The spleen microenvironment influences disease transformation in a mouse model of \( \text{KIT}^{\text{D816V}} \)-dependent myeloproliferative neoplasm

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Activating mutations leading to ligand-independent signaling of the stem cell factor receptor KIT are associated with several hematopoietic malignancies. One of the most common alterations is the D816V mutation. In this study, we characterized mice, which conditionally express the humanized KIT\(^{\text{D816V}} \) receptor in the adult hematopoietic system to determine the pathological consequences of unrestrained KIT signaling during blood cell development. We found that KIT\(^{\text{D816V}} \) mutant animals acquired a myeloproliferative neoplasm similar to polycythemia vera, marked by a massive increase in red blood cells and severe splenomegaly caused by excessive extramedullary erythropoiesis. Moreover, we found mobilization of stem cells from bone marrow to the spleen. Splenectomy prior to KIT\(^{\text{D816V}} \) induction prevented expansion of red blood cells, but rapidly lead to a state of aplastic anemia and bone marrow fibrosis, reminiscent of post polycythemic myeloid metaplasia, the spent phase of polycythemia vera. Our results show that the extramedullary hematopoietic niche microenvironment significantly influences disease outcome in KIT\(^{\text{D816V}} \) mutant mice, turning this model a valuable tool for studying the interplay between functionally abnormal hematopoietic cells and their microenvironment during development of polycythemia vera-like disease and myelofibrosis.

The blood forming system is characterized by a remarkable regenerative capacity, required for the continuous replacement of mature blood cells. During that process, the balance between cell proliferation and differentiation has to be tightly controlled to prevent hematopoietic malignancies. The KIT receptor, expressed by hematopoietic stem cells (HSCs) and progenitor cells (HPCs) and several lineage-committed precursors, is involved in cell maintenance, proliferation, survival and terminal differentiation\(^1\text{–}^6\). Uncontrolled KIT signaling is associated with several myeloproliferative disorders\(^7\text{–}^9\).

KIT belongs to the type III subfamily of tyrosine kinases and gets activated by its ligand stem cell factor (SCF), which is expressed as a membrane bound or soluble form\(^10\text{–}^12\). SCF is produced by stromal cells in the hematopoietic bone marrow (BM) niche. For HSCs, interaction of KIT with membrane bound SCF was shown to be important for positioning to the niche\(^13\text{,}^14\). Furthermore, KIT was described to be important for the maintenance of long-term HSCs\(^5\). In most lineages, KIT is downregulated during differentiation\(^2\), while in mast cells high KIT expression is maintained\(^15\). KIT deficient mice die in utero due to defects in fetal liver erythropoiesis, demonstrating its important function in red blood cells\(^16\). In erythroid progenitors, KIT regulates proliferation and maintenance of the undifferentiated state\(^17\).

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Several KIT mutations have been described that cause constitutive receptor activation without ligand binding. The D816V substitution is one of the most commonly described mutations associated with hematopoietic neoplasia8,10,11. We previously described the generation of a humanized transgenic mouse model for conditional KITD816V expression and analyzed effects of KITD816V signaling on fetal liver erythropoiesis15. Here, we used R26-LSL-KITD816V mice to investigate sustained KITD816V signaling in the adult hematopoietic system and found development of a myeloproliferative neoplasm (MPN) reminiscent of polycythemia vera (PV), which was transplantable and characterized by massively increased red cell mass and splenomegaly. Furthermore, stem cells were mobilized from BM to the spleen. Splenectomy of KITD816V mutants prevented the increase in red cell mass but promoted BM failure and myelofibrosis, clinical features observed upon transformation of PV to post polycythemic myeloid metaplasia. The fact that course of disease in KITD816V mutants is influenced by splenectomy demonstrates the relevance of the niche and provides a unique model to study the interdependency of hematopoietic cells and the microenvironment.

Results

KITD816V induces a polycythemia vera-like disease. We previously described the generation of the R26-LSL-KITD816V mouse line, harboring a conditional knock in of a humanized KITD816V receptor linked to a green fluorescent protein (GFP) in the ROSA26 genomic locus. The D816V mutation has been implicated in the pathology of acute myeloid leukemia, mastocytosis and other oncogenic malignancies7,9,12,20. To extend the knowledge on how KIT regulates hematopoiesis and contributes to myeloproliferative disorders, we studied the effects of ectopic KITD816V expression in the adult hematopoietic system. We mated R26-LSL-KITD816V with HSC-SCL-Cre-ER mice, which express a tamoxifen-inducible Cre recombinase under control of the stem cell enhancer of the Scl gene locus15. HSC-SCL-Cre-ER mice were viable and developed normally. For induction of KITD816V expression (Fig. 1a), we treated adult HSC-SCL-Cre-ER mice with 1 mg tamoxifen (TX) for 5 consecutive days. TX-treated wildtype and single transgenic littermates were used as controls. Quantitative real-time PCR validated KITD816V expression in hematopoietic compartments of HSC-SCL-KITD816V animals after induction, with transcript levels comparable to endogenous Kit expression in controls. To validate GFP co-expression, GFP-positive and -negative fractions were also analyzed for KITD816V expression (Supplementary Fig. S1).

