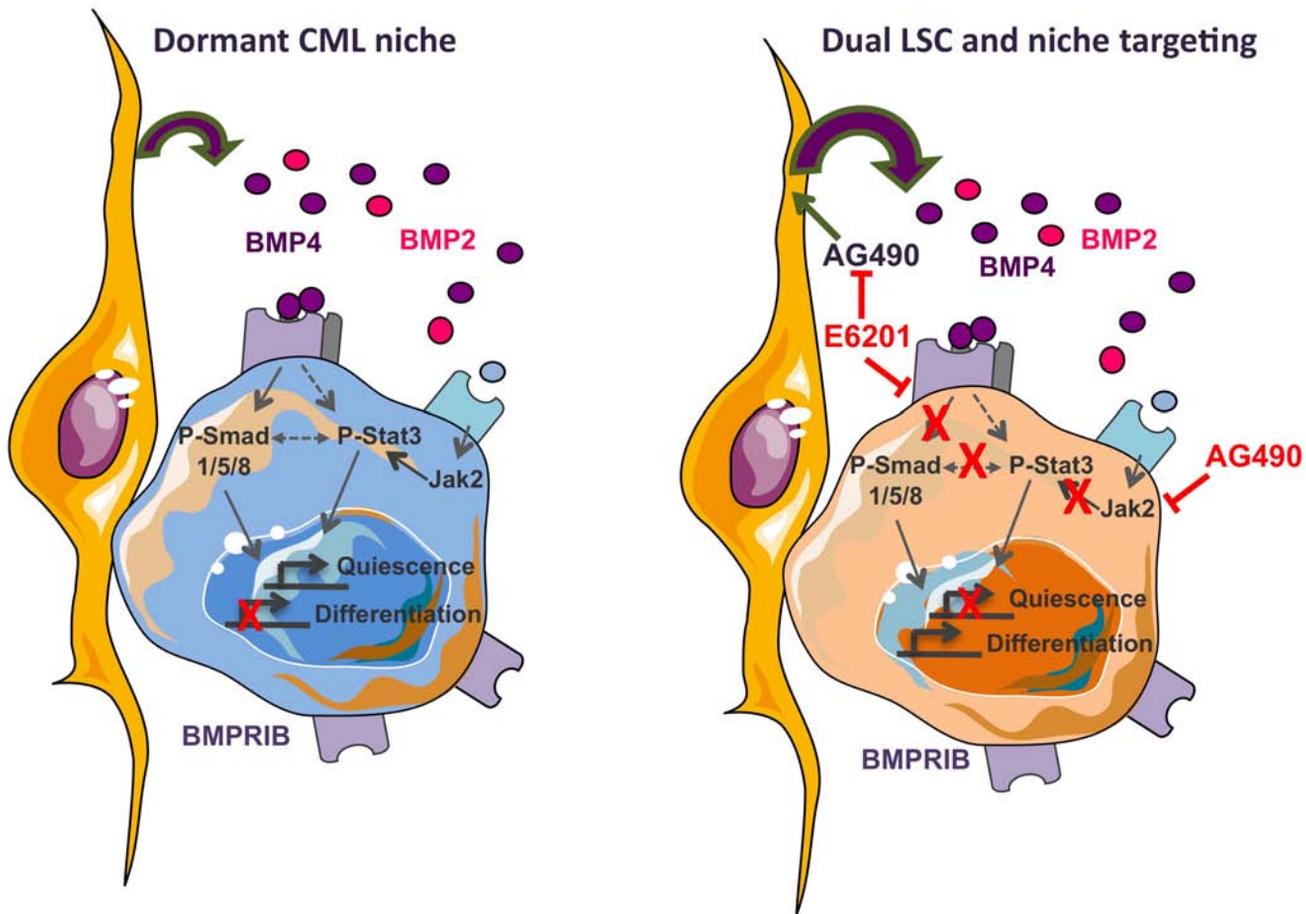


Figure 6



The quiescent fraction of chronic myeloid leukemic stem cells depends on BMPR1B, Stat3 and BMP4-niche signals to persist in patients in remission

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Supplemental figures legends

Supplemental Figure 1. (A) Beeswarm plots of *ID1*, *ID2*, *RUNX1* and *RUNX2* expression between group-A *BCR-ABL*⁺ SCs from patients at remission (light blue; *n* = 122) and group-B *BCR-ABL*⁺ SCs from patients at remission (purple; *n* = 123). Numbers of cells analyzed and numbers showing amplification for the selected genes are shown below the plot. The average gene expression level is indicated by red squares, and the boxes represent the median and quartiles of gene expression levels. *P*-values were calculated using the non-parametric Wilcoxon test. (B) Heat map shows hierarchical clustering of *ID1* and *RUNX1* genes in *BMPR1B*⁺ cells (*n* = 8) from group-A *BCR-ABL*⁺ SCs (left) or group-B *BCR-ABL*⁺ SCs (right). (C) Heat map shows hierarchical clustering of *IL2_Stat5* signaling pathway genes in *BMPR1B*⁺ cells (*n* = 8) from group-A *BCR-ABL*⁺ SCs (left) or group-B *BCR-ABL*⁺ SCs (right).

