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Iron- and erythropoietin-resistant anemia in a spontaneous breast cancer mouse model

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Data Sharing Statement:
All data generated or analyzed during this study are included in this published article and its supplementary information files. Large images are stored on servers of the University of Zurich and are available upon request.

Keywords: anemia of cancer, erythropoiesis, hematopoiesis, red blood cell maturation, breast cancer, granulocytes

Short Summary:
Hepcidin upregulation by inflammation is a key event in the development of functional iron deficiency that ultimately leads to anemia of cancer. However, the few mouse models established by injecting cancer cells report non-uniform hepcidin expression. Therefore, we characterized a spontaneously breast cancer-developing mouse model to test how well it reflects upon human pathology. While iron deficiency rather coincided with anemia than causing it, our breast cancer mouse model displayed a shift in hematopoiesis that resulted in increased myelopoiesis while erythropoiesis was irreversibly suppressed, i.e., erythropoietin (EPO) treatment could not prevent anemia. Thus, we propose our model to study mechanisms and treatment options of EPO-resistant anemia.

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**Author Contribution**

M.G., M.U.M. and M.T. initiated this project and M.T. further developed it.

N.F.B. contributed to designing experiments, performed mouse and wet lab experiments, analyzed and interpreted data, provided intellectual input, and helped writing the manuscript.

M.R. prepared tissue samples, supported iron measurements and evaluated tumor sections.

M.C.d.S., O.M. and S.A. supported tissue iron measurements, iron staining in tissue sections, and immunohistochemical staining of ferroportin. S.A. performed hepcidin measurements.

M.A.A., H.A., N.v.B. and J.A. supported animal experiments and molecular analyses. M.S. quantified bone marrow smears, J.M.M.R. performed Ki67 stainings, and V.S. provided intellectual input. R.P.S. and B.W. analyzed bone marrow hematopoiesis, and B.W. also contributed to writing the manuscript. M.U.M. and M.G. provided intellectual input and helped writing the manuscript. M.T. designed experiments, contributed to animal experiments, analyzed, and interpreted data, and wrote the manuscript.

**Conflict of Interests**

The authors declare no conflict of interests.

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Supplemental Figures: 9

Supplemental Tables: 5
Abstract

Anemia of cancer (AoC) with its multifactorial etiology and complex pathology is a poor prognostic indicator for cancer patients. One of the main causes of AoC is cancer-associated inflammation that activates mechanisms, commonly observed in anemia of inflammation, where functional iron deficiency and iron-restricted erythropoiesis is induced by increased hepcidin levels in response to IL-6 elevation. So far only a few AoC mouse models have been described, and most of them did not fully recapitulate the interplay of anemia, increased hepcidin levels and functional iron deficiency in human patients. To test if the selection and the complexity of AoC mouse models dictates the pathology or if AoC in mice per se develops independently of iron deficiency, we characterized AoC in Trp53^{floxWapCre} mice that spontaneously develop breast cancer. These mice developed AoC associated with high IL-6 levels and iron deficiency. However, hepcidin levels were not increased and hypoferremia coincided with anemia rather than causing it. Instead, an early shift in the commitment of common myeloid progenitors from the erythroid to the myeloid lineage resulted in increased myelopoiesis and in the excessive production of neutrophils that accumulate in necrotic tumor regions. This process could neither be prevented by iron nor erythropoietin (EPO) treatment. Trp53^{floxWapCre} mice are the first mouse model where EPO-resistant anemia is described and may serve as a disease model to test therapeutic approaches for a subpopulation of human cancer patients with normal or corrected iron levels that do not respond to EPO.
**Introduction**

Anemia of cancer (AoC) is a common comorbidity and an independent poor prognostic factor in cancer patients. One of the most frequent types of AoC is caused by inflammation associated with cancer. Among the proinflammatory cytokines especially interleukin 6 (IL-6) can cause anemia by different mechanisms. IL-6 can either directly suppress erythropoiesis or inhibit erythropoiesis by interfering with iron homeostasis, the latter effect being well studied in models of anemia of inflammation (AI). During inflammation, IL-6 induces the hepatic expression of hepcidin, the master regulator of iron metabolism. Under physiological conditions, hepcidin expression is induced by high iron levels via the BMP/HJV/SMAD pathway. In contrast, hepcidin is suppressed by the EPO-induced release of erythroferrone (ERFE) from red blood cell precursors. Hepcidin regulates iron trafficking by binding and degrading the cellular iron exporter ferroportin (FPN1) to prevent duodenal iron absorption and iron release from hepatocytes and macrophages. Thereby, hepcidin can cause functional iron deficiency by reducing serum iron levels and causing iron-restricted erythropoiesis even if tissue iron levels are normal or elevated.

AoC in humans is associated with increased hepcidin levels. Mouse models with AoC displayed inconsistent hepcidin expression despite inflammation, where the ablation of hepcidin did not change tissue and plasma iron levels. Mice with tumors overexpressing IL-6 showed increased liver hepcidin expression associated with anemia. However, inhibiting IL-6 prevented anemia without improving plasma iron levels, indicating that IL-6 may suppress erythropoiesis in a hepcidin- and iron-independent manner in mice. Also 10-30% of human cancer patients do not respond to combination therapies with iron and erythropoiesis-stimulating agents (ESA), indicating that a subpopulation of cancer patients may be resistant to ESAs. Such resistance is better described in anemic patients with kidney damage, where chronic inflammation and proinflammatory cytokines can directly suppress erythropoiesis. For example IL-6 and interleukin 1 suppress erythropoiesis by inhibiting downstream signaling of the EPO receptor (EPOR) in erythroid precursors, e.g., through activation of the suppressor of cytokine signaling-3 (SOCS-3). Interferon gamma (INFγ) and tumor necrosis factor alpha (TNFα) induce apoptosis of erythroid precursors or reduce their life span. Granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) also inhibit erythropoiesis in mice with AoC by interfering with RBC production, rather than by increasing hepcidin levels and dysregulating iron homeostasis.
However, the chosen model may determine the AoC pathology in mice. Because most mouse models analysed so far were established by cancer cell injection, we characterized AoC in Trp53\textsuperscript{flox}WapCre mice carrying a breast tissue-specific ablation of tumor suppressor p53 that gives rise to spontaneously occurring mammary carcinomas. In this study we asked i) whether Trp53\textsuperscript{flox}WapCre mice develop AoC due to functional iron deficiency or due to direct inhibition of erythropoiesis and ii) whether Trp53\textsuperscript{flox}WapCre mice mirror human pathology thereby representing an adequate model to study AoC mechanisms and treatment options.
Methods

Animals

Mouse experiments were performed in accordance with the Swiss animal law and with the approval of the ethical committee (license 128/2012 and 100/2018) of the local veterinary authorities. Trp53^flx^WapCre mice on a clean FVB background, obtained from Thomas Rülicke (University of Veterinary Medicine, Vienna, Austria), express Cre under the murine whey acid promoter (WAP) to delete p53 specifically in mammary tissue giving rise to spontaneous breast tumors (Table 1). Housing conditions and tumor size measurements are described in supplemental information. Mice were euthanized by CO₂ at the indicated time points or when either one single tumor reached a size of 2 cm³ or multiple tumors reached a total volume of 3 cm³. We defined this time point as terminal stage (TS). As controls we used age-matched tumor-free female Trp53^flx^WapCre mice (TF), i.e., mice that had not developed tumors at that point. Tissue sampling is further described in supplemental data.

Additional methods

Methods describing hematology, plasma cytokine measurements, treatment with EPO and iron, and qPCR analyses including primer list (Supplemental Table 1) are described in supplemental information. Statistical and flow cytometry analysis are also described there, including antibody cocktails for hematopoiesis (Supplemental Table 2), lineage-positive cell blocking (Supplemental Table 3) and erythroid maturation (Supplemental Table 4) as well as gating strategies for hematopoietic (Supplemental Figure 1A) and late-stage erythroid cells (Supplemental Figure 1B)²⁷, ²⁸.
Results

Female Trp53\textsuperscript{lox}\textsuperscript{WapCre} mice developed subcutaneous mammary tumors between 20 and 36 weeks of age and reached terminal stage (TS), as defined in material and methods, 18 to 43 days following tumor onset (Table 1). Tumor free (TF) Trp53\textsuperscript{lox}\textsuperscript{WapCre} female mice served as control.

