Non-canonical Hedgehog signaling mediates profibrotic hematopoiesis-stroma crosstalk in myeloproliferative neoplasms

Highlights

- Gli1 protein levels in blood cells mark fibrotic progression in MPN patients
- Hematopoietic Gli1 is upregulated in a smoothened-independent manner in MPNs
- Gli1 mediates profibrotic crosstalk with stromal cells via MIF-CD74 axis
- Inhibiting Gli1 or MIF reduces MPN phenotype and fibrosis grade

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In brief
Pritchard et al. show that increased Gli1 protein levels in MPN patient blood cells mark fibrotic progression. By systematically interrogating the role of canonical vs. non-canonical Hedgehog signaling, they show that smoothened-independent hematopoietic Gli1 signaling is critical for profibrotic crosstalk with stromal cells via an MIF-CD74 axis.
Non-canonical Hedgehog signaling mediates profibrotic hematopoiesis-stroma crosstalk in myeloproliferative neoplasms

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SUMMARY

The role of hematopoietic Hedgehog signaling in myeloproliferative neoplasms (MPNs) remains incompletely understood despite data suggesting that Hedgehog (Hh) pathway inhibitors have therapeutic activity in patients. We aim to systematically interrogate the role of canonical vs. non-canonical Hh signaling in MPNs. We show that Gli1 protein levels in patient peripheral blood mononuclear cells (PBMCs) mark fibrotic progression and that, in murine MPN models, absence of hematopoietic Gli1, but not Gli2 or Smo, significantly reduces MPN phenotype and fibrosis, indicating that GLI1 in the MPN clone can be activated in a non-canonical fashion. Additionally, we establish that hematopoietic Gli1 has a significant effect on stromal cells, mediated through a druggable MIF-CD74 axis. These data highlight the complex interplay between alterations in the MPN clone and activation of stromal cells and indicate that Gli1 represents a promising therapeutic target in MPNs, particularly that Hh signaling is dispensable for normal hematopoiesis.

INTRODUCTION

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm (MPN) characterized by the replacement of hematopoietic tissue in the bone marrow (BM) with fibrotic scar tissue. Despite extensive research into genetic alterations in hematopoietic stem cells (HSCs) that lead to MPNs and PMF,1,4 the mechanisms by which the fibrosis-driving niche cells are transformed and activated are not yet fully understood.

Recent work has demonstrated that Gli1, a downstream effector transcription factor (TF) of the Hedgehog (Hh) signaling pathway marks a population of non-hematopoietic stromal cells as a critical population of fibrosis-driving cells, not only in PMF5 but also in solid organs.5,7 The Hh signaling pathway is highly conserved, with a critical role in development,8 and includes three ligands (Indian, sonic, and desert Hh), the receptor PTCH1, the transmembrane protein smoothened (Smo), and the downstream TF family of Gli proteins. Dysregulation of this
RESULTS

GLI1, but not SHH, is deregulated on a protein level in peripheral blood from patients with MPNs

First, we examined the activity of Hh signaling in the peripheral blood (PB) of patients with MPNs, given that increased GLI1 mRNA levels have been found in MPN stromal cells and granulocytes. We performed an ELISA for sonic Hh protein (SHH), a canonical ligand of the Hh pathway, on plasma samples from healthy donors (HDs) and patients with MPN (Table S1). We found no correlation between SHH concentration and disease status (Figure 1A).

We next asked whether there is a link between the JAK2V617F mutation and increased GLI1 mRNA. The JAK2V617F mutant SET-2 cell line had significantly increased GLI1 transcripts compared with the JAK2WT MEG-01 cell line (Figure 1B). This link between the mutation and increased transcript levels was confirmed in induced pluripotent stem cell (iPSC) lines derived from patients with MPNs when comparing JAK2WT and Jak2V617F clones (Figure 1C).

To investigate whether transcriptional changes seen in granulocytes from JAK2V617F patients with MPNs are reflected in altered protein levels, we performed intracellular fluorescence-activated cell sorting (FACS) on PB mononuclear cells (PBMCs) to determine GLI1 protein levels (Figure S1A). The CD66b+ population had a significantly increased mean fluorescence intensity (MFI) for GLI1 in patients with fibrosis (grade 1) and marked the onset of fibrosis compared with both HDs and patients without fibrosis (grade 0) (Figure 1D). Importantly, the increased GLI1 protein expression did not solely correlate with the increased numbers of CD66b+ cells (Figure S1B).

In CD88+/89+ monocytes, which are decreased in number in patients with MPNs (Figure S1C), a similar pattern of GLI1 increase occurs at onset of fibrosis (Figure 1E). There was a trend for GLI1 protein to be expressed at lower levels in MF0 than in HDs and significantly lower than in MF1.

Altered lymphocyte levels in patients with MPNs have been reported previously and were confirmed in our cohort (Figures S1D and S1E). In our cohort, GLI1 levels in T cells were significantly correlated with onset of fibrosis, and the same trend was seen in the B cell population (Figure 1F).

To link the increased GLI1 protein levels seen in PBMCs from patients with MPNs to non-canonical stimulation of the Hh pathway, we isolated PBMCs from HDs and stimulated them with MPN-relevant cytokines: TGFβ1 or interleukin-6 (IL-6). After 72 h of stimulation, TGFβ1 and IL-6 resulted in increased GLI1 levels in CD66b+ granulocytes (Figure 1G), and TGFβ1 increased GLI1 levels in CD19+ B cells (Figure S1F).

Our data indicate that GLI1 expression is cell type specific, is differentially regulated throughout the course of MPNs, and increases with the onset of BMF. The increase in GLI1 protein levels between MF0 and MF1 in multiple hematopoietic lineages independent of cell number changes suggests that Hh signaling via GLI1 within hematopoietic cells plays a role in the progression of non-fibrotic MPNs to overt fibrotic disease. These data, when taken together with previous data showing an increase in GLI1 expression in fibrosis-driving stromal cells, suggest a role of GLI1 in both the hematopoietic and stromal niches in MPNs.

Knockout of GLI1 in hematopoietic cells reduces BMF

To investigate the role of hematopoietic GLI1 in MPNs, we utilized two methods of BMF induction: overexpression of thrombopoietin (TPO) and Jak2V617F. After lethal irradiation, mice received a transplantation (Tx) of either wild-type WT or Glil−/− germline knockout cKit+ hematopoietic stem and progenitor cells (HSPCs) transduced with either the overexpression/mutation vector or its corresponding control (Figures 2A and S2A).

Jak2V617F overexpression first recapitulates the myeloproliferative phase of MPNs mainly characterized by polycythemia. When the disease progresses to fibrosis, blood counts and hemoglobin (Hgb) levels drop reflecting the inability of fibrotic BM to produce mature blood cells. Although Glil knockout in Jak2V617F-overexpressing HSPCs did not ameliorate increased Hgb levels, it prevented the progressive anemia typically seen in this model with advanced fibrosis (Figure 2B). In line, Glil knockout in HSPCs in the Jak2V617F setting also prevented a decrease in platelet (PLT) levels over time. Although GLI1 levels are increased in granulocytes in patients with MPN with progressive disease grades, Glil knockout in HSPCs did not affect white blood cell (WBC) counts.

Our analysis of the PB indicated that absence of Glil is not sufficient to fully ameliorate the Jak2V617F-induced MPN phenotype but that mice do retain more normal hematopoietic function than those with Glil. Jak2V617F overexpression in HSPCs lead to changes in the HSC compartment, specifically an increase in long-term (LT) HSCs (Lin−Sca1+ckit+ [LSK], CD48−CD150+) (Figure 2C). Inhibition of Glil reduced this expansion to normal levels. Using GFP as a marker, we quantified the number of Jak2V617F mutant LT HSCs in the BM (Figure 2D). These are
**Figure 1.** SHH levels remain normal in patients with MPNs, while GLI1 RNA and protein level increases are linked to the JAK2^{V617F} mutation

(A) Plasma from patients with MPNs (n = 12, IDs MPN1–MPN12) and healthy donors (HDs) was isolated, and SHH protein levels were determined by ELISA. (B) GLI1 expression in megakaryocytic cell lines was quantified with qPCR relative to GAPDH, and the JAK2^{V617F}-containing SET2 cell line was normalized to the JAK2^{WT} MEG-01 cell line (n = 3).