Supplemental Figure 2. (A) *CD34*, *CD38*, *CD123*, *TPOR*, *BMPR1B*, *NANOG*, *c-Myc*, *FOXO1*, *FOXO3a*, *HIF2A*, *ALOX5*, *TWIST1*, *BCL2* and *BCL-XL* mRNA levels from TF1-BAP cells. TF1-BA cells were used as comparators for each sample; *N* = 4. T-test significant *P*-values are indicated by red asterisks in the figure. (B) Dot plot showing BMP4 secretion, analyzed by ELISA, from TF1-BA and TF1-BAP supernatants.

Supplemental Figure 3. (A) Representative pictures of myeloid-derived (top), Erythroid-derived (middle) or mixed (bottom) CFC colonies from primary Normal Bone Marrow (NBM) CD34⁺, TF1 WT, TF1 BA or TF1-BAP cells. (B) Representative pictures derived LTC-IC colonies from TF1 BA or TF1-BAP cells.

Supplemental Figure 4. (A) Western blots showing BCR-ABL, CRKL and SMAD1/5/8 phosphorylation levels in TF1-BA and TF1-BAP cells following 2 h of TKI treatment; Scatter plot showing P-BCR-ABL/BCR-ABL ratio and P-CRKL (GAPDH used as internal control), *n* = 5-6. (B) Western blots showing Stat3 phosphorylation (Tyr705) levels (relative to Stat3) in TF1-BA and

TF1-BAP cells; Scatter plot showing P-Stat3/Stat3 ratio (GAPDH used as internal control) n = 3.

Supplemental Figure 5. (A) TF1-BAP cells treated with AG490 (25 μ M) for 24 h and analyzed for their content of P-Smad1/5/8 (left) or P-Stat3 (right). (B-C) TF1-BAP cells treated with Imatinib (IM) (2 μ M) or AG490 (25 μ M) or E6201 (100 nM) for 24 h and analyzed for their P-CRKL (B), P-ERK (B) or cleaved caspase3 (C) contents. (D) TF1-BAP cells treated with AG490 (25 μ M) + E6201 (100 nM) for 24 h and analyzed for their P-Smad1/5/8 (left) or P-Stat3 (right) contents.

Supplemental Figure 6. (A-B) ELISA quantification of BMP2 (A) and BMP4 (B) in BM plasma from healthy donors, CML patients at diagnosis and CCyR patients (at remission). Results from individual samples are expressed in picograms per milliliter, and horizontal lines represent mean values \pm SEM of the indicated number of analyzed samples. (C) Mesenchymal stem cells from normal bone marrow donors were treated with Imatinib, AG490 or E6201 for 3 days. Comparative transcriptional expression of BMP4 gene, results from 2 individual samples analyzed are expressed in arbitrary units, and the horizontal line represents mean values \pm SEM, determined compared to untreated controls. Unpaired t-test significant *P*-values are indicated by asterisks in the figures. (D) TF1-BA and TF1-BAP cells treated or not with BMP4 (20 ng/ml for 24 h) were analyzed for their content in P-Stat3.

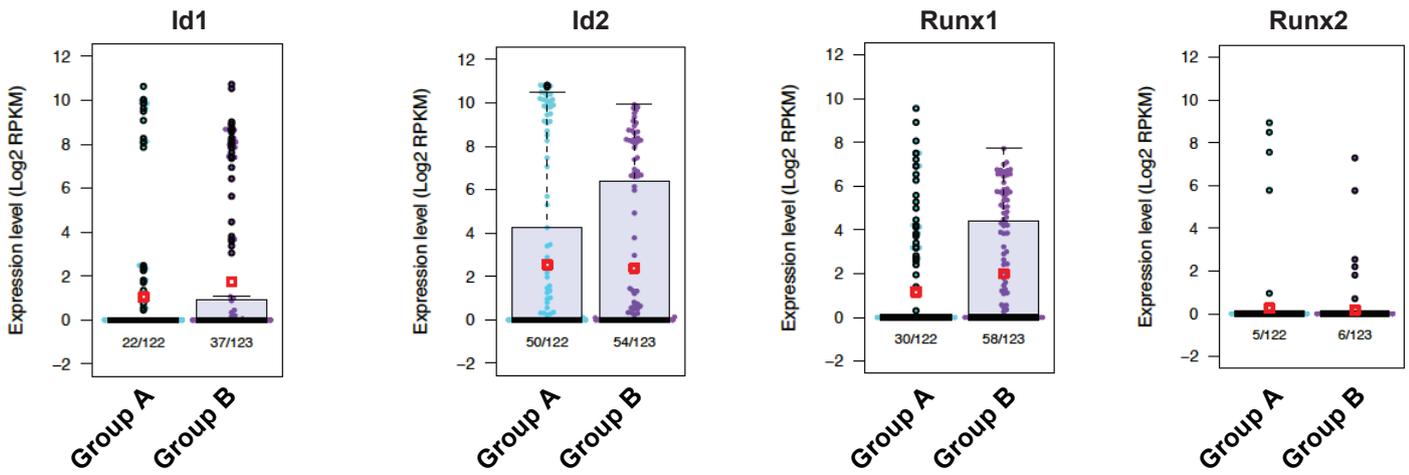
Supplemental Figure 7. (A) ELISA quantification of BMP4 in supernatants from HS5 and HS27A MSC cells. (B) TF1-BAP cells were seeded on top of a HS5 layer and then treated for 3 days with Imatinib (IM) (2 μ M), with or without AG490 (25 μ M) + E6201 (100 nM). Then TF1-BAP (GFP tagged) cells adherent fraction was harvested and analyzed for their content of Ki67-PE. Unpaired t-test significant *P*-values are indicated in the figure, *P*<0.05, **P*<0.01, **