\textit{Trp53\textsuperscript{lox}\textsuperscript{WapCre} mice develop breast cancer associated with anemia}

Hematocrit, hemoglobin levels and number of erythrocytes dropped from a mean of 41.1\%, 13.3 g/dl, and 9.8*10\textsuperscript{6}/µl in TF to 35.4\% (p<0.001), 11.0 g/dl (p<0.001) and 7.7*10\textsuperscript{6}/µl (p<0.001) in TS mice. While the proportion of overall circulating reticulocytes tended to increase, the immature reticulocyte fraction increased from 51 \% in TF to 56.5 \% in TS mice (p<0.05), but the mature reticulocyte fraction decreased from 49 \% in TF to 43.5 \% in TS mice (p<0.05) (Figure 1A, Supplemental Figure 2A). The mean corpuscular hemoglobin (MCH) did not differ between TF and TS mice. The mean corpuscular hemoglobin concentration (MCHC) decreased from 33.2 g/dl in TF to 31.3 g/dl in TS mice (p<0.01) and the mean corpuscular volume (MCV) increased from 42.7 fl in TF to 45.2 fl in TS mice (p<0.01). The red cell distribution width (RDW) decreased from 23.0\% in TF to 22.1\% in TS mice (p=0.06) (Figure 1B, Supplemental Figure 2B). Overall, Trp53\textsuperscript{lox}\textsuperscript{WapCre} tumor mice developed hypochromic, macrocytic anemia.

\textit{Trp53\textsuperscript{lox}\textsuperscript{WapCre} mice develop AoC associated with inflammation}

Liver mRNA levels of the inflammation marker serum amyloid A1 (\textit{Saa1}) increased 100-fold (p<0.001) and plasma IL-6 levels increased approximately 300-fold in TS mice (p<0.05), suggesting ongoing inflammation (Figure 2A). The number of circulating leukocytes of TS mice was 2.2 times higher than in TF mice (p<0.001). While the number of lymphocytes did not change, the number of monocytes increased 9 times in TS mice (p<0.001). Basophils were detected in TF but not in TS mice and eosinophils did not differ between TF and TS mice. Neutrophils increased 9.5 times in TS mice (p<0.01) (Figure 2B, Supplemental Figure 2C) and accumulated, together with monocytes and other leukocytes, in suppuratively inflamed tumor regions, probably contributing to tumor necrosis (Figure 2C).

\textit{Trp53\textsuperscript{lox}\textsuperscript{WapCre} mice develop EPO and iron resistant AoC}

Trp53\textsuperscript{lox}\textsuperscript{WapCre} mice showed no evidence of metastasis (Table 1) or kidney, spleen, and liver damage (Supplemental Table 5). However, the sizes of liver and spleen increased 1.5 times (p<0.001) and 2.5 times (p<0.001) (Figure 3A), respectively, the former due to
increased hepatocyte proliferation (Supplemental Figure 3) and the latter probably due to increased extramedullary erythropoiesis. Epo mRNA levels in the kidney were 2-times higher in TS than in TF mice (p<0.05) and mean EPO plasma levels increased from 448 in TF to 1109 pg/ml in TS mice (p=0.06) (Figure 3B), indicating that TS Trp53floxWapCre mice increased renal EPO synthesis. We next tested if exogenous EPO administration protects Trp53floxWapCre mice from anemia. EPO treatment did not alter tumor progression and increased, as expected, the hematocrit in TF mice. However, EPO did not prevent most tumor-bearing Trp53floxWapCre mice from developing AoC (Figure 3C). Only 2 of 14 EPO-treated TS mice probably responded with increased erythropoiesis (Figure 3C, red arrows).

Because Trp53floxWapCre mice did not respond to EPO, we determined plasma iron levels, which can be limiting for erythropoiesis especially during inflammation. The mean iron concentration in blood plasma dropped from a 240.5 µg/dl in TF to 186.7 µg/dl (p<0.01) in TS mice, the mean transferrin saturation decreased from 66% to 34% (p<0.001), and plasma ferritin was reduced by 54% (p<0.001) (Figure 3D, Supplemental Figure 4A). Tumor sections stained for iron as well as iron measurements in tumor samples indicated that tumors were largely iron spared and that TS mice had less iron in bone marrow, spleen, liver, and kidney than TF mice (Supplemental Figure 4B and C). Despite the high IL-6 plasma levels, plasma hepcidin did not differ between TS and TF mice. In fact, hepcidin (Hamp1) mRNA levels in TS mice were even 2.9 times lower (p<0.05) than in TF mice (Figure 3D). Hepcidin expression at earlier stages of tumor development was also not altered. Among the genes that regulate hepcidin, we observed that Bmp6 RNA levels were reduced while Bmp2, Hfe and Hjv were not differentially regulated in the liver of TS mice (Supplemental Figure 4D). However, TS mice showed 1.7 times (p=0.07) and 5 times higher (p<0.05) mRNA levels of erythroferrone (Erfe), a suppressor of hepcidin mRNA expression, in bone marrow and spleen, respectively, compared to TF mice (Figure 3D). Notably, expression of hepcidin-suppressing platelet derived growth factor BB (PDGF-BB) and its downstream target Crebl was not altered (Supplemental Figure 5). Unchanged Ferroportin (Fpn1) mRNA and immunohistochemically assessed FPN1 protein levels in the liver as well as increased FPN1 mRNA and protein levels in the spleen of TS mice (Supplemental Figure 4E) further indicated that hepcidin was not activated in Trp53floxWapCre mice. Because commercial diets exceed the daily iron demand of mice and may therefore blunt hepcidin expression, we fed mice with an iron sufficient diet. However, the diet did neither altered hepcidin expression, nor the extend of anemia and iron deficiency in TS mice (Supplemental Figure 6).
We concluded that TS mice developed iron deficiency and suppressed hepcidin expression by a mechanism that increases iron availability. Therefore, we tested if iron supplementation prevents iron deficiency and anemia in Trp53^{flox}WapCre tumor mice. Because tumor growth and time points of reaching the terminal stage varied between 18 and 43 days among tumor mice, we performed two iron supplementation experiments to control either the time after iron supplementation or tumor size. We i.v. injected mice with a single dose of iron or saline immediately after tumor onset and examined i) if anemia and iron levels changed 15 days after treatment independent of tumor size (15 DAT) as well as ii) if anemia and iron levels changed in terminal stage (TS) mice. In both experiments, iron treatment did neither alter tumor proliferation nor hematocrit (Figure 3E) and hemoglobin levels (Supplemental Figure 7A). While plasma iron levels were increased 15 days after iron supplementation, indicating that supplemented iron was available in the plasma at early time points, plasma iron levels in saline or iron treated TS mice were not altered (Supplemental Figure 7B). Accordingly, we concluded that hypoferremia coincided rather than caused AoC in Trp53^{flox}WapCre mice.