(C) GLI1 expression in undifferentiated patient-derived iPSC lines was quantified with qPCR relative to GAPDH, normalized to respective JAK2^{WT} clones (n = 3).

(D) PBMCs isolated from patients with MPNs compared with HD samples (HDs, n = 4, n = 5; MF0, n = 3; MF1, n = 4; MF2 and MF3, n = 3, IDs MPN13–MPN22). Shown are MFI quantification of GLI1 levels in CD66b^{HI} granulocytes per group normalized to fluorescence minus one (FMO) controls and a representative GLI1 histogram, with count normalized to mode.

(E and F) MFI quantification of GLI1 levels per group normalized to FMO controls in CD88^{+}CD89^{+}HLADR^{+} monocytes (E), CD3^{+} T cells, and CD19^{+} B cells (F).

(G) MFI quantification of GLI1 levels in CD66b^{+} granulocytes after PBMCs from HDs (n = 3) were stimulated with 10 ng/mL rTGFb or 50 ng/mL rIL-6 for 72 h, normalized to FMO controls.

Bar chart data are shown as mean ± SEM with unpaired Student’s t test. Boxplot data are shown as minimum (min) to maximum (max) with line at mean, one-way ANOVA followed by Tukey’s post hoc test. *p < 0.05, ** = p < 0.01, **** = p < 0.0001. See also Figure S1 and Table S1.
reduced in the absence of Gli1, suggesting that Gli1 plays a role in the clonal expansion of a Jak2V617F HSCs BM. The absence of Gli1 in normal HSPCs only leads to a minor, nonsignificant reduction of the LT HSC fraction, indicating that it may be an attractive therapeutic target for the specific reduction of the disease-causing clone.

Another typical feature of Jak2V617F-induced MPNs is increased megakaryocyte number and size. H&E staining showed this pathogenic megakaryocyte morphology with clustering of hyperlobulated megakaryocytes in atypical locations (Figure 2E). In striking contrast, in the absence of Gli1, the BM showed erythroid hyperplasia, in line with increased Hgb levels, but megakaryocytes that were more comparable in morphology and frequency with healthy BM (Figures 2E and 2F). The absence of Gli1 in the non-diseased background affected neither morphology nor cellularity (Figure 2E). Consistent with the progressed MPN phenotype, in WT Jak2V617F mice, the spleen-to-body weight ratio was significantly increased, indicating increased levels of extramedullary hematopoiesis (Figure 2G), and hematopoietic knockout of Gli1 in Jak2V617F-medicated MPNs reduced this.

As expected from the advanced splenomegaly in the presence of Gli1 in Jak2V617F-induced disease, the BM showed progressed fibrosis (Figure 2H). In contrast, Gli1−/−Jak2V617F BM had low-grade fibrosis (Figure 2I). This was also phenocopied in the ThPO model, where the absence of Gli1 resulted in fibrosis no greater than grade 1 compared with grade 2–3 with Gli1 (Figure S2E), although there was no accompanying effect on other measures of disease severity in the PB or BM (Figures S2B–S2D). Hematopoietic Gli1 knockout also preserved the cellularity of the BM in Jak2V617F-induced disease (Figure S2F). Importantly, there was no significant difference in the overall GFP+ percentage observed in the BM at sacrifice between the WT JAK2V617F and Gli1−/−JAK2V617F conditions, indicating that the observed phenotype reduction was not due to a reduced number of transduced cells in the BM (Figure S2H).

To definitively demonstrate that Gli1−/− results in less advanced fibrosis, we performed Sirius red/fast green staining, which stains collagen I and, thus, highlights more advanced fibrosis. Strikingly, only sections of femora from WT Jak2V617F mice stained positive, while in the Gli1−/− setting, red-stained collagen fibers were only observed around the central arteries itself (Figure 2J). This difference was significant (Figure 2K), and the absence of hematopoietic Gli1 in Jak2V617F-induced fibrosis normalized the amount to baseline levels. This is further evidence that, in the absence of Gli1, fibrosis is reduced in both Jak2V617F and ThPO overexpression models of BMF.

Important for future therapeutic prospects, we observed no negative hematopoietic phenotype in mice transplanted with Gli1−/− HSPCs compared with WT HSPCs. While changes were observed in the stem cell compartment of the BM (Figure S2G), engraftment was unaffected (Figure S2H). To further confirm that targeting Gli1 would not result in reduced stem cell fitness, we performed a competitive transplantation using a 50:50 mix of Gli1−/− and WT BM (Figure S2K). After 152 days, we observed no significant difference in engraftment or stem cell compartments between the two BM genotypes (Figures S2L and S2M), confirming that loss of Gli1 does not have a detrimental effect on HSC function.

**Neither knockout of Gli2 nor SMO in hematopoietic cells is sufficient to ameliorate the PMF phenotype**

We asked whether the reduction in Jak2V617F-mediated fibrosis seen in the absence of Gli1 also occurred in the absence of Gli2, another effector TF in the Hh signaling pathway shown to play a critical role in kidney fibrosis.27 We investigated whether the reduction of fibrosis seen in the absence of Gli1 could be recapitulated by knocking out the upstream canonical regulator of Gli1 and Gli2, Smo, which would strongly argue for a role of canonical Hh signaling via the three Hh ligands and Ptc1. WT mice were transplanted with either WT, Gli2, or Smo knockout HSPCs that had been transduced with the Jak2V617F patient-relevant mutation plasmid or the WT control (Figure S3A).

Gli2−/− mice still developed an MPN phenotype comparable with the WT Jak2V617F condition. Hgb, WBC, and PLT levels remained increased compared with controls and, in the case of WBCs, even trended higher throughout the experiment (Figure 3A), suggesting that hematopoietic Gli2 is not a key player in myelofibrosis. Smo knockout in the Jak2V617F setting did not significantly ameliorate the MPN phenotype, and the onset of anemia and reduction in PLTs indicated worsening BM function (Figure S3A). Neither knockout affected engraftment of the BM (Figure S3B).

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**Figure 2. Loss of Gli1 in HSPCs reduces BMF**

(A) C57BL/6 mice received lethal irradiation followed by WT or Gli1−/− cKit+ HSPCs transduced with Jak2V617F or WT control retroviral vectors (n = 5/group). Mice were sacrificed at 112 days after transplantation.

(B) Hgb, PLT, and WBC counts from PB over the course of the experiment. Mean ± SEM, two-way ANOVA with Geisser-Greenhouse correction followed by Tukey’s post hoc test; significance for values at sacrifice is indicated.

(C) FACS quantification of the long-term hematopoietic stem cell (LT HSC; Lin−Sca1+cKit+ CD48−CD150+) population as a percentage of the Lin−Sca1+cKit− (LSK) fraction in BM.

(D) FACS quantification of GFP+ LT HSC population as a percentage of all single cells (SCs) in the BM.

(E) Representative images of H&E staining. Scale bar, 60 μm.

(F) Quantification of megakaryocyte number and area in BM from H&E staining of the femur.

(G) Weight of the spleen at sacrifice as a percentage of total body weight.

(H) Quantification of myelofibrosis grade based on reticulin staining of the femur.

(I) Representative images of reticulin staining. Scale bar, 200 μm.

(J) Representative images of Sirius red/fast green staining of the femur. Scale bar, 60 μm.

(K) Quantification of Sirius red staining as a percentage of total area from Sirius red/fast green staining of the femur.

Bar chart data are shown as mean ± SEM, one-way ANOVA followed by Tukey’s post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Figure S2.
Figure 3. Loss of Gli2 or Smo in HSPCs does not reduce MPN or fibrotic phenotype

C57BL/6 recipient mice received lethal irradiation followed by WT, Gli2<sup>−/−</sup>, or Smo<sup>−/−</sup> cKit<sup>+</sup> HSPCs, isolated 4 weeks after final poly(I:C) injection, transduced with Jak2<sup>V617F</sup> or WT control retroviral vectors (n = 5/group). Mice were sacrificed 112 days after transplantation.