Supplemental Table1. Results from BMP signaling pathway GSEA between group-A BCR-ABL+ SCs from patients at remission and group-B BCR-ABL+ SCs from patients at remission.

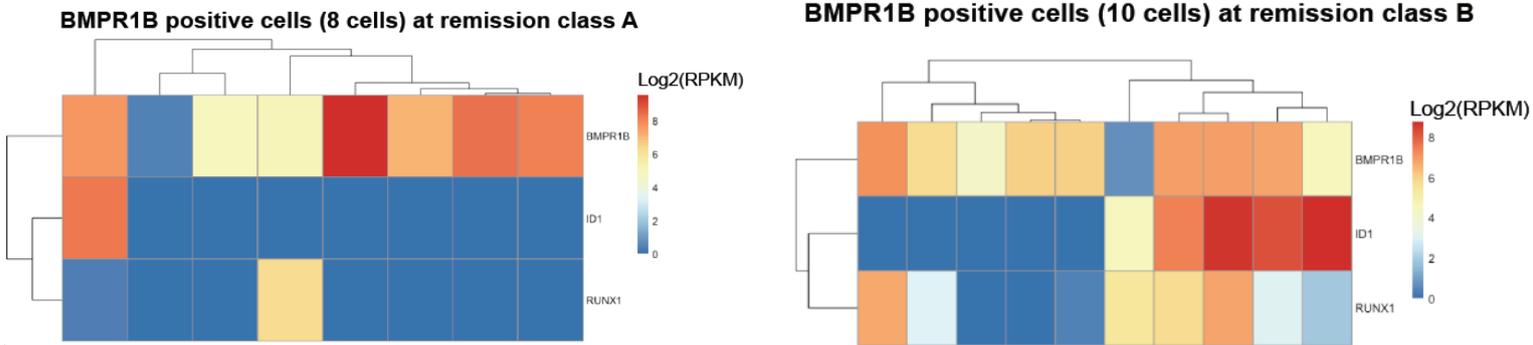
Supplemental Table 2. Chemical structure of E6201 (Top) and IC50 on top target genes with 100nM E6201 (Bottom).