Impaired erythropoiesis in bone marrow of Trp53^{flox}WapCre mice
To assess erythropoiesis, we quantified five different erythroid maturation stages from proerythroblast to mature red blood cells of Ter119^{+} erythroid progenitors in bone marrow and spleen (Figure 4A, Supplemental Figure 8A). The bone marrow proportion of total Ter119^{+} erythroid progenitors decreased 2 times (p<0.05) in TS mice (Figure 4B, Supplemental Figure 8B). The relative proportion of the different erythroid maturation stages did not significantly change, although proerythroblasts showed an increasing trend (p=0.06) (Figure 4C, Supplemental Figure 8B). In contrast, the proportion of total Ter119^{+} cells in spleens of TS mice did not change but the relative proportion of the erythroid maturation stages increased from 0.8% to 3.2% in proerythroblasts, from 4.7% to 11.7% in basophilic erythroblasts, from 16.0% to 27.2% in polychromatic erythroblasts, from 6.0% to 14.9% in orthochromatic erythroblasts but decreased from 72.5% to 43.0% in mature red blood cells (p<0.05) (Figure 4C, Supplemental Figure 8B). In parallel mRNA levels of \( Tfrc, Trf2, \) and \( Dmt1 \) as well as of the erythroid progenitor genes \( Epor, Gypa, Hba, Hbb \) increased in the spleens of TS mice, while their expression in bone marrow was reduced (Supplemental Figure 8C). To test if erythropoiesis can be boosted by acute iron supplementation during advanced tumor progression, we injected iron into mice harboring tumors larger than 1.5 cm\(^3\) and quantified bone marrow and spleen Ter119^{+} erythroid
precursors 48 h later. While the Ter119\(^+\) bone marrow proportion of iron-treated tumor mice was 2 times lower (p<0.05) than in iron-treated TF mice, the Ter119\(^+\) spleen proportion increased from 41.6\% (p<0.05) in TF to 53.8\% (p<0.05) in tumor mice after iron treatment (Figure 4D). Thus, spleen but not bone marrow erythropoiesis profited from iron supplementation, implying that splenic stress erythropoiesis was induced to compensate impaired bone marrow erythropoiesis.

**Impaired hematopoiesis in bone marrow of Trp53\(^{lox}\)WapCre mice**

While bone marrow erythroid precursors decreased, the bone marrow cellular density did not differ between TF and TS mice (Supplemental Figure 9A) indicating that other cells filled the bone marrow compartment. Indeed, myeloid precursors and mature myeloid cells were enriched in bone marrow smears of TS mice (Supplemental Figure 9B). To assess hematopoiesis, we analyzed hematopoietic cells by flow cytometry \(^3\). Our data show that neither CD48\(^-\)/CD150\(^+\) hematopoietic stem cells (HSC) nor CD48\(^+\)/CD150\(^-\) multipotent progenitors (MMP) differed between TF and TS mice. However, while the bipotent CD105\(^-\)/CD150\(^+\) pre-megakaryocyte-erythrocyte progenitors (Pre MgE) decreased from 0.25\% in TF to 0.16\% in TS mice (p=0.06) the CD105\(^-\)/CD150\(^-\) pre-granulocyte-monocyte lineage cells (Pre-GM) increased from 0.8\% in TF to 1.3\% in TS mice (p<0.05) (Figure 5A). This suggests that the differentiation of common myeloid progenitors (CMP) was shifted from the erythroid lineage towards the granulocyte-monocyte lineage (Figure 5B). This was further supported by an elevated proportion of CD45\(^+\)/GR1\(^+\) neutrophil progenitors in bone marrow that increased from 72.5\% in TF to 86.5\% in TS mice (p<0.05). Although the proportion of Pre-MgE was reduced in TS mice, we observed no difference in the proportion of CD150\(^+\)/CD41\(^+\) megakaryocyte progenitors (MkP) as well as CD105\(^+\)/CD150\(^+\) pre-colony forming units erythrocyte (pre-CFUe) between TF and TS mice. However, the CD105\(^+\)/CD150\(^-\) colony forming units erythrocyte (CFUe) decreased from 0.76\% in TF mice to 0.24\% in TS mice (p<0.01) (Figure 5A). To investigate why erythropoiesis was inhibited in bone marrow but not spleen we analyzed gene expression levels of stem cell factor (Scf), which stimulates erythropoiesis \(^3\), and suppressor of cytokine signaling (Socs-3), which inhibits erythropoiesis \(^2\). While Scf mRNA levels in bone marrow of TS mice were 4.5 times lower (p<0.01) and Socs-3 mRNA levels 2.3 (p<0.05) times higher than in TF mice, their mRNA levels in the spleen did not differ between TF and TS mice (Figure 5C). Additionally, autophagy genes, which play a critical role cell organelle removal during erythroid maturation \(^3\), were downregulated in the bone marrow of TS mice (Supplemental
Further, we analyzed cytokine mRNA levels in tumors of TS and in healthy mammary tissue of TF mice. Tumor necrosis factor alpha (Tnfα) mRNA levels were 9.2 times lower (p<0.001), interleukin 1 beta (Il-1β) levels were 5.3 times higher (p<0.001), granulocyte-macrophage colony-stimulating factor (Csf2) levels were 2.4 times higher (p=0.07) in tumor tissue, while granulocyte colony-stimulating factor (Csf3) levels did not differ between tumor tissue and mammary tissue (Figure 5D). However, the plasma cytokine levels of TNFα increased from 14.6 pg/ml in TF to 40.0 pg/ml in TS mice (p<0.05), the plasma IL-1β levels did not differ between TF and TS mice and the plasma levels of the myelocytic differentiation regulator GM-CSF increased from 2.0 pg/ml in TF mice to 3.1 pg/ml in TS mice (p=0.053). Additionally measured interferon gamma (INF γ) plasma levels did not differ between TF and TS mice (Figure 5E). Our data suggest that while erythropoiesis may be actively suppressed (e.g., by TFNα), myelocytic differentiation regulators (e.g., GM-CSF) alter the fate of early hematopoietic precursors and prevent erythropoiesis by upregulating myelopoiesis.
Discussion

We examined if Trp53\textsuperscript{flox}\textsuperscript{WapCre} mice that spontaneously develop breast cancer mirror the human AoC pathology. Terminal stage Trp53\textsuperscript{flox}\textsuperscript{WapCre} mice developed hypochromic AoC together with a strong inflammation and iron deficiency. However, hepcidin levels were not induced and iron deficiency rather coincided with AoC than caused it. Treatment with iron as well as with EPO did not restore normal hemoglobin levels. During early hematopoiesis, the progenitor fate of CMPs shifted from erythroid towards the myeloid lineage, resulting in an inadequate bone marrow production of red blood cells and an excessive production of granulocytes and neutrophils. Proinflammatory cytokines such as TNF\textgreek{a} and GM-CSF as well as IL-6 may block erythropoiesis and stimulate myelopoiesis independent of hepcidin and iron levels.

Trp53\textsuperscript{flox}\textsuperscript{WapCre} mice developed AoC associated with an increased number of monocytes and neutrophils in blood, which accumulated in suppuratively inflamed tumor regions. Anemic mice showed slightly upregulated renal \textit{Epo} mRNA as well as EPO plasma levels, probably in response to reduced blood oxygenation. Surprisingly, EPO treatment did not increase hematocrit and hemoglobin levels in TS mice. Non-responsiveness to ESAs is also observed in up to 30-40\% of human cancer patients\textsuperscript{33} either due to inhibited EPO signaling or due to reduced iron availability for erythropoiesis caused by proinflammatory cytokines. Especially, IL-6 upregulates hepcidin in the liver causing functional iron deficiency\textsuperscript{14, 15} or it directly inhibits erythropoiesis in patients or mice\textsuperscript{20, 21, 34}. In anemic Trp53\textsuperscript{flox}\textsuperscript{WapCre} mice, IL-6 showed by far the highest upregulation of all cytokines determined. However, while plasma iron levels were reduced, plasma hepcidin levels did not differ between TF and TS mice, and hepcidin mRNA levels in the liver were even reduced. We explain this discrepancy by the enlarged liver in TS mice with more hepcidin-producing cells that compensate the reduced cellular hepcidin synthesis rate. While hepcidin-inducing PDGF-BB\textsuperscript{29} may not play a major role in our model, the reduced expression of BMP6\textsuperscript{6, 7} and the low iron levels \textit{per se}\textsuperscript{35-37} may overwrite the hepcidin-inducing effect of IL-6 that is known from anemia of inflammation\textsuperscript{3}. Moreover, the increased bone marrow and spleen mRNA levels of \textit{Erfe}\textsuperscript{8} and the increased renal EPO mRNA levels suggest that hepcidin may have also been suppressed by the HIF2-EPO-ERFE axis\textsuperscript{38, 39}. Hepcidin downregulation in other AoC mouse models was suggested to be a consequence of activated erythropoiesis at late tumor stages, while at early stages, hepcidin expression may be increased by inflammation\textsuperscript{16}. In our study, no increase of hepcidin mRNA levels at early or late stages of tumor progression was observed. Also
feeding mice with an iron sufficient (50 mg/kg iron) diet, to exclude the possibility that high iron levels in commercial diets blunt acute hepcidin expression, did neither alter hepcidin expression nor anemia in our model. We conclude that hepcidin is not the main driver for AoC in Trp53\textsuperscript{flox}WapCre mice. However, we cannot exclude that inappropriately high hepcidin plasma levels (i.e., no decrease in response to iron deficiency) may contribute to the development of AoC in Trp53\textsuperscript{flox}WapCre mice.