(A) Hgb, PLT, and WBC counts from PB over the course of the experiment. Mean ± SEM, two-way ANOVA with Geisser-Greenhouse correction followed by Tukey’s post hoc test; significance for values at sacrifice is indicated.

(B) Quantification of megakaryocyte number and area in BM from H&E staining of the femur.

(C) Representative images of H&E staining of the femur. Scale bar, 60 μm.

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Significantly altered BM architecture was observed in Jak2V617F-induced MPNs in all genotypes. Megakaryocyte size was reduced in the absence of Smo and Gli2, but typical features of dysplasia remained (Figures 3B and 3C). In healthy BM, the knockout of Gli2 and Smo did not significantly influence the BM architecture compared with the control condition (Figure 3C).

Neither knockout of Gli2 nor Smo was sufficient to reduce Jak2V617F-induced extramedullary hematopoiesis, as measured by spleen-to-body weight ratio. (Figure 3D). In line with this, neither Gli2 nor Smo knockout ameliorated reticulin fibrosis in the BM, and all mice with Jak2V617F-induced fibrosis, regardless of genotype, presented with grade 1–2 fibrosis (Figures 3E and 3F). Quantification of collagen I in these mice also did not indicate any reduction in fibrosis level in the absence of Gli2 or Smo. The finding of no fibrosis or MPN phenotype improvement was recapitulated in the ThPO model (Figures S3D–S3F).

While the lack of fibrosis reduction without Gli2 or Smo eliminates them as potential therapeutic targets, the absence of an effect on normal hematopoiesis is additional evidence that targeting the pathway, via modulating Gli1 activity in patients, is a viable option. These findings further indicate that Gli1 can be activated independent of canonical Hh signaling via Smo because only knockout of Gli1 but not Smo ameliorates the MPN phenotype and fibrosis. Therefore, our data strongly indicated that non-canonical activation of Gli1 is an important mechanism driving myelofibrosis.

Gli1 mediates transcription of MPN-associated genes in HSPCs

Because Gli1 seems to play a role in the regulation of MPNs independent of canonical Smo activation, we next dissected the role of Gli1 in hematopoiesis by performing single-cell RNA sequencing (scRNA-seq) on BM cells in Jak2V617F-induced fibrosis and control, both in the presence and absence of Gli1. To understand the transcriptional changes occurring in the HSPC populations, we analyzed lineage-depleted, whole BM obtained at the end of a transplantation experiment in MPNs (Figure 2A). At time of sacrifice, all Jak2V617F mice in the presence of Gli1 in HSPCs showed progressed MPNs and high-grade BMF, while mice in the absence of Gli1 had only low-grade BMF (Figure 2B).

Unsupervised clustering revealed 13 hematopoietic cell types and one non-hematopoietic stromal cluster (Figure 4A; Tables S2 and S3). The top five differentially expressed genes (DEGs) per cluster demonstrate the commonalities between related clusters (Figure 4A; Table S4), and each condition (Gli1 WT vs. Gli1−/−) was represented in each cluster (Figure 4B). Investigation of the stromal compartment revealed two subpopulations: a mesenchymal stromal cell (MSC)-like and a fibroblast-like population (Figure 4C). This observation is consistent with heterogeneity seen in previously published stromal scRNA-seq datasets.29,31

We first explored the role of Gli1 in normal hematopoiesis by examining the DEGs in HSPCs. Our previous work demonstrated that S100A8 is elevated in MPNs and that targeting of S100A8/S100A9 signaling ameliorates BMF.36 Strikingly, S100a8 was among the top differentially downregulated genes with significantly reduced expression in the HSC and common myeloid progenitor (CMP) 3 cluster in the absence of Gli1 (Figure 4B); additionally, this is also seen across myeloid-lineage clusters (Figure S4D). We did not observe an impairment of myeloid differentiation in the absence of Gli1 in our mouse models (Figures S2I and S2J). Furthermore, in the CMP3 cluster, S100a11 was significantly downregulated in the absence of Gli1 (Figure 4B). S100A11 is linked to activation of the WNT/β-catenin pathway35 and the release of IL-6 from neutrophils,33 factors that have been linked to MPNs.34,36 Among the differentially upregulated genes in some of the most stem-cell-like populations were many ribosome-associated genes (Figure 4B), suggesting increased protein translation in the absence of Gli1.

Next, we investigated changes associated with Gli1 in the Jak2V617F-induced MPN setting, focusing on the primitive HSPC clusters. Notably, Hhb subunits (Hbb-bs and Hbb-bt) were downregulated in the absence of Gli1 in the HSC, CMP1, and CMP3 clusters (Figure 4C). Increased expression of Hbb has been linked to increased survival of metastasis-competent circulating tumor cells in solid organs,36 perhaps indicating a link between Gli1 and the increased proliferation and survival of the malignant clone in MPN. This also correlates with compensatory erythropoiesis seen in advanced fibrosis in MPNs. Another striking alteration is the downregulation of Lgals1 (Galectin-1) in Jak2V617F-induced MPNs in without Gli1. Galectin-1 has been linked to inflammation and extracellular matrix production, both signatures key to solid organ fibrosis and MPNs.37-39 Similar patterns of expression of ribosome-associated genes were associated with the absence of Gli1 (Figure 4C).

These data so far suggest that Gli1−/−, in addition to mediating transcription of MPN-associated genes, does not lead to detrimental expression changes in the most primitive HSPC compartments under normal conditions, providing further evidence that modulating GLI1 in patients with MPNs would specifically target the malignant clone.

TF analysis reveals cell-type-specific Gli family member expression patterns

Functional redundancy exists within the Gli TF family,40 and a systematic examination of the role of the members (GLI1, GLI2, and GLI3) in MPNs has not been done previously. Therefore, we performed network analysis using the Discriminant Regulation Enrichment Analysis (DoRothEA) tool41 to analyze TF activity in the HSC and CMP3 clusters (Figure 4D). There was little differential activity in the HSC cluster between the control and

(D) Weight of the spleen at sacrifice as percentage of total body weight.
(E) Quantification of myelofibrosis grade based on reticulin staining of the femur.
(F) Representative images of reticulin staining. Scale bar, 200 μm.
Bar chart data are shown as mean ± SEM, one-way ANOVA followed by Tukey’s post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Figure S3.
Figure 4. scRNA-seq analysis reveals the role of Gli1 in pro-fibrotic gene expression and pathway activation

(A) Unsupervised clustering of scRNA-seq of whole BM collected at sacrifice (112 days post Tx) from mice transplanted with either WT or Jak2V617F+/C0 retroviral vectors (n = 3/group, n = 19,897 cells) and identification of 13 hematopoietic clusters and 1 non-hematopoietic cluster. The most striking repeated signatures was the upregulation of gene sets linked to the phosphatidylinositol 3-kinase (PI3K) pathway, such as mTORC1 signaling, which is significantly associated with the presence of Gli1 in Jak2V617F+/- mediated fibrosis (Figures 4E–4G). The PI3K/akt/mTOR pathway has been shown previously to be activated by Gli1 in poor-prognosis acute myeloid leukemia patients and, conversely, PI3K inhibition has been shown to activate Gli1 in a noncanonical fashion, providing evidence that targeting PI3K rather than SMO might also be an attractive strategy to inhibit Gli1 activation and the Hh signaling cascade in MPNs.

Each of these patterns is indicative of the milder phenotype seen in Jak2V617F+-induced fibrosis in the absence of Gli1 and provides insight into possible benefits of targeting Gli1 independent of canonical Hh signaling in MPNs.

Pathway changes occur in stromal cells in response to the presence of hematopoietic Gli1

Importantly, due to the transplant model and techniques used, the stromal compartment in this dataset is both unmaturated and WT for Gli1 expression. Therefore, any transcriptional changes identified must occur due to alterations in Gli1-dependent communication from the transplanted donor HSPCs. To investigate the downstream effects of hematopoietic Gli1 onJak2V617F+-induced fibrosis, we also performed GSEA on this stromal cluster (Figures 4H and 4I; Table S5) using Hallmark gene sets.