Supp Figure 1

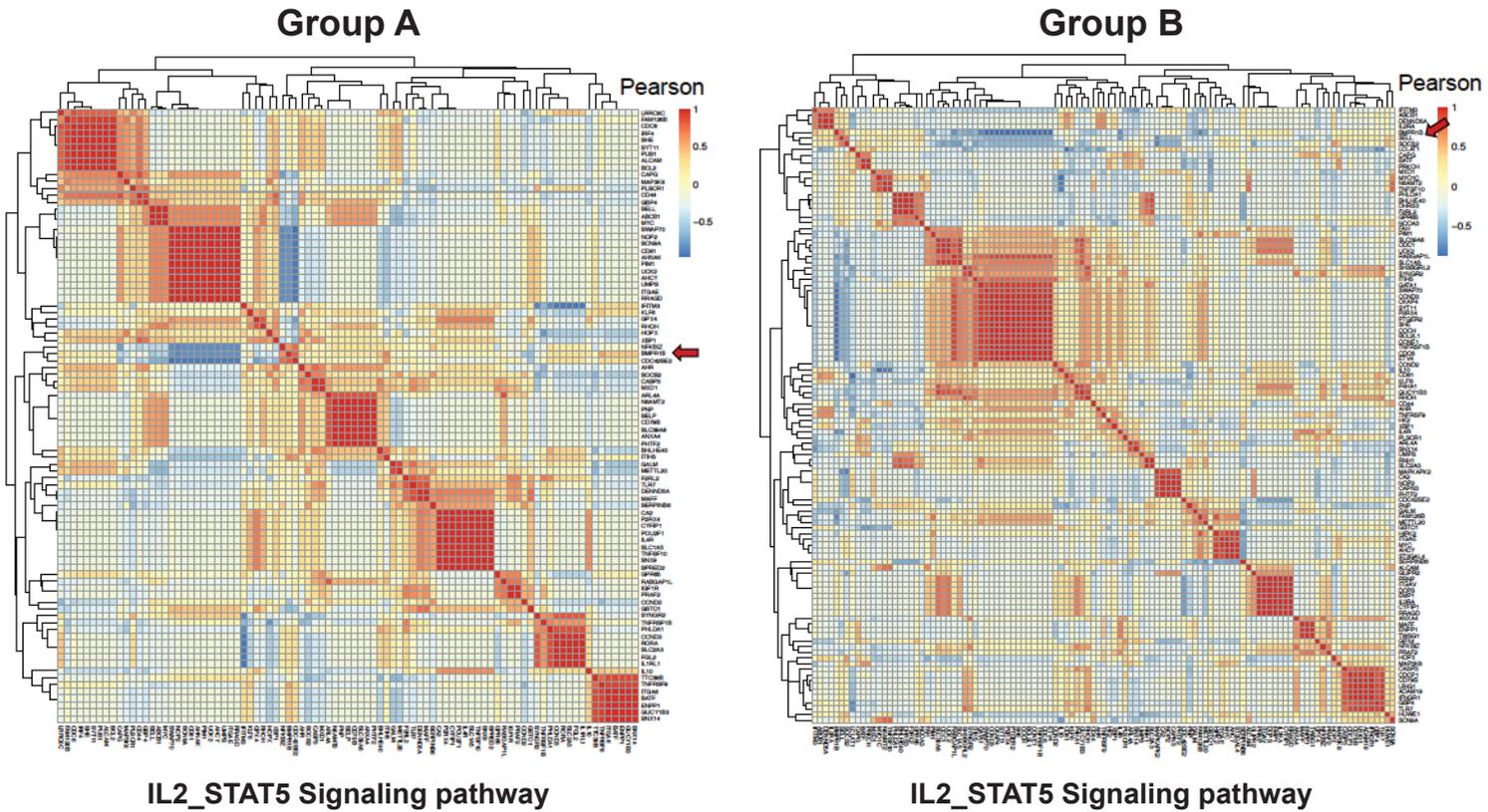
A



B

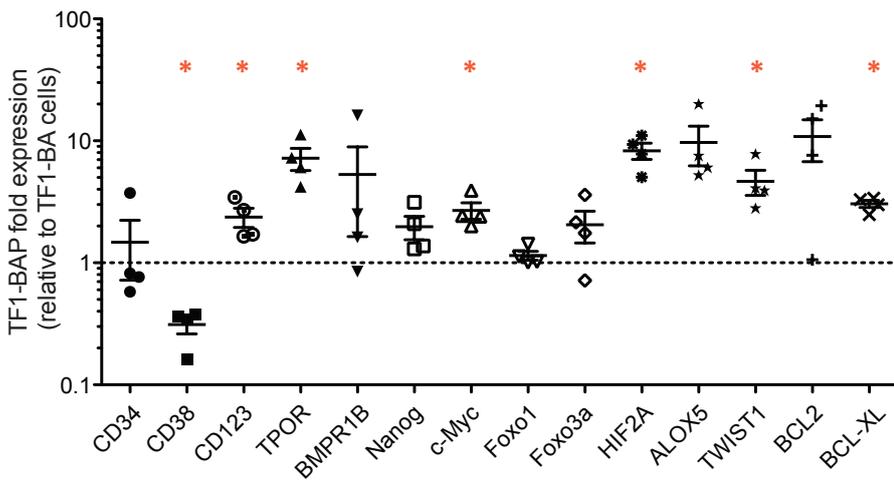


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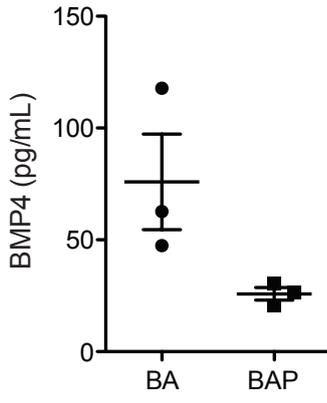