In total, 44 HSC-SCL-KITD816V and 45 control mice were monitored for 4–10 weeks after treatment. Only a limited group of 2 HSC-SCL-KITD816V and 4 control animals was monitored up to 18 weeks, as we observed a high rate of spontaneous mortality for HSC-SCL-KITD816V animals (29.55%; 13/44 mice) within the first 10 weeks after induction. Aside from moderate enlargement of the abdomen in some cases, HSC-SCL-KITD816V mice showed no signs of morbidity before death. From the control group, all animals survived the observation period.

Cell counts in peripheral blood (pB) were analyzed 4, 8, 10 and 18 weeks after induction. The red blood cell count (RBC), hemoglobin (Hb), mean platelet volume (MPV) and white blood cell count (WBC) were significantly elevated in HSC-SCL-KITD816V mice compared to controls (Fig. 1b, Supplementary Fig. S2). The hematocrit (Hct) was initially elevated to high levels and slightly decreased thereafter. The mean corpuscular volume (MCV) and the platelet count (PLT) were decreased in HSC-SCL-KITD816V mice.

We further investigated alterations in pB cell lineages via flow cytometry. Analyses demonstrated elevated amounts of CD45-positive cells in pB of HSC-SCL-KITD816V mice, caused by increased monocyte and B-cell populations and a mild increase in granulocytes. Staining for erythroid markers CD71 and Ter-119 revealed the presence of CD45-negative erythroblasts, which are normally not released into circulation (Fig. 1c, Supplementary Fig. S2). In addition, the reticulocyte frequency was increased (Fig. 1d). Figure 1d shows pB smears of HSC-SCL-KITD816V and control mice 8 weeks after induction. Accumulation of erythrocytes and mature myeloid cells is a hallmark of PV23. PV furthermore often includes thrombocytosis, but similar to murine PV models harboring the JAK2V617F mutation24–26 we found no elevation of platelets in HSC-SCL-KITD816V mice. As PV is additionally marked by endogenous erythroid colony formation and decreased serum erythropoietin23, we further analyzed these parameters. Indeed, for HSC-SCL-KITD816V mice markedly decreased serum erythropoietin levels and high numbers of splenic colony forming unit-erythroids (CFU-Es) in assays with low erythropoietin concentration were found, indicating erythropoietin hyper-responsiveness (Fig. 1d). Thrombopoietin serum levels were also reduced in HSC-SCL-KITD816V mice (Supplementary Fig. S2).

HSC-SCL-KITD816V mice develop splenomegaly with massive extramedullary erythropoiesis. BM and spleen were analyzed 10 weeks after KITD816V induction or when enlargement of the abdomen was observed. Hematoxylin and eosin (HE) staining revealed no apparent differences in BM histology of HSC-SCL-KITD816V and control animals. However, siderophages (mononuclear phagocytes containing hemosiderin, a product of hemoglobin catabolism) were scattered throughout control BM, whereas HSC-SCL-KITD816V mutants showed almost no siderophages, indicating reduced iron storage (Fig. 2a). Quantification further demonstrated a slight reduction in BM megakaryocytes. Immunohistochemical staining for active Caspase3 revealed a slight elevation of apoptosis in HSC-SCL-KITD816V BM, while Ki67 staining showed no differences in proliferation (Supplementary Fig. S3). Compared to controls, total BM cellularity of HSC-SCL-KITD816V mice was moderately increased (Fig. 2b). GFP-fluorescence was examined to estimate efficiency of KITD816V induction in HSC-SCL-KITD816V animals, demonstrating transgene expression in 57.40 ± 6.41% of total BM cells (Fig. 2b, dashed bar). While the frequency of CD45-positive cells was decreased in HSC-SCL-KITD816V BM, the overall frequency of CD45-negative erythroblasts was elevated in comparison to controls. Flow cytometric quantification of discrete developmental stages19,27 demonstrated a trend towards increased early and late erythroblasts, whereas reticulocytes were reduced, indicating a mild shift to more immature cells (Fig. 2b, Supplementary Fig. S3). To
also investigate early BM erythropoiesis, we performed assays for CFU-E progenitors, showing no differences between HSC-SCL:KITD816V and control mice (Supplementary Fig. S3).