Of note, stromal cells exposed to Jak2V617F+ BM cells had significantly upregulated hypoxia-associated genes. This is a known signature of fibrosis and was reduced in the absence of hematopoietic Gli1 (Figure 4H). Stromal cells also showed upregulation of Hallmark gene sets associated with epithelial-to-mesenchymal transition, TNF-α, and apoptosis (Figure 4H). This supports a more fibrotic, dysregulated stromal niche in the absence of hematopoietic Gli1, stromal cells are less primed for fibrotic transformation.

Under both the disease and healthy conditions, the adipogenesis hallmark gene set in the stromal cluster was more associated with the absence of hematopoietic Gli1. We thus used MarrowQuant to quantify adipose tissue in the marrow from these mice and did not find any significant differences in adipose tissue area (Figure S4E). However, we found a significant reduction in adipocyte density in the non-disease marrow in the absence of hematopoietic Gli1 compared with the WT condition (Figure S4F), an interesting topic for future studies.

Based on data from the hematopoietic clusters (Figures 4E–4G), we hypothesized that the mechanism of action behind PI3K inhibitor success in patients with MPN is not solely due to the effect on the disease-causing malignant clone because PI3K is upstream of mTORC1/2 signaling. To validate the role of the PI3K/mTORC1 signaling axis in fibrosis driving stromal cells, we used the robust in vitro model of stimulating
Figure 5. Ligand-receptor analysis shows change in interactions via the disease-relevant MIF-CD74 axis

(A–C) Network plots of ligand-receptor (LR) activity comparing (A) the GLI1+/Jak2wt condition and WT in the non-disease setting, (B) the Jak2V617F and WT conditions, and (C) the GLI1+/Jak2V617F and WTJak2V617F conditions.

(D) Top 35 downregulated interactions based on stromal cluster receptors in GLI1+/Jak2V617F compared with WT Jak2V617F; interactions are ordered based on LRScore.

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BM-derived MSCs (BM-MSCs) with recombinant transforming growth factor β1 (rTGFβ1). Stimulation of BM-MSC with rTGFβ1 for 48 h led to a significant 3-fold increase in expression of C01a1 (collagen 1), which is a well-known readout of fibrosis in BM-MSCs (Figure 4J), relative to unstimulated controls. When the cells were treated with 5 μM of the pan-PI3K and mTORC1/2 inhibitor apilisib, C01a1 was significantly downregulated even after stimulation with rTGFβ1 compared with stimulated, untreated BM-MSCs.

Ligand-receptor analysis shows rewired hematopoietic-stromal cell communication in the absence of hematopoietic Gli1

Previous research has indicated an important role for the hematopoietic-stromal cell crosstalk in MPN and fibrosis initiation and progression. Thus, we wanted to investigate what these interactions are and how they change with hematopoietic Gli1 expression.

Using CrossTalker, we compared cell-cell interactions under non-disease conditions to examine the role of Gli1 in homeostasis (Figure 5A; Table S6). In general, there were more significant interactions between almost all cell clusters in the absence of Gli1, and of particular interest was the highly increased level of self-interaction within the stromal cell cluster. This finding could indicate that hematopoietic Gli1 regulates a wide range of cellular crosstalk even in homeostasis.

We next evaluated cell-cell interaction changes in Jak2V617F-induced disease progression in the presence of Gli1 (Figure 5B). The general pattern was a loss of many interactions, particularly between hematopoietic cell clusters and the stromal cluster, indicative of the complete dysregulation of normal BM function and loss of stroma-derived hematopoietic support that occurs in Jak2V617F-induced fibrosis. Interestingly, the clusters with more increased interactions were the neutrophil progenitors (NPs) and CMP1 and CMP2, indicative of a myeloid-derived immune cell involvement in fibrosis. This is in contrast with the CMP3 cluster, which appears to have significantly fewer interactions with many cell types. Given previous evidence of this cluster having reduced S100a8 expression in the absence of Gli1 (Figure 4B) and also the significant differences in Gli-family TF activity (Figure 4D), these data highlight the importance of this CMP3 population in the progression of BMF in MPNs.

To evaluate the contribution of hematopoietic Gli1 to these Jak2V617F-mediated changes in cell-cell interaction, we compared the Gli1 knockout Jak2V617F and WT Jak2V617F conditions (Figure 5C). Decreased intercellular interactions seem to be associated with worse fibrosis grade, and increased interactions are associated with the milder phenotype seen in the absence of hematopoietic Gli1. These data show that the absence of Gli1 results in increased interactions between the stromal cluster and various hematopoietic cell types, suggesting a less deregulated marrow. It also shows the same pattern of increased self-interaction in the stromal cluster. Of particular note are the increased interactions between the stromal cell cluster and the previously noted CMP3 cluster.

The disease-relevant hematopoietic MIF-to-stromal CD74 axis is modulated by Gli1

To further interrogate the mechanisms behind the role of Gli1 in Jak2V617F-mediated fibrosis, we focused on comparing the two disease states, where downregulated interactions are associated with worse fibrosis grade. Interrogating a ranking of the top downregulated ligand-stromal receptor pairs (Figure 5D, Table S6), we identified 6 receptors and 10 ligands shortlisted for further investigation (Figure 5E). The strongest downregulated interaction was the CMP3-MIF-CD74 stalk-axis. The CMP3 cluster has highly altered interactions with stromal cells in normal disease development (Figure 5B). MIF expression was increased in Jak2V617F-mediated fibrosis in the HSC, CMP3, and CMP2 clusters, and this was significantly reduced in the absence of Gli1. Additionally, in the Jak2WT settings, absence of Gli1 reduced Mif expression in the HSC and CMP2 clusters. Cd74 expression was reduced in stromal cells in fibrosis in the presence of hematopoietic Gli1 (Figure 5F).

To confirm the disease relevance of the MIF-CD74 axis, we stimulated human MSCs with recombinant MIF and observed a significant increase in expression of the fibrosis-associated genes TNFa and TGFB1 compared with unstimulated controls (Figure 5G). At the other side of the axis, when Cd74−/− murine MSCs were cultured with ThPO conditioned medium, they expressed significantly reduced levels of aSMA and Gli1, both known key markers of fibrotic stromal cells (Figure 5H).

MIF protein levels are relevant in patients with MPNs, and expression is linked to JAK2 and PI3K

We next validated that the Gli1-mediated MIF-CD74 axis is relevant to patients with fibrosis and may represent an attractive therapeutic target by collecting serum from patients with MPNs (Table S1) and performing a MIF ELISA (Figure 6A). MIF protein levels were indeed significantly increased in patients with MPNs compared with HDs, trending up in patients diagnosed with polycythemia vera and specifically significantly increased in patients with essential thrombocytopenia and PMF. Serum MIF levels were significantly correlated with PLT levels (Figure 6A), although no link to allele burden or fibrosis grade was observed (Figure S5A).

(E) Representation of LR interactions shortlisted for further interrogation.
(F) Violin plot of MIF expression across HSPC and myeloid primed clusters and of Cd74 in the stromal cluster across all conditions. One-way ANOVA followed by Tukey’s post hoc test.
(G) A human MSC cell line, MSOD, was stimulated with 100 ng/mL recombinant MIF for 24 h. TNFa and TGFB expression relative to HPRT1 expression was quantified by qPCR and normalized to the unstimulated condition. Unpaired Student’s t test with Welch’s correction (n = 3).
(H) BM-MSCs isolated from WT or Cd74−/− mice were treated with conditioned medium collected from an immortalized BM progenitor cell line overexpressing ThPO to induce a fibrotic phenotype. aSMA and GLI1 expression levels were quantified by qPCR and normalized to the WT stromal cells (n = 3). Unpaired Student’s t-test with Welch’s correction.

*p < 0.05, **p < 0.01, ****p < 0.0001. See also Table S6.
exclusive to patients harboring a JAK2<sup>V617F</sup> mutation, and MIF protein levels are also significantly increased in patients with a calreticulin (CALR) mutation (Figure 6B), raising the possibility that therapeutic intervention targeting MIF would benefit a wide range of patients with MPNs.