When supplementing tumor mice with iron, hematocrit and hemoglobin values did not increase, although the iron plasma levels were elevated at least until 15 days after treatment. The majority of mice did not fully establish an anemic phenotype during the first 15 days of tumorigenesis and iron supplementation may not have had the desired effect at such early stages. We cannot exclude that higher iron dosages may have protected Trp53\textsuperscript{flox}WapCre mice better from anemia, despite calculating iron supplementation so that normal hemoglobin values should have been restored (Ganzoni formula\textsuperscript{40}). Moreover, iron supplementation may also increase tumor proliferation\textsuperscript{41} but the calculated iron dosages did not cause tumor progression. When mice with advanced 1.5 cm\textsuperscript{3} large tumors were treated with iron, they acutely increased Ter119\textsuperscript{+} erythroid progenitors in the spleen but not in the bone marrow. This suggested that RBC production was maintained by the spleen, while it was blocked in the bone marrow where hypoferremia was not a limiting factor for erythropoiesis and thus, rather coincided with AoC in our model.

At late-stage erythropoiesis, the proportion of Ter119\textsuperscript{+} erythroid precursors was reduced in the bone marrow of Trp53\textsuperscript{flox}WapCre mice, while the composition of the erythroid maturation stages from proerythroblasts to red blood cell (stage I-V)\textsuperscript{27} were not significantly altered. In contrast, the proportion of erythroid precursors in the spleens of TF and TS mice did not differ, while the proportion of stages I-IV (proerythroblasts to orthochromatic erythroblast) were increased, and the proportion of the red blood cells (stage V) decreased. We interpret these results in the spleen and the associated splenomegaly as a consequence of stress erythropoiesis and the active release of RBCs. Similar data have been reported in spleens of mice with abscess-induced inflammation. Those authors suggested that the accumulation of the maturation stages I–IV results from a blockade of erythroid maturation stage V\textsuperscript{42}.

Both, EPO, and iron treatment, did not prevent AoC in Trp53\textsuperscript{flox}WapCre. Therefore, our data suggest that bone marrow erythropoiesis was inhibited prior to iron-dependent maturation stages (erythroblast – reticulocyte) as well as prior to EPO-dependent maturation stages.
(CFUe – proerythroblast). At early hematopoiesis, bone marrow HSC, MPP and CMP did not differ between TF and TS mice. While HSCs may be recruited from bone marrow to the spleen resulting in a bone marrow depletion of HSC during AoC \(^{26}\), the stem cell niche in our model appeared not affected. Instead, the commitment of CMP into either Pre-MgE or Pre-GM was shifted in mice. While the proportion of Pre-MgE including downstream CFUe that give rise to erythroid lineage was reduced, the proportion of the myeloid precursors Pre-GM, that give rise to granulocytes \(^{43}\), was increased, explaining the excessive neutrophil production in Trp53\(^{flox}\)WapCre mice. Increased granulocytosis is also observed in human (breast \(^{44}\)) cancer patients \(^{45}\), where it associates with poor survival, as well as in transplanted tumor mouse models \(^{46, 47}\). Especially tumor-produced colony stimulating factors G-CSF and GM-CSF \(^{25, 26}\), which block erythropoiesis by depleting erythroblastic island macrophages \(^{48}\) and increasing the number of granulocytes, can cause excessive myelopoiesis \(^{25}\). Some tumors in cancer patients express G-CSF and GM-CSF associated with an increased number, survival, and activation of neutrophiles \(^{49, 50}\). While the mRNA levels of both factors in tumors of Trp53\(^{flox}\)WapCre mice were hardly higher than in healthy mammary tissue, plasma protein levels of myelopoiesis-stimulating GM-CSF were increased, probably contributing to excessive granulocytosis. Increased TNF\(\alpha\) levels in Trp53\(^{flox}\)WapCre mice suggest that, in addition to promoting myelopoiesis, erythropoiesis suppressing mechanisms were activated \(^{23}\). Indeed, Trp53\(^{flox}\)WapCre mice showed increased expression of the EPOR signaling inhibitor SOCS3 \(^{22}\) in bone marrow but not in spleen. Additionally, we observed a decreased expression of erythropoiesis stimulating SCF \(^{51}\). However, the plasma levels of TNF\(\alpha\) as well as GM-CSF increased less than 3 and less than 2 times, respectively, which challenges their significance in dysregulating bone marrow hematopoiesis in anemic Trp53\(^{flox}\)WapCre mice. We speculate that a cocktail of several cytokines, potentially including GM-CSF and TNF\(\alpha\), is required to cause iron and EPO-resistant AoC. It may be also possible that AoC in Trp53\(^{flox}\)WapCre mice is predominantly caused by IL-6 that directly suppresses erythropoiesis without increasing hepcidin.

Thus, AoC in Trp53\(^{flox}\)WapCre mice differs from one of the most common forms of AoC in humans, where iron deficiency plays a key role \(^{52}\) and where increased hepcidin levels may serve as a serological biomarker for absolute or functional iron deficiency \(^{53}\). Iron deficiency AoC is often associated with either normocytic , (75%) or microcytic (21%) anemia \(^{52}\). We observed macrocytic anemia in Trp53floxWapCre mice (corresponding to observations in 4% of human patients \(^{52}\)), which may be a consequence of the severe leukocytosis \(^{54}\). Thus,
Trp53floxWapCre mice mimic a rare type of AoC in human patients, where medullar erythropoiesis is irreversibly suppressed while myelopoiesis is induced.

**Conclusion**

We characterized AoC in breast cancer Trp53floxWapCre mice and show that hypoferremia does not cause AoC despite high IL-6 plasma levels that are expected to activate hepcidin mRNA expression. While the IL-6-induced pathways during anemia of inflammation and AoC largely overlap in humans, IL-6 in mice may activate distinct pathways during anemia of inflammation and AoC. In fact, multiple mouse models, including the herein characterized Trp53floxWapCre mice, develop AoC in response to excessive myelopoiesis suggesting that tumor-induced inflammation alters hematopoiesis prior to erythroid maturation. Trp53floxWapCre tumor mice are, to our knowledge, the first mouse model where AoC with excessive myelopoiesis cannot be prevented by iron or EPO treatment. Thus, this model will be suitable to study mechanisms and treatment options of EPO-resistant anemia.
References


Table 1. Clinical characterization of terminal stage Trp53\textsuperscript{flox}\textsuperscript{WpCre} mice

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* TS: Terminal stage, when either one tumor reached a size of 2 cm\textsuperscript{3} or multiple tumors reached a total volume of 3 cm\textsuperscript{3}.
Figure Legend

Figure 1. Anemia of cancer in Trp53\textsuperscript{flox}WapCre breast cancer mice. Blood of tumor bearing Trp53\textsuperscript{flox}WapCre mice (grey boxes) was isolated when the tumor reached maximal permitted size (defined as terminal stage; TS). Age-matched tumor free (TF) Trp53\textsuperscript{flox}WapCre mice (white boxes) served as control. (A) Shown are hematocrit (n=14-15) and hemoglobin (n=16-33) analyzed by microcentrifugation and by ABL800, respectively, as well as erythrocyte count (n=6), proportion of overall circulating reticulocytes and proportion of immature and mature reticulocyte fractions (n=4) analyzed by Sysmex XT-2000iV from whole blood. (B) Shown are mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), and red cell distribution width (RDW) (n=4-6), analyzed by Sysmex XT-2000iV from whole blood. Data are shown as box plot with min to max whiskers and were analyzed by a Student’s t-test (black symbols) or a Mann-Whitney test (red symbols or p-values) (*p<0.05, **p<0.01, ***p<0.001).