Supp Figure 2

A

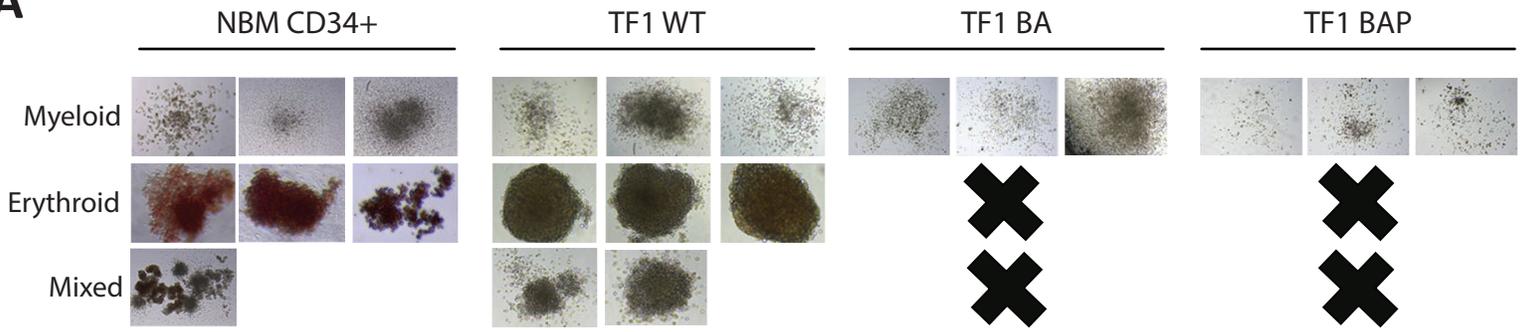


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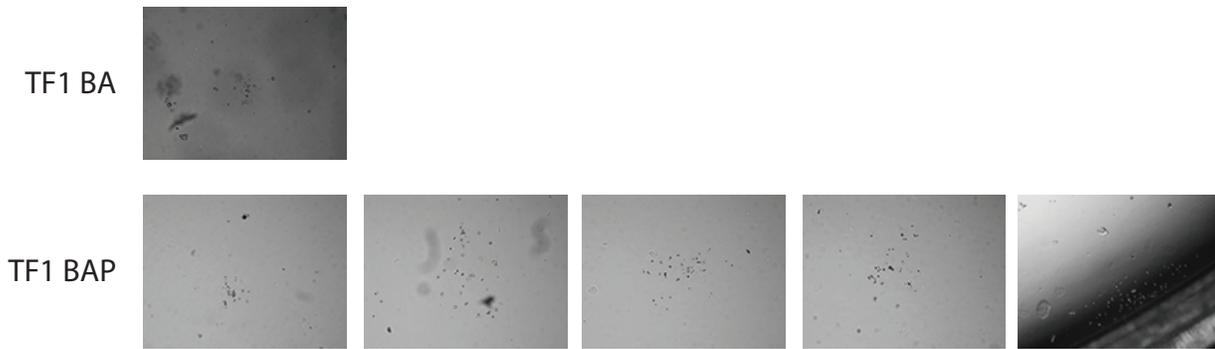


Supp Figure 3

A

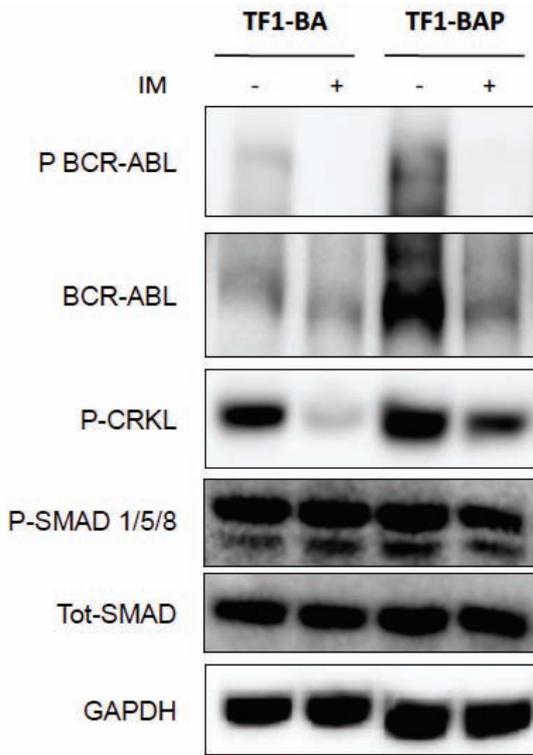


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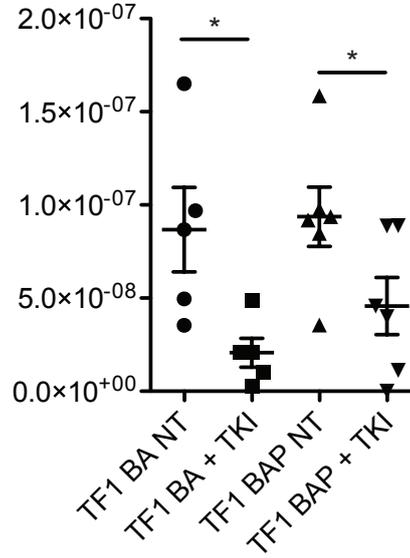