To better understand the mechanism behind MIF expression in patients with MPNs, we utilized JAK2<sup>V617F</sup> mutant HEL cells and treated them with either the JAK2 inhibitor ruxolitinib or the dual PI3K/mTOR inhibitor BEZ235. In both cases, Mif mRNA expression was significantly reduced compared with untreated controls (Figure 6C).

**Genetic or pharmacological perturbation of the MIF-CD74 axis reduces disease phenotype**

Having shown the relevance of MIF in patients with MPNs and illustrated a potential mechanism of regulation, we next wanted to validate its potential as a therapeutic target. Utilizing the previously described ThPO overexpression MPN model, WT mice were transplanted with either WT or Mif knockout HSPCs that had been transduced with the ThPO overexpression vector or empty vector control (Figure S5B).

In the absence of MIF, mice transplanted with THPO overexpression BM exhibited many typical aspects of the MPN phenotype (Figures S5C–S5G). However, there were significant reductions in both monocytes and macrophages in the PB over time (Figure 6D) and in the BM at sacrifice (Figure 6E). The monocyte expansion typically seen in this model was ameliorated.

Whilst the absence of MIF in the ThPO overexpression mouse model did not completely normalize the MPN phenotype (Figures S5C–S5G and 6F), reticulin staining demonstrated a quantifiable reduction in reticulin fibrosis (Figures 6G and 6H), and this was coupled with normalization of BM cellularity and BM viability in the absence of MIF (Figure 6I). Importantly, there was no difference in engraftment of GFP expression in the marrow collected at sacrifice, confirming that the reduction of fibrosis is an MIF-dependent effect and not due to reduced gene marking (Figure S5D). Thus, we demonstrated that Mif knockout has a specific effect on the fibrosis-driving stroma and on reducing the pathognomonic monocytosis.

To confirm that pharmacologically targeting MIF can reduce the fibrotic transformation, we cultured a human MSC cell line with conditioned medium collected from iPSCs during myeloid differentiation of JAK2<sup>WT</sup> or JAK2<sup>V617F</sup> patient-derived iPSCs. When cultured with the JAK2<sup>V617F</sup>-conditioned medium, key markers such as collagen 1 were significantly upregulated in the stromal cells compared with the control. With the addition of the MIF antagonist, ISO-1, this was normalized, indicating that MIF released from hematopoietic cells upregulates fibrosis genes in stromal cells and, importantly, that the upregulated MIF in patients is an attractive therapeutic target, either alone or in combination with JAK inhibition (ruxolitinib), to increase the inhibitory effect on MIF itself but also fibrosis (Figure 6J).

**DISCUSSION**

Our studies reveal a key role of non-canonical Hh signaling in the development and progression of PMF. We provide evidence that GLI1 can be a biomarker for disease progression and also a therapeutic target. While SHH protein levels in MPN patient serum are no different from that from HDs, GLI1 expression levels are specifically increased in a JAK2<sup>V617F</sup> mutant megakaryocytic cell line and patient-derived iPSCs compared with the JAK2<sup>WT</sup> equivalents. In addition, GLI1 protein levels are increased in both myeloid and lymphoid subsets of JAK2<sup>V617F</sup> PBMCs isolated from patients with MPNs. Importantly, this change marks the transition from non-fibrotic MPNs to overtly fibrotic disease. While this finding requires validation in larger cohorts, intracellular GLI1 flow cytometry could be implemented in follow-up appointments of patients to assess progression.

Previous data indicate that Hh signaling is dispensable for normal hematopoiesis. However, these studies either made use of Smo knock out mice or analyzed hematopoietic cells in steady-state Glil-null mice. Because it is now widely accepted that the Gl family of Tfs can be activated independent of Hh ligands, the use of upstream Hh pathway knockouts does...
not fully consider the potential role of non-canonical Hh signaling. Additionally, without transplantation into Gli1-null mice, analysis of the hematopoietic compartment in steady-state Gli1-null mice cannot rule out the contribution of non-hematopoietic cell types to observed phenotypes. By using Gli1, Gli2, or SMO knockout mice as donors and transplanting into WT, Hh signaling competent recipients, we here systematically deciphered the role of hematopoietic canonical and non-canonical Hh signaling in normal hematopoiesis and in MPNs.

Inhibition of the canonical Gli protein activator SMO has been proven to be an effective treatment in some cancer types and is FDA approved for BCC. Similar attempts to target it in MPNs has had varied success. In line, our data show that the genetic knockout of Smo is insufficient to reduce disease progression. Two MPN-relevant cytokines, TGFβ1,21,22 and IL-6,23–25 increase in GLI1 protein levels in PBMCs from HDs, showing that GLI1 activation can occur in a non-canonical fashion in MPNs and that it can be propagated to non-mutated cells by the inflammatory environment caused by the mutant hematopoietic clone. Knockout of Gli2 was unable to rescue the MPN phenotype or ameliorate fibrosis, pointing to a specific role of Gli1 rather than general activation of the Hh pathway. Recent studies using Gli1−/− mice indicate a specific role of Gli1 in regulation of HSPC function and cycling. Gli1−/− mice exhibit increased numbers of LT HSCs and increased engraftment in competitive transplantation studies, in line with our findings that the absence of Gli1 does not have any detrimental effects on normal HSPCs.

Our data show that hematopoietic Gli1 is crucial for malignant hematopoietic-stromal cell crosstalk in MPNs and affects key pathways in the disease pathogenesis. A key hallmark of MPNs and PMF is inflammation. Proinflammatory cytokines, such as TNF-α, are linked to the survival of the Jak2V617F malignant clone and to stromal cell activation in fibrosis. Our data show that TNF-α signaling via nuclear factor ιB (NF-κB) is significantly downregulated when hematopoietic Gli1 is inhibited in MPNs. Additionally, stromal cells under this condition also show the same pattern of downregulation, indicating a role of Gli1 in the communication of this signal between cell types.

PI3K/AKT signaling is important in PMF because it is critical for Jak2V617F-mediated cellular dysregulation. Our data show that inhibition of hematopoietic Gli1 reduces upregulation of this pathway in both hematopoietic and stromal cells. Given that the PI3K/AKT pathway has been shown previously to both activate Gli1 in a noncanonical fashion and also be activated itself by Gli1, this is one mechanism by which Gli1 signaling is perpetuated and transmitted between cell types in MPNs, in particular because the application of a PI3K inhibitor inhibited the fibrotic transformation of fibrosis-driving cells.

The Gli1-MIF/CD74 axis is relevant in this context because it also both activates and is regulated by the PI3K/AKT pathway and is linked to inflammation and fibrosis. MIF is interesting to be followed up on as a diagnostic marker because it is a secreted factor, and protein levels in serum of patients show a significant association with MPNs. We have shown that genetically inhibiting hematopoietic MIF reduces fibrotic transformation in vivo in a murine MPN model. We thus conclude that targeting MIF in MPNs would interrupt crucial hematopoietic-stromal, pro-fibrotic crosstalk. Our data also indicate the application of combinatorial strategies by pointing out a JAK2V617F-PI3K/GLI1-MIF-CD74 axis that exerts cell-intrinsic and -extrinsic effects on the MPN clone and the fibrotic transformation.

Activation of GLI1 can occur independent of canonical Hh signaling and is directly associated with the JAK2V617F mutation in MPN. Therefore, specific, direct targeting of this TF is needed rather than inhibiting upstream nodes of the Hh pathway. Downstream of GLI1 in MPNs, multiple pathways and genes are activated and upregulated, including PI3K and MIF; the complex paracrine and autocrine interactions between these nodes result in increased secretion of MIF, which, in turn, increases expression of fibrosis-relevant genes in stromal cells. Our data highlight the importance of combinatorial strategies to interrupt pro-fibrotic intracellular signaling and intercellular crosstalk, which might be more effective than either alone.