Gross examination of the mice revealed massive splenomegaly in HSC-SCL:KITD816V animals, another diagnostic criterion for PV. KITD816V mutants showed a 19.81 ± 10.09-fold increase in spleen weight and altered spleen histology (Fig. 2c). Lymphoid nodules, normally marked by clusters of CD3-positive cells, were virtually absent in the spleen of mutant mice (Supplementary Fig. S3).
**Figure 2.** KIT<sup>D816V</sup> induces excessive splenic stress erythropoiesis in HSC-SCL-KIT<sup>D816V</sup> mice. Hematopoietic organs of HSC-SCL-KIT<sup>D816V</sup> and control mice were analyzed 7–10 weeks after induction of KIT<sup>D816V</sup> expression with TX. (a,b) Analysis of BM. (a) Paraffin sections were subjected to HE staining and Prussian Blue staining for hemosiderin. (b) Graphs show quantification of indicated cell populations. For HSC-SCL-KIT<sup>D816V</sup> mice, the frequency of GFP-positive cells within each population is depicted. Right bar graph in Log<sub>10</sub> scale. BM Cellularity - Control: N = 7. HSC-SCL-KIT<sup>D816V</sup>: N = 5. CD45-positive cells - Control: N = 9. HSC-SCL-KIT<sup>D816V</sup>: N = 7. Erythroid populations - Control: N = 11. HSC-SCL-KIT<sup>D816V</sup>: N = 8. (c,d) Analysis of the spleen. (c) Left: Macroscopic view. Scale bars: 1 cm. Middle: Scatter plot shows spleen weight in g. Control: N = 15. HSC-SCL-KIT<sup>D816V</sup>: N = 11. Right: HE stainings of paraffin sections. (d) Quantification of indicated cell populations in the spleen. Right graph in Log<sub>10</sub> scale. CD45-positive cells - Control: N = 9. HSC-SCL-KIT<sup>D816V</sup>: N = 7. Erythroid populations - Control: N = 10. HSC-SCL-KIT<sup>D816V</sup>: N = 8. EryB: Erythroblasts; ProE: Proerythroblasts; BasoE: Basophilic EryB; PolyE: Polychromatic EryB; OrthoE: Orthochromatic EryB; Retic: Reticulocytes. Data are presented as mean ± standard deviation. P-values were determined using two-tailed, unpaired Student’s t-test.

Flow cytometric examination demonstrated CD45 expression in almost 80% of control spleen cells. In HSC-SCL-KIT<sup>D816V</sup> mice, this frequency was reduced to less than 20% (Fig. 2d). This reduction was due to a massive increase in splenic erythropoiesis, as CFU-Es and erythroblast frequency were significantly elevated in HSC-SCL-KIT<sup>D816V</sup> mice (Figs 1c and 2d, Supplementary Fig. S3). The high ratio of GFP-positive erythroblasts compared to controls (Fig. 3a, Supplementary Fig. S4). To investigate the reasons for this expansion, we sorted HSC-SCL-KIT<sup>D816V</sup>: N = 11. Right: HE stainings of paraffin sections. (d) Quantification of indicated cell populations in the spleen. Right graph in Log<sub>10</sub> scale. CD45-positive cells - Control: N = 9. HSC-SCL-KIT<sup>D816V</sup>: N = 7. Erythroid populations - Control: N = 10. HSC-SCL-KIT<sup>D816V</sup>: N = 8. EryB: Erythroblasts; ProE: Proerythroblasts; BasoE: Basophilic EryB; PolyE: Polychromatic EryB; OrthoE: Orthochromatic EryB; Retic: Reticulocytes. Data are presented as mean ± standard deviation. P-values were determined using two-tailed, unpaired Student’s t-test.

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In summary, these data demonstrate that chronic KIT<sup>D816V</sup> signaling causes a PV-like disease based on moderately increased BM erythropoiesis and a massive induction of splenic erythropoiesis. As PV is often associated with high incidence of thrombosis due to elevated Hct<sup>23</sup>, we assume that abrupt death of HSC-SCL-KIT<sup>D816V</sup> animals is a consequence of thrombotic events.

**KIT<sup>D816V</sup> signaling causes stem cell mobilization from BM and a shift of hematopoiesis to extramedullary sites.** We next examined effects of KIT<sup>D816V</sup> signaling on stem cells by analyzing the frequencies of LK (KIT<sup>pos</sup>Sca-1<sup>neg</sup>Linneg), LSK (KIT<sup>pos</sup>Sca-1<sup>pos</sup>Linneg) and HSC (LSK-C드48<sup>pos</sup>CD150<sup>pos</sup>) populations among CD45-positive cells. BM HSC and LSK populations were increased in HSC-SCL-KIT<sup>D816V</sup> animals compared to controls (Fig. 3a, Supplementary Fig. S4). To investigate the reasons for this expansion, we sorted LinnegKITpos BM cells and analyzed expression of important transcriptional regulators. Increased Gata2 transcript was found in the stem cell-enriched compartment of KIT<sup>D816V</sup> mutants (Fig. 3b, Supplementary Fig. S4). Consistently, Gata2 has been shown to mediate proliferation and survival in hematopoietic stem cell compartments and in vitro differentiation of Gata2-deficient embryonic stem cells demonstrated impaired SCF-responsiveness<sup>25,26</sup>.