Figure 2. Inflammation in anemic Trp53\textsuperscript{flox}WapCre breast cancer mice. Blood and liver tissues of tumor bearing Trp53\textsuperscript{flox}WapCre mice (grey boxes) were isolated when the tumor reached maximal permitted size (defined as terminal stage; TS). Age-matched tumor free (TF) Trp53\textsuperscript{flox}WapCre mice (white boxes) served as control. (A) Shown are liver mRNA levels of serum amyloid A 1 (Saa-1) (n=14-15) determined by qPCR and normalized to \(\beta\)-actin (Actb) mRNA levels (left panel) as well as plasma IL-6 levels (n=4), determined by ELISA (right panel). (B) Shown are the cell counts per µl blood of leukocytes, lymphocytes, monocytes, neutrophiles, eosinophil, and basophils from tumor free (TF, white boxes) and terminal stage (TS, grey boxes) Trp53\textsuperscript{flox}WapCre mice, analyzed by Sysmex XT-2000iV. (C) Representative image of suppuratively inflamed tumor regions with massive neutrophile invasion (black arrows representative examples). Scale bar 250 µm. Data are shown as box plot with min to max whiskers and were analyzed by a Student’s t-test (black symbols) or a Mann-Whitney test (red symbols) (*p<0.05, **p<0.01, ***p<0.001).

Figure 3. Iron and Erythropoietin resistance in hypoferremic Trp53\textsuperscript{flox}WapCre breast cancer mice with anemia of cancer. (A, B and D) Tissue and blood of tumor bearing Trp53\textsuperscript{flox}WapCre mice (grey boxes) were isolated when the tumor reached maximal permitted size (defined as terminal stage; TS). Age-matched tumor free Trp53\textsuperscript{flox}WapCre mice (TF, white boxes) served as control. (A) Shown are the wet weight of spleen (left panel) and liver (right panel) normalized to the bodyweight. (B) Shown are qPCR-quantified
erythropoietin (Epo) mRNA levels in the kidney normalized to β-actin (Actb) (n= 9-10) (left panel) as well as EPO plasma levels determined by ELISA (n=9-12) (right panel). (C) Immediately after tumor onset, Trp53floxFapCre mice were subcutaneously injected with either 1000 U/kg EPO (purple) or saline (light blue) thrice a week until tumors reached maximal size. Shown is a modified Kaplan-Meier curve showing the percentage of saline (light blue) and 1000 U/kg EPO treated (purple) mice, which reached maximal tumor size (n=11-13) (left panel). Further shown are hematocrit levels of saline (light blue) or 1000 U/kg EPO (purple) treated tumor mice (Tumor⁺) as well as tumor free controls (Tumor⁻) (n=6-14) (right panel). Red arrows indicate two EPO treated mice that showed increased hematocrit values. (D) Shown are plasma iron and transferrin saturation (n=19-20) analyzed by a bathophenanthroline assay (upper panels). Further shown are plasma ferritin (n=6) and plasma hepcidin levels (n=5-7) analyzed by ELISA (middle panels). Liver mRNA levels of hepcidin (Hamp1) (n=9-11) as well as erythroferrone (Erfe) (n=4-6) in bone marrow (BM) and spleen were determined by qPCR and normalized to β-actin (Actb) (lower panels). (E) Trp53floxFapCre mice were intravenously injected with a single dose of iron (dark blue boxes) or saline (light blue boxes) immediately after tumor onset. In experiment 1, mice received 20 mg/kg Ferinject® and blood as well as tissues were isolated 15 days after treatment (15 DAT). In experiment 2, mice received 13.28 mg/kg Ferinject® or saline and blood as well as tissues were isolated when the tumor reached maximal permitted size (defined as terminal stage; TS). Shown are a modified Kaplan-Meier curve that shows the percentage of saline (light blue) and 13.28 mg/kg iron treated (dark blue) mice, which reached maximal tumor size (n=9-10) (left panel) as well as hematocrit (n=8-10) (right panel). Data are shown as box plot with min to max whiskers and were analyzed by a Student’s t-test (black symbols) or a Mann-Whitney test (red symbols) (*p<0.05, **p<0.01, ***p<0.001). Modified Kaplan-Meier curves were analyzed with a Log-rank (Mantel-Cox) test.

Figure 4. Late-stage erythropoiesis in anemic Trp53floxFapCre mice. Bone marrow and spleen cells of age-matched tumor free (TF) and terminal stage (TS) Trp53floxFapCre mice were isolated when the tumors reached maximal permitted size and analyzed by flow cytometry. (A) Shown is a representative image of Ter119⁺ cells (late erythroid precursors) gating in Ter119⁺ vs. SSC-Area plots (left panel). Different clusters of Ter119⁺ cells in an FSC-Area vs. CD44 plot (right panel) identified erythroid maturation stages (I proerythroblasts, II basophilic erythroblasts, III polychromatic erythroblasts, IV
orthochromic erythroblasts (including reticulocytes) and V mature erythrocytes) based on cell size and CD44 expression levels. (B) Shown is the average proportion of Ter119⁺ (red) and Ter119⁻ (yellow) cells in bone marrow (upper panels) and spleen (lower panels) from tumor free (left) and terminal stage (right) Trp53<sup>fl</sup>WapCre mice analyzed by flow cytometry (n=4). (C) Shown is the average proportion of the five maturation stages of erythrocytes (I proerythroblasts, II basophilic erythroblasts, III polychromatic erythroblasts, IV orthochromic erythroblasts (including reticulocytes) and V mature erythrocytes) identified by flow cytometry in an FSC-Area vs. CD44 plot in bone marrow (upper panels) and spleen (lower panels) from tumor free (left) and terminal stage (right) Trp53<sup>fl</sup>WapCre mice analyzed by flow cytometry (n=4). (D) Age-matched tumor free (TF) and tumor bearing (TB) mice received a single i.v. 20 mg/kg iron injection when tumors reached a size of 1.5 cm³. Bone marrow and spleen were harvested 48 after injection and analyzed by flow cytometry. Shown are the proportion of bone marrow (left panel) and spleen Ter119⁺ cells (right panel) in iron treated mice (n=4). Data in (B and C) are shown as average values in pie charts (n=4), data in (D) are shown as box plot with min to max whiskers. Data were analyzed by a Student’s t-test (black symbols) or a Mann-Whitney test (red symbols, p-values) (**p<0.01; *p<0.05). The black and red dotted lines in panel (D) indicate the average values of untreated tumor free (black dotted line) and untreated terminal stage (red dotted line) mice. # in panel (D) indicates a difference (p<0.05) between iron treated TS and untreated TS (red dotted line) mice.