Supp Figure 4

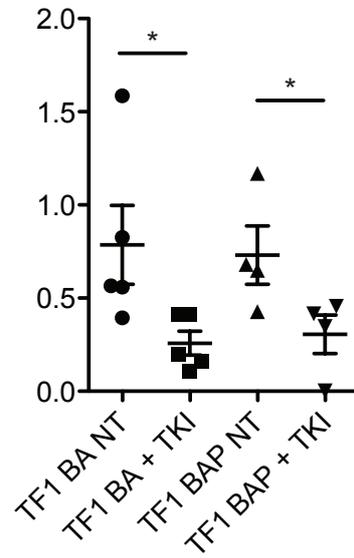
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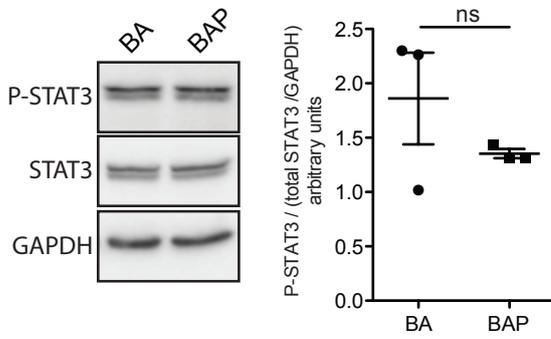
P-BCR-ABL / BCR-ABL / GAPDH



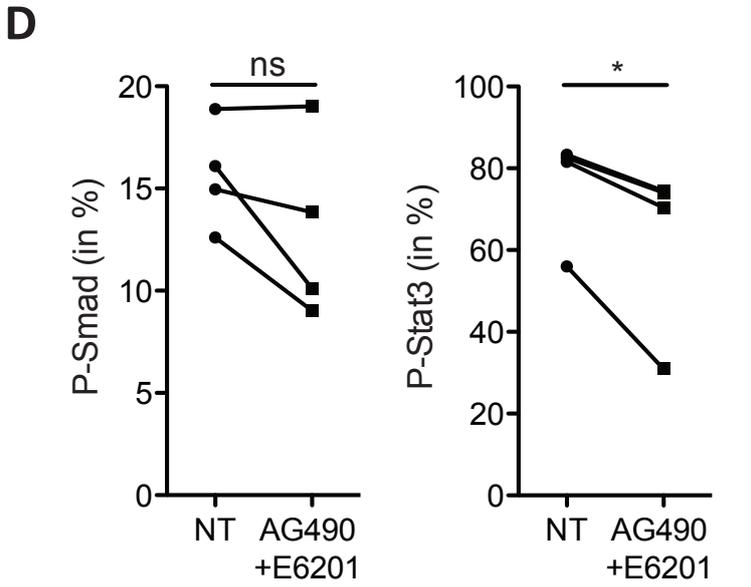
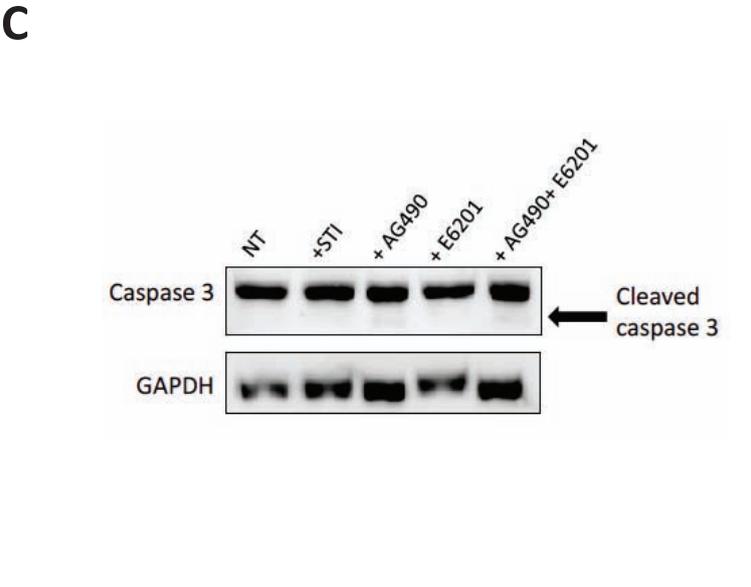
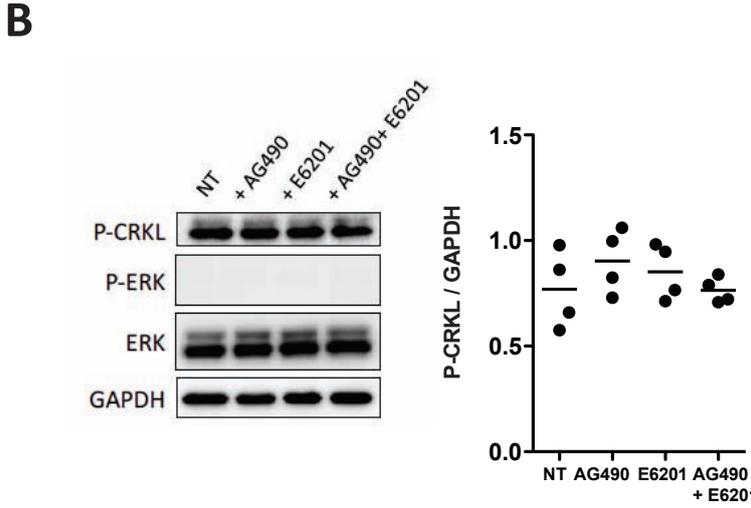
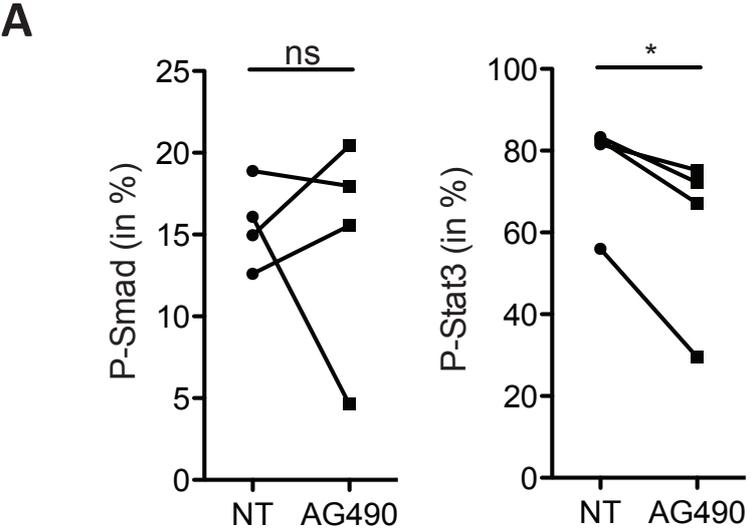
P-CRKL / GAPDH