To examine if KIT<sup>D816V</sup> signaling confers a proliferative advantage to stem cells, we analyzed Ki67 expression in BM stem cell populations (Fig. 3c, Supplementary Fig. S4). Interestingly, we found that the effects varied between the different populations. While actively cycling cells were increased in the HSC population of HSC-SCL-KIT<sup>D816V</sup>
In the spleen, HSCs showed a mild but not significant elevation, while the LSK and LK populations bearing lower stem cell potential were significantly increased (Fig. 3a, Supplementary Fig. S4).

We further examined distribution of long-term (LT)-HSCs, short-term (ST)-HSCs and multipotent progenitors (MPPs) between BM, pB and spleen of HSC-SCL:KITD816V and control mice. We found BM LT-HSCs slightly increased in HSC-SCL:KITD816V mice, while the other populations in BM were not significantly altered. In contrast, ST-HSC and MPP frequencies were considerably elevated in pB and spleen (although changes were not significant for pB), indicating increased HSC activation and mobilization from BM to circulation. Consistently, progenitor cell populations were slightly reduced in BM but increased in spleen (Fig. 3d, Supplementary Fig. S4).
HSC analysis includes Kit as a cell surface marker. It is thus important to note that the KIT^D816V mutant receptor localizes to intracellular compartments. In line with this, we found the fraction of cells positive for Kit surface expression similar in GFP-positive and GFP-negative BM populations in HSC-SCL:KIT^D816V mice, suggesting that only the endogenous receptor reaches the cell surface and ectopic KIT^D816V expression does not affect HSC analysis (Supplementary Fig. S5).

Unexpectedly, the frequency of GFP-positive cells was quite low in KIT D816V HSCs, while it increased with progressive differentiation. To examine potential loss of KIT^D816V-positive HSCs or high Cre-recombination frequencies in differentiated cells, we analyzed kinetics of reporter gene expression in different hematopoietic populations 2, 4 and 6 weeks after induction. The GFP-frequency was stable in the LT-HSC compartment and gradually increased in ST-HSC and MPP populations, indicating no negative influence of KITD816V signaling on stem cell survival (Supplementary Fig. S5). Initially, the GFP-frequency in most progenitor and mature populations was lower than in stem cells, demonstrating Cre-recombination primarily in HSCs, albeit at low frequency. However, analyses suggested also low recombination in granulocytes and B-cells. As the GFP-positive fraction was especially high in the erythroid compartment, we analyzed erythroblasts directly after TX-induction, further revealing recombination in proerythroblasts to a comparatively high degree (Supplementary Fig. S5).

As we found alterations in stem cell distribution, we also analyzed distribution of mature blood cells. In BM, we found no alterations in granulocyte, B-cell and monocyte populations, while in the spleen we found a reduction in lymphocyte frequency for HSC-SCL:KIT^D816V animals (Fig. 4a, Supplementary Fig. S6). Analysis of dendritic cells (DCs) revealed a decrease in BM and an increase in the spleen of HSC-SCL:KIT^D816V mice. As KIT^D816V is strongly associated with mastocytosis, we checked for mast cell infiltrations. Surprisingly, flow cytometry revealed a reduced number of peritoneal and skin mast cells in mutants, although changes were not significant (Fig. 4b, Supplementary Fig. S6).

Taking together, our analyses demonstrate that stem cell and differentiated blood cell populations tend to be reduced in BM but increased in spleen (with the exception of lymphocytes), indicating a shift in hematopoiesis to extramedullary sites (Fig. 4c).
The KIT\(^{D816V}\)-mediated PV-like phenotype is transplantable. In addition to the hematopoietic compartment, HSC-SCL-Cre-ERT\(^{T}\)-mediated recombination also occurs in endothelial cells\(^{15}\). Hence, KIT\(^{D816V}\) signaling might also be activated in cells of the perivascular niche. Thus, we investigated whether the PV-like phenotype was a secondary event due to changes in the hematopoietic microenvironment. We treated HSC-SCL:KIT\(^{D816V}\) and control animals with TX and performed transplantation of unfractionated BM 6 weeks later. Recipients were analyzed 3 and 6 weeks after transplantation. In HSC-SCL:KIT\(^{D816V}\) transplanted mice we observed a gradual increase in RBC, Hb, Hct and WBC (Fig. 5a, Supplementary Fig. S7). Moreover, HSC-SCL:KIT\(^{D816V}\) recipients developed splenomegaly and progressively elevated erythroblast numbers (Fig. 5b, Supplementary Fig. S7). While splenic stem cell frequencies were generally elevated post-transplantation, absolute numbers were markedly higher for HSC-SCL:KIT\(^{D816V}\) recipients compared to controls (Fig. 5c, Supplementary Fig. S7). In summary, these data demonstrate that early PV-like disease develops autonomously from the medullary hematopoietic compartment.