**Figure 5. Early-stage hematopoiesis in anemic Trp53<sup>fl</sup>WapCre mice.** (A) Bone marrow and spleen of age-matched tumor free (TF, white boxes) and terminal stage (TS, grey boxes) Trp53<sup>fl</sup>WapCre mice were isolated when the tumors reached maximal permitted size and analyzed by flow cytometry. Shown are the proportion of CD48⁺/CD150⁻ hematopoietic stem cells (HSC), CD48⁺/CD150⁻ multipotent progenitors (MPP), CD105⁻/CD150⁺ pre-megakaryocyte erythrocyte progenitors (Pre-MgE), CD105⁺/CD150⁻ pre-granulocyte-monocyte lineage cells (Pre-GM) (n=4). Further shown are the proportion of CD45⁺/GR1⁺ positive leukocyte precursors in bone marrow (BM) and spleen as well as CD150⁺/CD41⁺ megakaryocyte precursors (Mkp), CD105⁺/CD150⁺ pre-colony forming units erythrocyte (Pre-CFUe), and CD105⁺/CD150⁺ colony forming units erythrocyte (CFUe) in bone marrow (n=4). Panel (B) schematically shows the steps of hematopoiesis. The red arrows indicate an up- or down regulation of hematopoietic precursors and the triple black arrows indicating a shift in the maturation of common myeloid progenitors (CMP) towards Pre-GM in the bone
marrow of TS Trp53\textsuperscript{flx}WapCre mice. (C) Shown are mRNA levels of stem cell factor (Scf) (left panel) and suppressor of cytokine signaling 3 (Socs3) (right panel) in the bone marrow (BM) and spleen, determined by qPCR and normalized to β-actin (Actb) mRNA levels (n=4-6). (D) Shown are mRNA levels of tumor necrosis factor alpha (Tnfa), interleukin 1 beta (Il-1b), granulocyte-macrophage colony-stimulating factor (GM-Csf) and granulocyte colony-stimulating factor (G-CSf) in mammary tissue of tumor free (TF Mamma) and in tumor tissue of terminal stage Trp53\textsuperscript{flx}WapCre mice (TS Tumor), determined by qPCR and normalized to β-actin (Actb) mRNA levels (n=4-8). Panel (E) shows plasma levels of tumor necrosis factor alpha (TNF\textalpha) and Interleukin 1 beta (IL-1\textbeta) (left panel) as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon gamma (INF \gamma) (right panel) quantified by ELISA (n=7-8). Data are shown as box plot with min to max whiskers and were analyzed by a Student's t-test (black o values panel E) or a Mann-Whitney test (red symbols, p-values) (***p<0.001; **p<0.01; *p<0.05).
Figure A: Flow cytometry graphs showing Ter119+ and Ter119-FITC-A, CD44-APC-A, and FSC-A. Regions I-V are labeled:
- I: Proerythroblasts
- II: Basophilic Erythroblasts
- III: Polychromatic Erythroblasts
- IV: Orthochromatocytic Erythroblasts
- V: Mature Red Blood Cells

Figure B: Pie charts for Tumor Free and Terminal Stage bone marrow and spleen. Percentages for each cell type are shown.

Figure C: Pie charts for Tumor Free and Terminal Stage bone marrow and spleen. Percentages for each cell type are shown with a p-value of 0.06.

Figure D: Bar graphs for bone marrow and spleen showing the percentage of Ter119+ cells with mean values and standard deviations.
Supplemental Information

Iron- and erythropoietin-resistant anemia in a spontaneous breast cancer mouse model
Fabregas Bregolat et al.

Running Title: Iron- and erythropoietin-resistant mice with AoC
Methods

Animals
Trp53floxWapCre mice \(^1\) were housed at 22±5 °C in a 12 h light/dark cycle and received water and food (standard rodent chow containing 250 mg/kg iron from Kliba Nafag, #3436, Switzerland) ad libitum. Tumor development in female Trp53\(^{\text{flox}}\)WapCre mice was monitored visually and by palpation when mice reached 18 weeks of age. Tumor length (L) and width (W) were measured using a caliper and tumor volume was calculated \([V=(LxW^2)/2]\) \(^2\). In addition to the standard rodent chow (containing 250 mg/kg iron) (Kliba Nafag, #3436, Switzerland), some mice were kept on an iron sufficient diet (containing 50 mg/kg iron) (Kliba Nafag, #2222 modified, Switzerland), which did not exceed the daily iron demand. Mice were placed on this low iron diet either directly after weaning or after 15 weeks of age. Prior tissue isolation, blood was obtained from the right ventricle and mice were transcardially perfused with PBS. Isolated tissue was either immediately processed, snap frozen in liquid nitrogen or incubated for 48 h in 4% paraformaldehyde (PFA) for paraffin embedding. For histological analyses a hematoxylin-eosin staining was performed.

Hemoglobin, hematocrit, and hematology
Hemoglobin was measured from fresh whole blood by ABL800 (Radiometer RSCH GmbH, Switzerland) and hematocrit was manually measured in blood-filled heparin capillaries by determining the ratio of the volume occupied by red blood cells to the volume of whole blood after 5 min at 120 rpm in the microcentrifuge (HETTICH, Switzerland). Hematology, including erythrocytes was analyzed from whole blood by the Sysmex XT-2000iV (Sysmex Swiss AG, Switzerland).

Plasma metabolites
Fresh whole blood containing heparin was centrifuged at 2'000 rpm for 10 minutes to isolate plasma that was processed immediately or snap frozen and stored at -80°C until analysis. Bilirubin, glucose, urea, creatinine, total protein, cholesterol, triglycerides, alkaline phosphatase, aspartate transaminase and alanine transaminase were analyzed by using the Roche Cobas Integra 800 device (Roche, Switzerland).

Erythropoietin treatment
Mice were subcutaneously (s.c.) injected with either 100 µl saline or with 1'000 U/kg erythropoietin (Epoetin-beta; Recormon®, Roche) diluted in 100 µl saline directly after tumor detection trice a week until mice reached maximal tumor size.
Iron treatment
Mice were intravenously (i.v.) injected with either 100 µl saline or 13.8 as well as 20 mg/kg ferric carboxymaltose (Ferinject®, Vifor Pharma, Switzerland) diluted in 100 µl saline directly after tumor detection. The dosage was calculated by the Ganzoni formula \(^3\) based on body weight (BW) and hemoglobin (Hb) levels as follows:

\[
\text{Total iron deficit [mg]} = \text{BW [kg]} \times (\text{target Hb - actual Hb} [\text{g/dl}] \times 2.4) + \text{storage iron [mg]}
\]

with BW at the moment of tumor onset, target Hb referring to the upper 75% percentile of Hb values in tumor free mice and actual Hb referring to the lower 25% percentile of Hb values in terminal stage tumor mice.

Plasma and tissue iron
Plasma iron concentration was measured using a colorimetric bathophenanthroline assay (SFBC, Biolabo, France) and the unsaturated iron binding capacity was measured by UIBC assay (Biolabo, France). Total iron binding capacity (TIBC) was calculated as

\[
\text{TIBC (µg/dl)} = \text{SFBC (µg/dl)} + \text{UIBC (µg/dl)}
\]

and transferrin saturation was calculated by

\[
\text{Tsat(%)} = \frac{\text{SFBC}}{\text{TIBC}} \times 100
\]

Non-heme iron content in tissues was measured by the bathophenanthroline method and normalized to weight of dry tissue \(^4\).

Perls’-DAB enhanced staining and immunohistochemistry
Tissues were paraffin embedded after fixation with 4% paraformaldehyde for 24 hours and 5 µm sections were cut. After deparaffinization slides were stained with a potassium ferrocyanide/HCl solution (Sigma-Aldrich, Switzerland) followed by washing in demineralized water. After blocking with H\(_2\)O\(_2\) (Sigma-Aldrich, Switzerland), slides were stained with 3,3-diaminobenzidinetetrahydrochloride (DAB) (Sigma-Aldrich, Switzerland) and counterstained with hematoxylin (Sigma-Aldrich, Switzerland). For Ki67 immunohistochemistry, tissue slides were incubated in 3% H\(_2\)O\(_2\) (Sigma-Aldrich, Switzerland) and stained in the automated staining system Discovery (Ventana Medical Systems, Inc., USA) using a rabbit-anti-Ki67 antibody (# 790-4286, Roche, Switzerland) and the Discovery DAB Map detection kit (#760-124, Roche, Switzerland). For morphometric quantification of Ki67 cells in the liver, slides were scanned (NanoZoomer 2.0-HT; Hamamatsu, Hamamatsu City, Japan) and evaluated with an image analysis software (Visiopharm 2020.08.1.8403; Visiopharm, Hoersholm, Denmark). First the whole liver tissue was detected by Decision Forest classification method and outlined as region of interest (ROI). Subsequently, the Cell classification method was used to detected negative cells (blue) and positive cells (brown) within the ROI. The results were expressed as percentage of positive cells. For ferroportin
immunohistochemistry, tissue slides were stained using a rabbit-anti-metal transport protein MTP1 (Ferroportin) (# MTP11-A, Alpha Diagnostic, USA) and the Vectastain ABC kit (Vector Labs, USA) as well as the Vector AEC substrate (Vector Labs, USA). Prior incubation with the primary antibody, slides were incubated in 3% H₂O₂ (Sigma Aldrich, Switzerland) to block endogenous peroxidases followed by an antigen retrieval with Citra Plus solution (BioGenex, USA). Slides were counterstained with hematoxylin.