Limitations of the study
Our findings of increased GLI1 protein levels in hematopoietic cells were only from patients with MPNs harboring a JAK2V617F mutation; thus, larger cohorts with varied mutational statuses and more controls are needed. The kinetics of the disease might also play a role because GLI1 knockout had more effect in the JAK2V617F model, which has slower kinetics of disease progression than Thpo overexpression. We acknowledge that Gli2 and Smo are floxed, conditional genetic knockout models needing poly(I:C) for excision, while the GLI1 model is a germline, non-inducible knockout. All experiments had a respective control cohort, and while we waited an appropriate amount of time prior to transplanting to ensure that any cycling effects on the HSPCs were mitigated, only the inducible knockout lines and their controls received poly(I:C), which may be a confounder. The frequency of GFP+ cells showed variability in the mice that was not reflected in the phenotype. In our scRNA-seq, a higher number of stromal cells might increase insights into GLI1-mediated crosstalk. Although our data link PI3K with GLI1 in MPNs, and we saw an effect of PI3K inhibition on the fibrotic transformation of stromal cells, clinical trials of inhibitors targeting this pathway were halted due to insufficient improvement.

STAR METHODS
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**QUANTIFICATION AND STATISTICAL ANALYSIS**

- Image processing and quantification
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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.113608.

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**REFERENCES**


**DECLARATION OF INTERESTS**

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pro-inflammatory stem cell niche drives myelofibrosis through a targetable galec tin 1 axis. Preprint at bioRxiv.


**STAR METHODS**

**KEY RESOURCES TABLE**

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### Critical commercial assays

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### Experimental models: Cell lines

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### Experimental models: Organisms/strains

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### Oligonucleotides

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(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rebekka K. Schneider, MD, PhD (reschneider@ukaachen.de).

Materials availability
This study did not generate new unique reagents. For specific details on availability please refer to the key resources table.

Data and code availability
- Single cell sequencing experiments are deposited at GEO: GSE218616. Seurat objects of dataset and sample markdown code is available at Zenodo: https://doi.org/10.5281/zenodo.1009072.
- This paper does not report original code.
- Tables with complete marker genes, differential expression, gene expression enrichment analysis, pathway and ligand receptor analysis can be found in the supplemental information of this paper.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human studies
Blood samples were obtained from patients with MPN at the Department of Hematology, Oncology, Hemostaseology and Stem Cell Transplantation of RWTH Aachen University, or from fully anonymized healthy individuals at the Department of Transfusion Medicine at RWTH Aachen University, after written informed consent as approved by the Ethikkommission an der Medizinischen Fakultät der Rheinisch-Technischen Hochschule (RWTH) Aachen. (EK127/12, EK206/09 and EK099/14 respectively). Further control samples were obtained via the Institute for Clinical Chemistry at Erasmus MC, NL. Surplus material was collected from dermatological, orthopedic or neurological patients after diagnostics according to the ethical vote MEC-2018-1445 by the METC Erasmus Medisch Centrum Rotterdam. Additional blood samples from patients with MPN and healthy cord blood samples...
wrote collected from fully anonymized donors at Memorial Sloan Kettering Cancer Center, NY, USA according to ethical vote MSK IRB 09–141 by the Memorial Sloan Kettering Cancer Center’s Institutional Review Board (IRB)/Privacy Board. Material was processed on day of acquisition. For hMSC isolation, a femur head biopsy from 66-year-old orthopedic patient who underwent hip replacement, was used (ethical approval EK300–13).

All patient data was deidentified prior to inclusion. All patients provided informed consent and the data collection was performed in accordance with the Declaration of Helsinki.

**Mouse experiments**

Mouse experiments were all approved by the Animal Welfare/Ethics committee of the EDC in accordance with legislation in the Netherlands (approval No. AVD1010020173387) Gli1tm3(re/ERT2)Alj/J (JAX Stock #007913), Gli2tm6Alj/J (JAX Stock #007926), SmoMt2Amc/J (JAX Stock #004526), B6.Cg-Tg(Mx1-cre)1Cgn/J (JAX stock #003556) and C57BL/6 (JAX Stock #00664) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). B6. SJL-PtprcaPepcb/BoyCrl mice were purchased from Charles River (Netherlands). MIF/C0 mice were kindly provided by Jürgen Bernhagen. All mice were maintained in specific-pathogen-free conditions, on a 12-h light/dark cycle and were provided with water and standard mouse chow ad libitum. Offspring were genotyped by PCR according to the protocols provided by the Jackson Laboratories or generating labs.

**METHOD DETAILS**

**Patient samples**

Peripheral Blood mononuclear cells (PBMCs) were isolated from whole blood via density gradient (Pancoll human, PAN-Biotec) according to manufacturer’s protocol. Isolated cells were frozen at −80°C in 90% FCS +10% DMSO. Serum from patients with MPN and healthy donors was isolated from whole blood, allowed to coagulate, plasma was isolated from whole blood collected in EDTA tubes. Samples were centrifuged at 2000g for 7 min and supernatant was stored at −80°C.

**Mouse experiments**

In experiments using MxCre (B6.Cg-Tg(Mx1-cre)1Cgn/J), Gt2mXCre (Gt2m6Alj/J; Tg(Mx1-cre)1Cgn) and SmoMxCre (Smotm2Amc/J; Tg(Mx1-cre)1Cgn) strains, donor mice received 3 times 200ug polyI:C (Invivogen) via intraperitoneal injection for one week, 4 weeks prior to harvesting. The Gli1tm3(re/ERT2)Alj/J line is germline knock out and therefore required no prior induction. BM from all donors (male and female) was harvested 48 h prior to transplantation and ckit+ enriched BM cells were transduced with ThPO lentivirus or control EV lentivirus, or Jak2 V617F retrovirus or control Jak2 WT retrovirus, depending on the model used. For all experiments, 8–10 week old B6.SJL recipient mice (female, n = 5/group) were lethally irradiated using 10.5Gy before receiving 4-5x10^5 ckit+ donor cells via intravenous injection. Mice were sacrificed at 7–8 weeks post-transplant for ThPO-induced fibrosis experiments and 16–17 weeks post-transplant for Jak2 V617F-induced fibrosis experiments. For all analysis, comparisons were made to the respective control cohorts in each experiment.

**Viral transduction**

For Jak2 V617F/Jak2 WT retroviral and ThPO/EV lentiviral transduction, BM cells from 12 to 20 week-old donor mice were isolated by crushing compact bones. Cells were magnetically enriched for CD117 (ckit) (Miltenyi Biotec). ckit+ BM cells were pre-stimulated for 24 h in CellGro media (Corning) supplemented with murine stem-cell factor (m-Scf, 50 ng/ml, Peprotech) and murine thrombopoietin (m-Tpo, 50 ng/ml, Peprotech). Retroviral particles were produced using standard protocols and an ectropic envelope using Fugene. Lentiviral particles were produced by transient transfection with the lentiviral plasmid together with pSPAX and VSVG packaging plasmids using Fugene. Lentivirus and retrovirus particles were concentrated via ultracentrifugation at 4°C. Retroviral transduction was performed on retroNectin (Takara Bio)-coated cell culture dishes pre-coated with unconcentrated virus with the addition of 4-5x10^5 ckit+ donor cells via intravenous injection. Mice were sacrificed at 7–8 weeks post-transplant for ThPO-induced fibrosis experiments and 16–17 weeks post-transplant for Jak2 V617F-induced fibrosis experiments. For all analysis, comparisons were made to the respective control cohorts in each experiment.

**Flow cytometry**

Patient PBMCs were defrosted, washed in 2%FCS/PBS (Gibco) and passed through a 70um cell strainer to remove clumps. Cells were stained with the following extracellular monoclonal, directly fluorochrome-conjugated antibodies; anti-human: CD19 (BB700, BD, 1:100), CD66b (PE, BD, 2.5:100), HLA-DR (PeCy7, BD, 1:100), CD45 (APC-H7, BD, 2.5:100), CD3 (BV510, BD, 1:100), CD88 (BV766, BD, 1:100), CD89 (BV786, BD, 1:100) in the dark at 4°C for at least 24 h. Lentivirus transductions were performed with concentrated lentiviral supernatant in the presence of 4 μg/ml polybrene at 37°C for at least 24 h. Lentivirus transductions were performed with concentrated lentiviral supernatant in the presence of 4 μg/ml polybrene at 37°C for at least 24 h.