Splenectomy protects HSC-SCL:KIT\(^{D816V}\) mice from increased red blood cell mass but promotes rapid hematopoietic failure. We assumed that the high mortality rate of HSC-SCL:KIT\(^{D816V}\) animals was a consequence of thrombotic events caused by the elevated Hct due to massive splenic erythropoiesis. We therefore hypothesized that splenectomy (SpIE) might have a protective effect and subjected HSC-SCL:KIT\(^{T816V}\) and control animals to SpIE prior to TX-treatment (afterwards termed SpIE HSC-SCL:KIT\(^{T816V}\) and SpIE control mice). Blood parameters determined 4 and 10 weeks after KIT\(^{T816V}\) induction demonstrated that SpIE HSC-SCL:KIT\(^{T816V}\) mice indeed were protected from excessive red blood cell production, as RBC, Hb, Hct, MCV and reticulocyte frequency were similar to SpIE controls (Fig. 6a, Supplementary Fig. S8). Moreover, although pb monocytes were significantly elevated after 18 weeks, the WBC and the overall number of CD45-positive cells were unaffected in SpIE HSC-SCL:KIT\(^{T816V}\) animals (Supplementary Fig. S8). Surprisingly, however, 18 weeks after induction SpIE HSC-SCL:KIT\(^{T816V}\) animals became anemic, with RBC, Hb, Hct and PLT values falling significantly below control values (Fig. 6a, Supplementary Fig. S8). Despite the decrease in erythrocytes, a considerable number of erythroblasts was found in pb of SpIE HSC-SCL:KIT\(^{T816V}\) mice (Supplementary Fig. S8).

Histologically, BM of SpIE HSC-SCL:KIT\(^{T816V}\) mice appeared hypocellular compared to SpIE controls. Consistently, the total cell number per femur and BM megakaryocyte counts were dramatically decreased (Fig. 6b, Supplementary Fig. S8). In SpIE controls BM siderophages were abnormally abundant, indicating partial take-over of red blood cell destruction after spleen removal. In contrast, siderophages were absent in KIT\(^{T816V}\) mutants after SpIE, again demonstrating affected iron storage (Supplementary Fig. S8).

After 18 weeks we found the frequency of CD45-positive cells in SpIE HSC-SCL:KIT\(^{T816V}\) BM considerably reduced in favor of a significant increase in erythroblast frequency (Fig. 6b). However, an elevated ratio of early and late erythroblasts to reticulocytes indicated ineffective cell maturation. Moreover, given the overall cell loss in BM, total cell numbers were reduced for all erythroid populations (Supplementary Fig. S8). In line with the increase in erythroblast frequency, CFC-assays revealed significantly more CFU-E colonies for SpIE HSC-SCL:KIT\(^{T816V}\) animals compared to SpIE controls. In contrast, the number of non-erythroid colonies was reduced for SpIE HSC-SCL:KIT\(^{T816V}\) mice (Fig. 6c). These data indicate, that KIT\(^{T816V}\) preferentially promotes erythropoiesis.

As extramedullary hematopoiesis can also occur in liver, SpIE animals were examined for liver erythropoiesis (Supplementary Fig. S8). Indeed, a mild induction of erythropoiesis was found in livers of SpIE HSC-SCL:KIT\(^{T816V}\) mice, but the overall extent was low and no hepatomegaly was observed.

Splenectomy is accompanied by stem cell loss and BM fibrosis in KIT\(^{D816V}\) mice. Analyzing stem cell populations of SpIE HSC-SCL:KIT\(^{D816V}\) animals, we found a strong progressive reduction in BM HSCs/HPCs compared to SpIE controls (Fig. 7a, Supplementary Fig. S9). This was in contrast to KIT\(^{D816V}\) mutants not subjected to SpIE, which showed no indication of stem cell depletion after 10 weeks (Fig. 3a). We also examined Erk1/2 and Akt phosphorylation in BM LK and LSK populations of SpIE animals, but again found no constitutive activation or altered reaction to SCF or thrombopoietin stimulation in KIT\(^{D816V}\) cells (Supplementary Fig. S9).