B

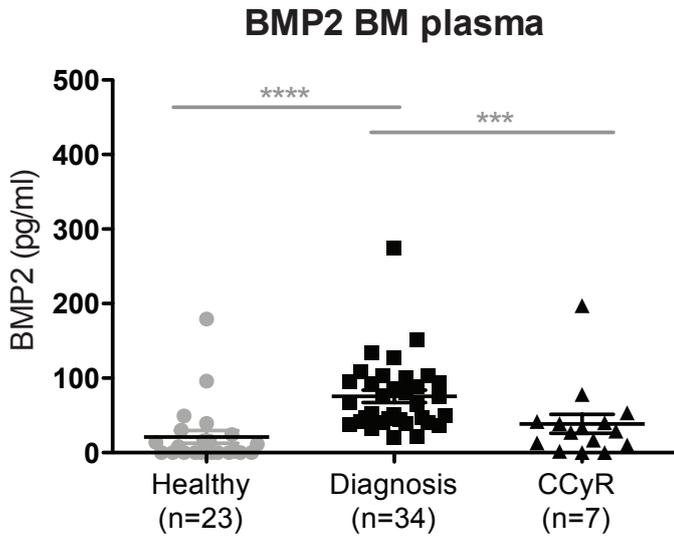


Supp Figure 5

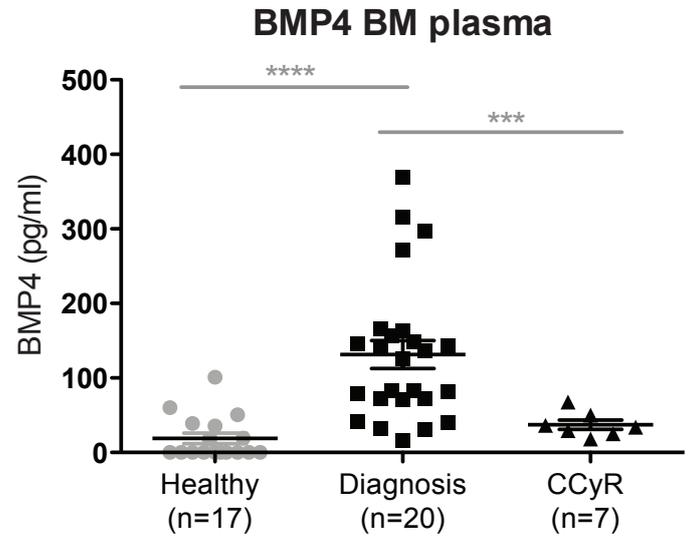


Supp Figure 6

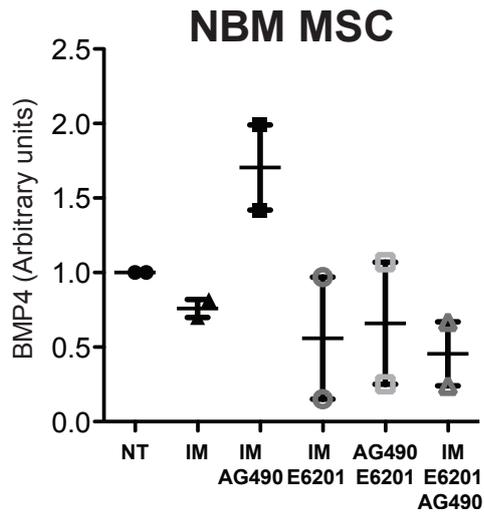
A



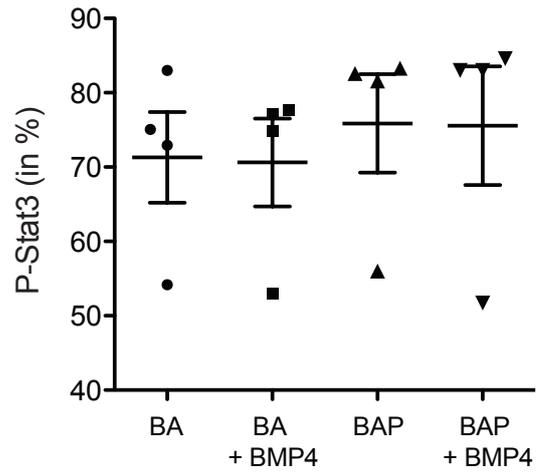
B



C

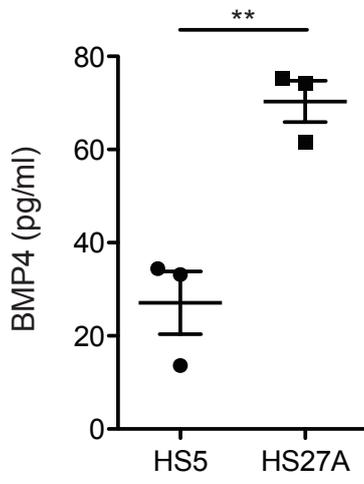


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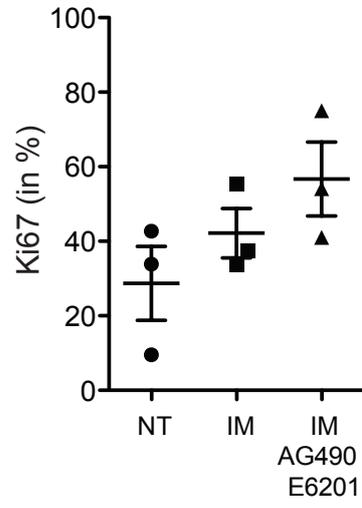


Supp Figure 7

A



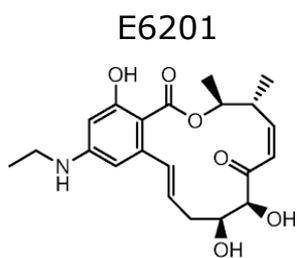
B



Supp Table S1. BMP Signaling pathway GSEA

PROBE	RANK IN GENE LIST	RANK METRIC SCORE	RUNNING ES	CORE ENRICHMENT
NOG	226	0,1885	0,0596	Yes
ID2	246	0,1850	0,1269	Yes
SMAD2	505	0,1511	0,1714	Yes
ACVR2B	618	0,1425	0,2190	Yes
BMP7	813	0,1303	0,2586	Yes
SMAD5	904	0,1269	0,3014	Yes
BMP8A	1382	0,1102	0,3215	Yes
ACVR2A	1395	0,1099	0,3614	Yes
RUNX3	1783	0,0991	0,3812	Yes
RUNX2	1789	0,0990	0,4174	Yes
ID3	1879	0,0962	0,4490	Yes
SMURF1	2069	0,0920	0,4747	Yes
ACVRL1	2663	0,0864	0,4811	Yes
BMPR1B	2892	0,0828	0,5017	Yes
SMAD7	3279	0,0756	0,5130	Yes
BMP2K	4437	0,0559	0,4839	No