**Bone marrow smears**

Bone marrow smears were obtained from the medullar cavity of femurs using the paint brush technique. Slides were air-dried and stained with a Giemsa staining. The cells were morphologically evaluated, and a differential count of the hematologic precursors was performed by light microscopy at 100x.

**mRNA expression analysis by real time PCR and digital droplet PCR**

RNA was extracted from snap frozen tissue by using the ReliaPrep® RNA Tissue Miniprep System (Promega) and RNA was either immediately processed or stored at -80°C. For cDNA synthesis we used the RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Switzerland) as well as RiboLock RNase inhibitor (Thermo Fisher Scientific, Switzerland). Primers were designed with Primer3 and all primer sequences are indicated in **Supplementary Table 1**. Real time PCR was performed in Thermocycler ABI7500 Fast (Applied Biosystems) using LightCycler 480 SYBR Green (Roche Applied Science, Switzerland) and relative gene expression was determined by the ΔΔCT method with β-actin (Actb) as a reference gene. In addition, we quantified hepcidin (Hamp1) expression by a digital droplet PCR (QX100, ddPCR™, Biorad) with mouse specific TaqMan probes (ThermoFisher Scientific, Switzerland) against Hamp1 (Mm04231240_s1) and Actb (Mm00607939_s1).

**Plasma cytokines, ferritin and hepcidin**

Fresh whole blood containing heparin was centrifuged at 2000 rpm for 10 minutes to isolate plasma that was processed immediately or snap frozen and stored at -80°C until analysis. Plasma interleukin 6 (IL-6) was measured by ELISA using U-PLEX Mouse IL-6 Assay® (Meso Scale Discovery, USA) and TNFα, IL1β, GM-CSF as well as INFγ were measured by ELISA using a V-PLEX custom mouse cytokine assay (#K152A0H-1, Meso Scale Discovery, USA). Ferritin was measured with a mouse Ferritin ELISA kit (Crystal Chem, Inc., USA) and hepcidin was measured in 12 µl of plasma (in duplicate) with the Hepcidin Murine-competitive ELISA Kit (Intrinsic Lifesciences, USA) following the manufacturer’s instruction.
FACS analyses of hematopoietic stem cells and erythroid progenitors in bone marrow and spleen

For analyzing bone marrow hematopoiesis, a single-cell suspension of isolated bone marrow cells pooled from 2 femurs and 2 tibiae was incubated with an antibody cocktail (Supplemental Table 2). Lineage-positive cells were excluded using a cocktail of biotinylated antibodies (Supplemental Table 3) and staining with streptavidin (SA; eF450; eBioscience). Fluorescence-activated cell sorting (FACS) analysis was performed on LSRII (Becton Dickinson), and sorting was done on Aria II (Becton Dickinson). Cell numbers were counted on MACS quant. Data were analyzed with DIVA (Becton Dickinson), MACS quantify (Miltenyi), or FlowJo (Tree Star, Ashland, OR, USA). The gating strategy adopted from Pronk et al., 2007 is described in Supplemental Fig.1 A. For analyzing erythroid progenitors in bone marrow and spleen, single-cell suspension of isolated spleen or bone marrow cells pooled from 2 femurs and 2 tibiae was incubated with an antibody cocktail (Supplemental Table 4) and with a Zombie Yellow™ Fixable Viability Kit (#423103, Biolegend). Cell suspension was measured by a Cytoflex S Flow Cytometer (Beckmann Coulter Life Science) and analyzed by FlowJo software (Tree Star, Ashland, OR, USA). The gating strategy adopted from Chen et. al, 2009 is described in Supplemental Fig.1 B.

Statistics

Statistics were performed in GraphPad Prism6 (GraphPad Software, San Diego, US) Data distribution was analyzed with the Kolmogorov-Smirnov test. We used the Student’s t-test for parametrically distributed and a Man-Whitney test for non-parametrically distributed data to compare two groups. For multiple comparisons, a one-way ANOVA with a Bonferroni’s multiple comparisons for parametrically distributed data or a Kruskal-Wallis test with a Dunn’s multiple comparisons for nonparametrically distributed data was performed. Kaplan-Meier curve was analyzed with a Log-rank (Mantel-Cox) test.
### Supplemental Tables

#### Supplemental Table 1. Sequences of primers used for semi-quantitative real time PCR.

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<td></td>
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<td></td>
<td>Rev: 5’GCT TCC GCT GTG TGT TG3’</td>
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<td>Cereb1 (Cyclic AMP-Responsive Element-Binding Protein 1)</td>
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<td>Rev: 5’TCTGATTGGTGCCGAGATG3’</td>
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<td></td>
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<td>Rev: 5’TGGAAAGCGAATGGAAGG3</td>
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<td>Rev: 5’CAATCCCTTCAGCCTATTC3’</td>
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<td>Forward Primer</td>
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<td><strong>Epo</strong> (Erythropoietin)</td>
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<tr>
<td><strong>Epor</strong> (Erythropoietin receptor)</td>
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<td><strong>Erfe</strong> (Erythroferrone)</td>
<td>5' AGCGAGCTCTTCACCATCTC 3'</td>
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<td><strong>Fpn1</strong> (Ferroportin)</td>
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<tr>
<td><strong>Fth1</strong> (Ferritin heavy chain 1)</td>
<td>5' GTCCTCATCTCGCTGTTGCT 3'</td>
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<td><strong>Gypa</strong> (Glycophorin A)</td>
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<td><strong>Hamp1</strong> (Hepcidin-1)</td>
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<tr>
<td><strong>Hba</strong> (Hemoglobin alpha chain)</td>
<td>5' TGAAGCCCTGGAAAGGATGTGTTG 3'</td>
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<td><strong>Hbb</strong> (Hemoglobin beta chain)</td>
<td>5' ATGCGAGCTCTTCACCATCTC 3'</td>
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<tr>
<td><strong>Hfe</strong> (Homeostatic iron regulator)</td>
<td>5' TGTGAGTGTTCCAGAGGTGAG 3'</td>
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<td><strong>Hjv</strong> (Hemojuvelin)</td>
<td>5' TGCCTTAGATAACGACTCTCTTGGT3'</td>
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<tr>
<td><strong>Il-1b</strong> (Interleukin-1 beta)</td>
<td>5' CCTGTGTAATGAAAGGCA 3'</td>
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<td><strong>Il-6</strong> (Interleukin-6)</td>
<td>5' CTCTGGGAATCGTGGAGAATATG 3'</td>
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<tr>
<td><strong>Il-6r</strong> (Interleukin-6 receptor)</td>
<td>5' TTT GGG TTG CT TCT CTC TGT GTC TT 3'</td>
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<tr>
<td><strong>Pdgfb</strong> (Platelet Derived Growth Factor Subunit B)</td>
<td>5' GCCAGAACTACCACCCAGGAC 3'</td>
</tr>
<tr>
<td><strong>Saa1</strong> (Serum amyloid A1)</td>
<td>5' CCAAGGACTATGAGTCGCTGCT 3'</td>
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<tr>
<td><strong>Scf</strong> (Stem cell factor)</td>
<td>5' TGGGAAATAGTGGGATACCTCGTG 3'</td>
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<td><strong>Socs3</strong> (Suppressor of cytokine signaling 3)</td>
<td>5' TCACCCACAGCAGTTCCC 3'</td>
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<td><strong>Tmprss6</strong> (Transmembrane serine protease 6)</td>
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<td><strong>Tnfa</strong> (Tumor necrosis factor alpha)</td>
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<tr>
<td>Gene Symbol</td>
<td>Description</td>
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<td>Tfrc</td>
<td>Transferrin receptor-1</td>
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<td>Tfr2</td>
<td>Transferrin receptor-2</td>
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<tr>
<td>Trf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>Zip14</td>
<td>Slc39a14 solute carrier family 39 (zinc transporter), member 14</td>
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**Supplemental Table 2.** Antibody cocktail to analyze bone marrow hematopoiesis.