Our results demonstrate, that although SpIE protects HSC-SCL:KIT\(^{D816V}\) mice from excessive red blood cell accumulation, it promotes rapid BM failure. Clinically, PV can progress to its spent phase polycythemic myeloid metaplasia (PPMM)\(^{13}\). There, the initially hypercellular BM becomes hypocellular and secondary myelofibrosis develops. Red blood cell production becomes ineffective and pb cell counts decrease, resembling aplastic anemia. In this study, HSC-SCL:KIT\(^{D816V}\) mice without SpIE developed a malignancy reminiscent of early PV, whereas the phenotype observed in SpIE HSC-SCL:KIT\(^{D816V}\) mice resembled PPMM. To further validate this, we performed staining for reticulin fibers to check for BM fibrosis. Indeed, fibrotic changes were found in SpIE HSC-SCL:KIT\(^{D816V}\) BM 10 and 18 weeks after KIT\(^{D816V}\) induction\(^{12}\). In contrast, HSC-SCL:KIT\(^{D816V}\) mice not subjected to SpIE neither displayed any signs of fibrosis in BM after 10 or 18 weeks nor increased reticulin deposition in spleen (Fig. 7b, Supplementary Fig. S9). In SpIE HSC-SCL:KIT\(^{D816V}\) BM we also observed considerably elevated apoptosis, analyzed by immunostaining for active Caspase3 (Fig. 7b). Ki67 staining showed no apparent differences (Supplementary Fig. S9).

Our results indicate that removal of the splenic hematopoietic niche in KIT\(^{D816V}\) mutants dramatically influences the clinical picture of the PV-like disease.

Discussion

In this study, we analyzed the consequences of oncogenic KIT\(^{D816V}\) expression in the adult hematopoietic system and found MPN development reminiscent of early and advanced forms of PV, depending on pre-treatment of animals with SpIE.
Figure 5. The KIT<sup>D816V</sup>-induced increase in red cell mass and extramedullary hematopoiesis is transplantable. Whole BM cells from HSC-SCL:KIT<sup>D816V</sup> and control mice were used for BM transplantation 6 weeks after KIT<sup>D816V</sup> induction. Recipients were analyzed 3 and 6 weeks post-transplantation. Control donors: N = 1. HSC-SCL:KIT<sup>D816V</sup> donors: N = 3. 3 W - Control recipients: N = 1. HSC-SCL:KIT<sup>D816V</sup> recipients: N = 3. 6 W - Control recipients: N = 2 for (a); N = 1 for (b,c). HSC-SCL:KIT<sup>D816V</sup> recipients: N = 5. (a) Indicated pB parameters were analyzed with a hematology analyzer. (b) Representative macroscopic picture of the spleens of control and HSC-SCL:KIT<sup>D816V</sup> recipients 6 weeks after transplantation. Graphs illustrate quantification of splenic erythroblasts (EryB) as analyzed by flow cytometry. (c) Quantification of splenic HSC/HPC populations shown as frequency of CD45-positive cells. For recipients of BM transplants, the frequency was calculated for the CD45.2-positive donor cell population. Data are presented as mean ± standard deviation.
Upon induction of KIT\textsuperscript{D816V} expression, we observed massively increased red blood cell production. Under normal conditions, the erythrocyte pool is maintained by BM steady-state erythropoiesis, whereas extramedullary stress erythropoiesis mediates its rapid expansion upon acute anemia\textsuperscript{33}. Different studies have reported...
a role of KIT in stress erythropoiesis. Accordingly, we found the increase in red blood cells to depend on splenic erythropoiesis. Similar observations have been reported for KitV558A,T669I/+ mice. While this clearly demonstrates involvement of KIT in stress erythropoiesis, effects on BM steady-state erythropoiesis are difficult to evaluate in our model, since analysis is impeded by splenic erythropoiesis or hematopoietic failure. However, the erythroid expansion in BM of Spiei KitD816V mice indicates that KIT signaling regulates proliferative expansion during both, steady-state and stress erythropoiesis.

HSC-SCL:KitD816V mice had significantly increased pB monocytes, which is likely based on increased proliferation rather than mobilization, as the monocyte frequency in HSC-SCL:KitD816V BM or spleen was not reduced and pB monocytes were also elevated after Spiei. Detailed investigation will be necessary to elucidate effects of KitD816V on the monocytic lineage. Untypical for PV, pB B-cells were also elevated in HSC-SCL:KitD816V mice. We suppose that this was primarily caused by displacement from the spleen, which showed markedly reduced B-cell frequency.