**Patient PBMCs**

Peripheral blood collected during animal experiments was first lysed with Pharm Lyse (BD) for 15 min at RT, washed with 2%FCS/PBS (Gibco) and stained using CD3 (PE, BioLegend), CD41 (PeCy7, BioLegend), GR1 (ef450 ThermoFisher), CD11b (APC, BioLegend), CD45.1 (APCCy7, BioLegend), CD45.2 (PerCP Cy5.5, BioLegend).
At sacrifice murine BM cells were isolated by crushing the pelvis, hind leg bone and spine in 2%FCS/PBS before being strained through a 70μm cell strainer. BM was lysed at RT for 10 min with red blood cell lysis buffer (BD Pharm Lyse) before being washed in 2%FCS/PBS. Cells were labeled 1:100 in 2%FCS/PBS for 15 min in the dark at 4°C with the following monoclonal directly labeled antibodies; anti-mouse: CD3 (PE, BioLegend), CD4 (PeCy7, BioLegend), GR1 (ef450, ThermoFisher), CD11b (APC, BioLegend), CD45.1 (APCcy7, BioLegend), CD45.2 (PerCPCy5.5, BioLegend), ckit (APC, BioLegend), Sca1 (PerCPCy5.5, Sony BioTech), CD48 (APCCy7, BioLegend), CD150 (PeCy7, BioLegend), CD45.2 (PE, Sony BioTech), CD3 (ef450, ThermoFisher), B220 (ef450, ThermoFisher), GR1 (ef450, ThermoFisher), Ter119 (ef450, ThermoFisher).

All samples were analyzed/sorted by flow cytometry using a FACScantoll, FACSFortessa FACSMelody or FACSAria (BD Biosciences) and data were analyzed using FlowJo Software (Version10, TreeStar Inc), MFI of Gli1 values were corrected for FMO values in all experiments.

Stimulation of HD PBMCs
PBMCs were cultured in RPMI with 10% FCS and 1% Pen/Strep with added SCF (100 ng/ml), IL3 (30 ng/ml), IL-6 (25 ng/ml) and FLT3L (50 ng/ml) as a base medium. Prior to and during stimulation the FCS concentration was reduced to 1%. Cells were stimulated with 50 ng/ml IL-6 or 10 ng/ml TGFb1 for 72 h and the stimulation was refreshed every 24 h. At the end of the experiment cells were collected, washed and processed for transcription factor FACS as described above.

Human megakaryocytic cell line culture
MEG-01 cells were maintained in 90% RPMI 1640 (Gibco, #21875-091), 10% FCS and 1% Pen/Strep at a seeding density of 5.0 x 10^5/mL. SET-2 cells were maintained in 80% RPMI 1640, 20% FCS and 1% Pen/Strep at a seeding density of 8.0 x 10^5/mL. Cells were collected and processed for RNA isolation as described below.

Patient-derived IPSC lines and JAK2 conditioned medium
Patient derived IPSC lines and paired JAK2WT and JAK2V617F clones were generated and characterized as described by Satoh et al. Briefly, iPS cells were harvested with Accutase (PAN-Biotech, Aidenbach, Germany) and a single cell suspension was plated at a density of 0.5x10^6 cells/ml for 72h before medium was collected centrifuged, filtered through a 0.2um filter to remove cell debris and frozen for use in stimulation experiments.

Murine hematopoietic progenitor cell lines and ThPO conditioned medium
HOXB8 multi potent progenitor cells were generated from WT whole bone and maintained according to the protocols described by Xu et al., they were subsequently transduced with ThPO lentiviral particles generated as described above and sorted for GFP positivity. Cells were cultured at a density of 0.5x10^6 cells/ml for 72h before medium was collected centrifuged, filtered through a 0.2um filter to remove cell debris and frozen for use in stimulation experiments.

Generation of hMSC cell line, subsequent recombinant cytokine and conditioned medium stimulation
BM stromal cells isolated from healthy patient femur head samples were isolated as previously described and immortalized using hTERT and LargeT over expression vectors. Cells were cultured in AlphaMEM (CytivialHyClone) supplemented by 10% FCS, 1% Pen/Strep, 1%Sodium Pyruvate (Gibco) and 1% HEPE (Gibco) (full medium). On Day 0, 90'000 cells were seeded in one well of a 24-well plate (TPP) (n = 3 per condition) in full medium. After allowing for attachment overnight, the full medium was exchanged for starvation medium containing 1% FCS for 24 h.

For TGFb stimulation and Apitosilib experiment: on day 2 cells were pretreated with 5μM Apitosilib (MedChemExpress) or DMSO (in equal proportions) for 1 h in full medium, prior to stimulation with 10 ng/ml TGFb (Invivogen). Medium (full medium containing +/- 5μM Apitosilib (dissolved in DMSO), +/- DMSO, +/- rTGFb) was refreshed on day 3 after 24 h and cells were collected for RNA isolation by trypsinization (Gibco) and pelleting, on day 4 after 48 h total treatment time.

For conditioned medium and ISO-1 experiment: on Day 2, starvation medium was replaced with conditioned medium generated as described above supplemented with an additional 10% FCS +/- 40μM ISO-1. Cells were cultured in these conditions before being collected for RNA isolation by trypsinization (Gibco) and pelleting, on day 4 after 48 h total treatment time.
Isolation of primary murine MSCs and subsequent stimulation with ThPO conditioned medium

CD74−/− or WT primary MSCs were isolated and cultured according to the protocol previously described by Zhu et al. and were maintained in AlphaMEM (CytiviaHyClone) supplemented with 20% FCS, 1% Pen/Strep, 1 ng/ml murine basic FGF (Peprotech) and 5 ng/ml murine EGF (Peprotech) (full medium). On Day 0, 50,000 passage 3 cells were seeded in one well of a 6-well plate (n = 3 per condition) in full medium. After allowing for attachment overnight, the full medium was exchanged for starvation medium containing 1%FCS for 24 h. On day 2, starvation medium was replaced with ThPO conditioned medium generated as described above supplemented with an additional 10% FCS. Cells were cultured in these conditions before being collected for RNA isolation by trypsinization (Gibco) and pelleting, on day 4 after 48 h total treatment time.

Culture and treatment of HEL cell line

JAK2V617F positive HEL cells were cultured in RPMI 1640 (GIBCO) supplemented by 10% FCS and 1% Pen/Strep (full medium) and maintained at a density of 1x10⁶ cells/ml. Prior to treatment on Day 0, cells were plated at a density of 1x10⁶ cells/ml (n = 3 per condition) and starved overnight in medium with only 2% FCS (starvation medium). On Day 1, cells were treated with either 500nM Ruxolitinib (Selleckchem), 250nM BEZ235 (Selleckchem) or DMSO. After 4 h, the BEZ235 and equivalent DMSO conditions were harvested and cells were processed for RNA isolation. After 24 h the Ruxolitinib and equivalent DMSO conditions were harvested, and cells processed for RNA isolation.

Culture and stimulation of MSOD cell line

MSOD cells were maintained in AlphaMEM (CytiviaHyClone) supplemented by 10% FCS, 1% Pen/Strep (Gibco), 1% L-Glutamine (Gibco) 1% Sodium Pyruvate (Gibco), 1% HEPES (Gibco) and 5 ng/ml FGF2 (Peprotech) (full medium). On Day 0, 6.6x10⁵ cells were seeded in a 6 well plate (TPP). On Day 1, full medium was exchanged with the addition of 100 ng/ml recombinant human MIF (Peprotech). On Day 2, cells were harvested for RNA isolation after 24h stimulation. MSODs were kindly provided by Ivan Martin.