TWSG1	5742	0,0344	0,4407	No
SMAD3	6223	0,0271	0,4301	No
RUNX1	6764	0,0195	0,4142	No
ACVR1B	7284	0,0132	0,3968	No
DAND5	7559	0,0102	0,3888	No
CHRD1	7964	0,0065	0,3739	No
BMPR2	8283	0,0042	0,3618	No
BAMBI	8780	0,0000	0,3405	No
BMP15	8805	0,0000	0,3395	No
BMP2	8806	0,0000	0,3395	No
BMP5	8807	0,0000	0,3395	No
BMP6	8808	0,0000	0,3395	No
BMPER	8810	0,0000	0,3394	No
BMPR1A	8811	0,0000	0,3394	No
CER1	9171	0,0000	0,3241	No
CHRD2	9198	0,0000	0,3230	No
GREM1	10032	0,0000	0,2873	No
SMAD6	13175	0,0000	0,1526	No
TNFRSF11B	13609	0,0000	0,1341	No
TNFSF11	13611	0,0000	0,1340	No
TWIST1	13699	0,0000	0,1303	No
ID1	14417	-0,0062	0,1018	No
SMAD9	16035	-0,0280	0,0429	No
SMAD1	16926	-0,0428	0,0204	No
SMURF2	17163	-0,0471	0,0277	No
BMP4	19096	-0,0836	-0,0244	No
FST	20088	-0,0944	-0,0321	No
SMAD4	20464	-0,1029	-0,0103	No
BMP1	22496	-0,1694	-0,0351	No
ENG	22856	-0,1986	0,0226	No

Supp Table S2



Gene	IC50@100nM
MEK1	0,1
MEK2	0,1
BMPR1B	0,45
BMPR1A	36
BMPR2	77
JAK1	89
JAK2	100