In HSC-SCL:KitD816V animals we observed mobilization of HSCs to the spleen. Several studies have indicated a role of KIT in mobilization of stem cells from the quiescent niche. Mobilization of HSCs/HPCs from the BM niche was shown to depend on the release of soluble SCF mediated by matrix metalloproteinase-9 (MMP9). Moreover, KIT signaling is involved in cell mobilization triggered by functionally blocking cytadhesion molecules VLA4/VCAM-1, suggesting an integrin/cytokine crosstalk. We also observed an increased frequency of cycling HSCs and increased ST-HSCs in KitD816V mutants. Differences in the KIT expression level within the LS-K-CD150+/CD48− HSC pool have been reported, with lower expression in quiescent cells. These data indicate that oncogenic KitD816V contributes to activation and mobilization of dormant HSCs. Interestingly, mobilization of HSCs/HPCs has also been associated with primary myelofibrosis and PPMN.

HSC-SCL:KitD816V animals pre-treated by Spiei showed stem cell loss and myelofibrosis already 10 weeks after KitD816V induction. Interestingly, no fibrosis was detected in HSC-SCL:KitD816V mice without Spiei, suggesting that disease pathogenesis depends not only on time but also on the interplay between KitD816V-positive cells and the medullary and extramedullary hematopoietic niches. This is in line with the “bad seeds in bad soil” concept proposed for primary myelofibrosis, presuming that an abnormal hematopoietic cell clone alters its microenvironment, resulting in niche dysfunctions. One may hypothesize, that abnormal KitD816V-positive cells cycle between hematopoietic niches and upon splenectomy remain in or repopulate the BM and produce high levels of fibrogenic cytokines, stimulating stromal reticulin production. Consequently, displacement from the niche caused by KitD816V-induced stem cell mobilization and myelofibrosis leads to hematopoietic failure. Studies which have shown that the spleen serves as a reservoir of aberrant stem cells in primary myelofibrosis patients support this assumption. Further, a study by Migliaccio et al. demonstrated that the spleen microenvironment is capable of supporting maturation of Gata1low mutant stem cells that fail to mature in the BM, suggesting the possibility that the BM niche may likewise not sustain maturation of KitD816V mutant cells.

Future experiments with Spiei HSC-SCL:KitD816V mice should investigate the disease-promoting cell population(s) and cytokine production to further substantiate this hypothesis. As Spiei can manipulate the course of disease, HSC-SCL:KitD816V mice provide an excellent model to study the interplay between hematopoietic cells and microenvironment in PV-like disease.

Noteworthy, while Philadelphia-negative MPN are highly associated with the JAK2V617F mutation found in hematopoietic and endothelial-like cells, there is only one study reporting an association of KIT mutations with PV. However, other sequencing studies did not confirm this association, raising the question for the relevance of activating KIT mutations in human MPN. It might be possible that alternative mechanisms lead to altered KIT signaling in human MPN. For instance, it was shown that cultured pB cells from primary myelofibrosis patients produce elevated levels of activated MMP9. As mentioned before, MMP9 can mediate release of soluble KIT ligand. Furthermore, phospho-proteomic analysis of erythroblasts from PV patients revealed reduced total KIT and phospho-KIT-Y719 protein content, indicating altered KIT signaling. It remains unclear if this results from hyper- or hypoactivation of the pathway, as the relative KIT-Y719 phosphorylation in PV and control groups was not compared. However, the reduction in total KIT on protein but not transcript level points to increased internalization upon activation in PV cells.

KITD816V represents a frequent mutation in mastocytosis in man and development of cutaneous mastocytosis with variable speed and degree has been demonstrated in a BAC transgenic KitD816V mouse model. Thus, it was surprising that peritoneal and skin mast cells were reduced in HSC-SCL:KitD816V mice and no mastocytosis was observed. This discrepancy might be due to differential expression levels, as the the KIT promoter exhibits strong physiological activity in the mast cell lineage compared to moderate ROSA26-mediated expression. Moreover, mastocytosis in man is associated with additional mutations, such as TET2, SRSF2, ASXL1, and RUNX1, which are not present in HSC-SCL:KitD816V mice. A recent study by Jawhar et al. has shown that mutations in TET2, SRSF2 or ASXL1 precede the KitD816V mutation in mastocytosis. Thus, in the absence of such seed mutations the KITD816V-mediated disease phenotype might well be different from the phenotype in KitD816V mastocytosis patients. In addition to mastocytosis, the KitD816V mutation is also frequently identified in the core binding factor leukemias, involving the AML1-ETO or CBFB/MYH11 genes. It has been suggested that KIT signaling regulates proliferation and differentiation of aberrant KitD816V cells with the hematopoietic microenvironment leading to PV-like disease and myelofibrosis.
Materials and Methods

Animal studies. All experiments were compliant with the German law of animal protection and the European Directive 86/609/EEC and were approved by the local institutional animal care committees (Landesamt für Natur, Umwelt und Verbraucherschutz, North Rhine-Westphalia; approval-ID: #84-02.04.2013.A491/#84-02.04.2012.A256).