Histological staining

After sacrifice murine organs were fixed in 4% paraformaldehyde for 24 h before being transferred to 70% ethanol. Femurs were decalcified in 10% EDTA/Tris-HCl (pH 6.6) solution for 7 to 10 days, dehydrated and paraffin embedded. H&E, Reticulin staining (Sigma-Aldrich) and Sirius red/Fast Green (Chondrex) were performed on 4μm sections according to routine protocols.

scRNA sequencing sample preparation

BM was isolated at sacrifice and frozen at −80°C in 90% FCS +10% DMSO, was defrosted, washed in 2%FCS/PBS before being strained through a 70μm cell strainer. Whole BM cells from each replicate (n = 3) within a group were incubated with 2.5-5μl of hashtag antibody (anti-mouse-TotalSeq, BioLegend) and lineage depletion antibodies (Miltenyi) for 10 min at 4°C. Subsequently, standard magnetic depletion protocol was followed. Eluted Lineage negative cells from each replicate were mixed per group and stained with Celltrace Calcein Red/Orange (ThermoFisher) (viability) for 15 min at RT and anti-mouse; CD3 (ef450, ThermoFisher), B220 (ef450, ThermoFisher), CD11b (ef450, ThermoFisher), GR1 (ef450, ThermoFisher), Ter119 (ef450, ThermoFisher) for 15 min at 4°C to check purity of lineage depletion. Two populations were sorted (BD FACSAriaII); GFP(Jak2) Lin−, to enrich for stromal cells, and GFP+. The populations were mixed 75%/25% respectively and used for the 10x platform.

scRNA library preparation and sequencing

RNA Libraries were prepared using the Chromium Single Cell 3′ Reagents kits (10x Genomics): Chromium Next GEM Single Cell 3′Kit v3.1 (PN-1000268), Chromium Next Gem Chip G Single Cell Kit (PN-1000127), Dual Index Kit TT Set A (PN-1000215). Additional libraries were generated for the hashtags using publicly available protocol. Quality of the libraries was determined using D1000 ScreenTape on a 2200 TapeStation system (Agilent Technologies). Libraries were sequenced on an Illumina Novaseq6000 targeting a read depth as suggested by 10x Genomics 3′ single-cell RNA kits v3.1.

scRNA-seq data—Alignment

Using cell ranger Version 4.0.0, reads were aligned to mouse genome mm10, with an added GFP sequence (5’ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCCAGCTGCTTGGCAGGGCGAGGCGATGCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGTCGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAGCGACGCAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGCAGACCACACTACCCGCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTTGGGCAACAGGCGAGGCATCTCTGAGCTCCGGCAGCAGGGCGAAAAAGAGGCGGACATCCTCGGGGCAAGAAGGGCGACATCCTCGGGGCAAGAAGGGCGGACATCCTCGGGGCAAGAAGGGCGACATCCTCGGGGCAAGAAGGGCGACATCCTCGGGGCAAGAAGGGCGACATCCTCGGGGCAAGAAGGGCGACATCCTCGGGGCAAGAAGGGCGACATCCTCGGGGCAAGAAGGGCGACATCCTCGGG

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scRNA-seq data—Initial processing
All primary scRNA-seq analysis was performed using the Seurat toolbox (v4.0.1) for R (v4.0.5).
Data from all cells underwent uniform preprocessing for quality control, using standard thresholds of <10% mitochondrial reads and <30000 total counts. Normalization was carried out, involving variance-stabilizing log2 transformation and regression of technical factors (number of counts and percentage of mitochondrial reads).
Cells were demultiplexed according to hashtag antibodies which were unique to each biological replicate. This was done using HTODemux (included in Seurat) according to kmeans clustering with a threshold of 0.99. Preliminary visualization of the data following hashtag demultiplexing revealed high lineage bias in one control (WT + Jak2) replicate. This replicate was not included in any subsequent downstream processing and analysis. Due to the overall low efficiency of hashtagging we did not separate the biological replicates for downstream analysis.
Ambient RNA contamination was estimated using the SoupX package (v1.5.0). Filtering was applied to eliminate the top 0.1% of genes identified as contamination, with the notable exception of S100a8 and S100a9 which were retained due to known biological relevance in the context of fibrosis. Mitochondrial genes and X-linked long non-coding RNA genes were also removed due to visible biases. We chose not to regress out cell cycle as no projections suggested a cluster specific cell cycle bias and we wanted to preserve any knockout mediated effects. Datasets were then integrated using rpca integration. See Table S2 for quality control thresholds, metrics and cell numbers for this dataset.

scRNA-seq data—Cluster annotation
Unsupervised clustering of cells was performed using UMAP dimensionality reduction, and clusters were annotated according to consensus across multiple differential expression analyses. Marker genes for each cluster were identified using the FindMarkers function included in Seurat. These markers were interrogated via gene enrichment using WebGestaltR (v0.4.4), as well as comparison to known markers using cellmarkersDB (v1.0.0) and previously published markers. The same FindMarkers function was used to identify the differential genes between individual conditions, statistics of which were used to generate plots in Figures 4 and S4.

scRNA-seq data—Transcription factor analysis
Transcription factor activity prediction was performed via enrichment analysis with the DoRothEA gene regulatory network (v1.7.2). Based on the expression matrix of the scRNA-seq data, transcription factor activity scores were retrieved using the statistical method provided within the DoRothEA R package. For each cell, the predicted transcription factors were returned with a corresponding activity score, in the form of z-scores for comparability. By grouping the cells based on the cell type or sample condition and averaging over the activity scores for each transcription factor, the analysis of differences between the groups was enabled.

scRNA-seq data—Pathway activity analysis
For pathway analysis, we used FindMarkers function with logfc.threshold >0.25 to find condition and cluster specific markers. Pathway enrichment analysis was performed using the enricher() function from clusterProfiler (v3.18.1) and Hallmark gene sets.

scRNA-seq data—Ligand-receptor analysis
Ligand receptor interactions representing cell-cell communication were identified for each individual normalized and preprocessed dataset using CellPhoneDB (v2.1.7) considering only the interactions with p value ≤ 0.05. CrossTalker (v1.3.2) was used to was used to build and analyze ligand-receptor derived networks and shed light on interactions which differ between conditions, as well as generating network plots.

RNA isolation and RT-qPCR analysis
Cell pellets were immediately processed using the NucleoSpin RNA Plus XS kit (Macherey-Nagel) or the RNAeasy kit (Quiagen) as per manufacturer’s instructions. 1μg eluted RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo-Fisher). Quantitative polymerase chain reactions were performed using Applied Biosystems Fast SYBR Green Master Mix (Applied Biosystems) on a CFX Opus 96 system (BioRad). HPRT1 was used as a house-keeping gene and the $2^{-ΔΔCt}$ method to calculate differences in RNA levels.

Primer sequences

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ELISA
Before use, samples were thawed gently on ice and centrifuged for 5 min at 2500xg. Serum samples were diluted 1 in 10 and MIF concentration was quantified using the LegendMax Human Active MIF ELISA kit (BioLegend) according to manufacturer’s instructions. For SHH concentration, Plasma samples were used undiluted and quantified using the Human Sonic Hedgehog/Shh N Termi-nus DuoSet ELISA (R&D).

QUANTIFICATION AND STATISTICAL ANALYSIS

Image processing and quantification
All plots were generated with GraphPad Prism Version 9.0.0 or R Version 4.2.0. To ensure readability line thickness, labels and font size were adjusted manually using Adobe Illustrator CC 2023. Size, count and area quantification from images was performed via ImageJ from a minimum of 3 representative fields per section. BM composition was analyzed from whole sample scans of H&E stained slides using MarrowQuant. Unless otherwise specified, images were representatively chosen and jointly rescaled before manual alignment.

Statistical analysis
Statistical analysis – excluding that for scRNAseq data – was performed using GraphPad Prism version 9.0.0. Correlation was assessed via simple linear regression testing. Comparison between two groups was performed using an unpaired t test with Welch’s correction. For multiple group comparison at a single time point, a one-way ANOVA with post-hoc Tukey correction was applied. For multiple group comparison over multiple time points, two-way ANOVA with Geisser-Greenhouse correction followed by Tukey’s post-hoc test was applied. p values lower or equal to 0.05 were considered statistically significant and data are shown as mean ± SEM.