R26-L-KitD816V mice were maintained on 129Sv/SvJ, HSC-SCL-Cre-ERT2 mice on C57BL/6 genetic background. For transgene induction animals received intraperitoneal injection of 1.5 mg TX/day for 5 consecutive days at a minimum age of 8 weeks. Peripheral blood samples were collected from the tail vein. Blood and BM cell counts were determined using an Hemavet 950 hematology analyzer (Drew Scientific, Miami Lakes, FL, USA). Studies were powered to detect a pre-specified RBC difference of 1 × 10^6 cells/μl between HSC-SCL-KitD816V and control animals (effect size = 1.48, two-sided α-value = 0.05/β-value = 0.2). For splenectomy, animals were anesthetized and an incision was made at the left abdominal wall. Ligatures were set around splenic vessels before spleen removal. The incision was closed with wound clips. Following surgery, animals obtained carprofen for pain relief and were allowed to recover for one week before TX-treatment. For transplantation assays, R26-L-KitD816V mice were backcrossed to C57BL/6. CD45.1 recipient mice were subjected to lethal irradiation (2 × 5.5 Gray in 4 h interval) and 2 × 10^8 unfractionated BM cells from HSC-SCL-KitD816V CD45.2 donor mice were transplanted via tail vein injection. Reconstitution of CD45.2 donor cells was analyzed by flow cytometry. Splenectomy/transplantation studies were powered to detect a RBC difference of 7 × 10^6 cells/μl, based on earlier results (effect size = 4.69).

 Colony forming cell assays and erythropoietin levels. Colony forming cell (CFC) assays were performed in technical triplicates using MethoCult methylcellulose medium (StemCell Technologies, Vancouver, Canada) according to manufacturers instructions with minor modifications. Per replicate, 5 × 10^5 nucleated cells were plated for BM and 2.5 × 10^5 for spleen. CFU-E colonies were scored after 2–4 days. If not stated otherwise, erythropoietin concentration was 3 U/ml. For GEMM and GM colonies, the medium was supplemented with 50 ng/ml SCF, 10 ng/ml IL3 and 10 ng/ml IL6 and the total colony number scored after 9–10 days. Serum erythropoietin and thrombopoietin levels were determined by ELISA (R&D Systems, Minneapolis, MN, USA).

Flow cytometry. For flow cytometry, cells were stained with fluorochrome-conjugated antibodies listed in Supplementary Table S1 or with 1 μg/ml Hoechst H33342 for staining of nucleated cells. For reticulocyte staining, whole EDTA blood was stained using anti-Ter119-antibody and 1 ng/ml thiazol orange. Flow cytometry was performed on a BD FACSCanto™ or a BD FACSCanto™ II Flow Cytometer (Becton Dickinson, Heidelberg, Germany). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA). For marker combinations defining cell populations see Figure descriptions and Supplementary Table S2.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated using RNeasy Mini or RNeasy Micro Kits (Qiagen, Hilden, Germany). cDNA synthesis was performed using RevertAid Premium reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). For amplification of target sequences Maxima SYBR Green/ROX (Fermentas) was used. Reference genes Gusb (glucuronidase) and Sdha (succinate dehydrogenase) were used for normalization. Primer sequences for qRT-PCR are given in Supplementary Table S3.

Histology and Immunohistochemistry. For histology, samples were fixed in 4% paraformaldehyde, dehydrated and paraffin-embedded. Immunohistochemistry was performed using the BrightVision Ultimate Plus Kit (Immunologic, Duiven, Netherlands) with antibodies listed in Supplementary Table 1. For Prussian Blue/hemosiderin staining, sections were deparaffinized, hydrated, stained in 1:2 potassium ferrocyanide (1–2%)/hydrochloric acid (1–2%) and counterstained with nuclear fast red. Reticulin fibers were stained using Gordon and Sweet’s silver staining protocol for reticulin.

Statistical analysis. All data are presented as mean ± standard deviation. P-values were determined using two-tailed, unpaired Student’s t-test. Studies were neither randomized nor blinded, as all animal experiments were performed with homogeneous age, strain and similar variance.

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Author Contribution

N.P., T.R., I.G., V.J., M.Z. and H.S. conceived and designed the experiments. N.P., T.R., M.K., C.R., J.L. and K.S. performed the experiments. N.P., T.R., I.G., M.K., C.R., K.S., M.Z. and H.S. analyzed the data. I.G. evaluated histologic sections. H.S., M.Z. and V.J. contributed reagents/materials/analysis tools. N.P. and H.S. wrote the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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