<table>
<thead>
<tr>
<th>Antibody/Supplier</th>
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<tr>
<td>c-kit (A780, 2B8; eBioscience)</td>
</tr>
<tr>
<td>Sca1 (PE-Cy5 or PE-Cy7 or PE-Cy5.5, D7; eBioscience)</td>
</tr>
<tr>
<td>CD34 (FITC, RAM34; eBioscience)</td>
</tr>
<tr>
<td>CD135 (PE or PE-Cy5, A2F10; eBioscience)</td>
</tr>
<tr>
<td>CD48 (APC, HM48-1; eBioscience)</td>
</tr>
<tr>
<td>CD150 (PE-Cy7, TC15-12F12.2; BioLegend)</td>
</tr>
<tr>
<td>CD105 (PE, 12-1051-82, ThermoFisher)</td>
</tr>
<tr>
<td>CD41 (FITC, 11-0411-82; ThermoFisher)</td>
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Supplemental Table 3. Antibody cocktail to block lineage-positive cells

<table>
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<tr>
<th>Antibody/Supplier</th>
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</thead>
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<tr>
<td>CD3 (145-2C11; eBioscience)</td>
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<tr>
<td>CD19 (eBio1D3; eBioscience)</td>
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<tr>
<td>NK1.1 (PK136; eBioscience)</td>
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<tr>
<td>Ter119 (Terr119; eBioscience)</td>
</tr>
<tr>
<td>CD11b (M1/70; eBioscience)</td>
</tr>
<tr>
<td>Gr1 (RB6-8C5; eBioscience)</td>
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<tr>
<td>B220 (RA3-6B2; eBioscience)</td>
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**Supplemental Table 4.** Antibody cocktail to analyze erythroid progenitors

<table>
<thead>
<tr>
<th>Antibody/Supplier</th>
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<tbody>
<tr>
<td>FITC anti- mouse TER-119 antibody (#16205, Biolegend)</td>
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<tr>
<td>APC-Cy™7 rat anti-mouse CD45 (#561037, BD Pharmingen™)</td>
</tr>
<tr>
<td>APC/Cyanine7 anti-mouse Ly-6G/Ly-6C (Gr-1)</td>
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<tr>
<td>APC/Cyanine7 anti-mouse Ly-6G/Ly-6C (Gr-1) antibody (#108423, Biolegend)</td>
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<tr>
<td>APC rat anti-mouse CD44 Clone IM7 (RUO) (#561862, BD Pharmingen™)</td>
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Supplemental Table 5. Clinical markers in plasma of tumor free and terminal stage tumor bearing (maximal permitted tumor size) Trp53<sup>flox</sup>WpCre mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tumor free</th>
<th>Terminal stage</th>
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<tbody>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>&lt; 0.18 ± 0.058</td>
<td>&lt; 0.24 ± 0.1261</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>186.20 ± 28.59</td>
<td>163.40 ± 30.84 *</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>36.18 ± 2.906</td>
<td>31.62 ± 4.155</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>&lt; 0.17 ± 0.0</td>
<td>&lt; 0.17 ± 0.0</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>40.43 ± 1.718</td>
<td>50.00 ± 7.371 **</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>106.30 ± 8.025</td>
<td>116.60 ± 42.14</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>191.90 ± 49.27</td>
<td>202.20 ± 182.7</td>
</tr>
<tr>
<td>Alkaline phosphatase (AP) (IU/l)</td>
<td>89.00 ± 7.321</td>
<td>30.50 ± 11.61 ***</td>
</tr>
<tr>
<td>Aspartate transaminase (AST) (IU/l)</td>
<td>496.70 ± 473.4</td>
<td>173.40 ± 97.57</td>
</tr>
<tr>
<td>Alanine transaminase (ALT) IU/l</td>
<td>39.57 ± 18.86</td>
<td>21.57 ± 6.705 *</td>
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</tbody>
</table>

Values presented as mean ± standard deviation; terminal stage is termination of experiment when maximal permitted tumor size was reached. A Mann Whitney test was performed for Bilirubin, Cholesterol and Triglycerides and all other parameters were analyzed with a student’s t test (n=7). *p<0.05; **p<0.01; ****p<0.0001.
Supplemental Figure 1. Flow cytometry analysis of early and late erythroid maturation. (A) Shown are representative images of gated plots and the gating strategy used for flow cytometry analysis of early hematopoietic precursors in bone marrow. (1) Lineage negative (Lin−) cells were selected for subsequent gating of (2) C-kit+/SCA-1- cells followed by gating of (3) CD48+/CD150+ hematopoietic stem cells (HSC) as well CD48+/CD150- multipotent progenitors (MPP). From (2) C-kit+/SCA-1- events, (4) CD150+/CD41+ cells were considered megakaryocyte precursors (Mkp). CD41- events were selected for a (5) CD16_32/CD105 plot.
and CD16\_32\^- cells were further selected for (6) a CD105/CD150 plot to identify CD105^-/CD150^- pre-granulocyte-monocyte lineage cells (Pre-GM), CD105^-/CD150^- pre-megakaryocyte-erythrocyte progenitors (pre-MgE), CD105^-/CD150^- pre-colony forming units erythrocyte (pre-CFUe) and CD105^+/CD150^- colony forming units erythrocyte (CFUe). (B) Shown are representative images of gated plots and the gating strategy for analyzing erythroid maturation (late erythroid progenitors) and leukocyte precursors in bone marrow and spleen by flow cytometry. Cells were gated in a forward scatter (FSC-Area, cell size) vs. side scatter (SSC-Area, cell complexity) (upper left panel) and single cells were subsequently gated in FSC-Area vs. FSC-Height plot (upper middle panel). Living cells were selected by a Zombie Yellow (dead cell marker) vs. SSC-Area plot (upper right panel) and Ter119^+ late erythroid progenitors were gated on living cells in a Ter119 vs. SSC-Area plot (lower left panel). Different clusters were identified in FSC-Area vs. CD44 plot and based on cell size and CD44 expression (both decrease during maturation) different stages of late erythroid maturation were gated: (I) proerythroblasts, (II) basophilic erythroblasts, (III) polychromatophilic erythroblasts, (IV) orthochromatophilic erythroblasts (including reticulocytes) and (VI) mature erythrocytes (lower middle panel). Additionally, shown is the gating of CD45/GR1+ leukocyte precursors in a CD45/GR1 vs. SSC-Area plot (lower right panel).
Supplemental Figure 2. Hematological analyses of anemic Trp53$^{flox}$WapCre mice. Blood of tumor bearing Trp53$^{flox}$WapCre mice (grey boxes) was isolated immediately after tumor onset (0d), 15 days after tumor onset (15d) and when the tumor reached maximal permitted size (defined as terminal stage; TS). Age-matched tumor free (TF) Trp53$^{flox}$WapCre mice (white boxes) served as control. Except hematocrit and hemoglobin, all parameters were analyzed by the Sysmex XT-2000iV. (A) Shown are hematocrit (n=11-37) and hemoglobin (n=3-39), analyzed by microcentrifugation and by ABL800, respectively. Further shown are erythrocyte count per µl blood as well as the proportion of low (LFR), middle (MFR) and high fluorescent reticulocytes (HFR) (n=4). The combined counts of HFR and MFR correspond to premature and LFR corresponds to mature reticulocytes (right panel). (B) Shown are mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean
corpuscular volume (MCV), and red cell distribution width (RDW) (n=4-6). (C) Shown are the number of leukocytes per µl blood as well as the cell count per µl blood of lymphocytes, monocytes, neutrophils, eosinophils, and basophiles (n=4-6). Data are shown as box plot with min to max whiskers and data were analyzed by a one-way ANOVA with a Bonferroni’s multiple comparison (black symbols) or Kruskal-Wallis test with a Dunn’s multiple comparisons (red symbols). Reticulocytes in panel (A) were analyzed by a Mann-Whitney test (red symbols) (*p<0.05, **p<0.01, ***p<0.001).

Supplemental Figure 3. Hepato- and splenomegaly in anemic Trp53<sup>flox</sup>WapCre mice. Liver of tumor bearing Trp53<sup>flox</sup>WapCre mice (grey boxes) was isolated, and immunohistochemically analyzed. Shown is a representative image of an immunohistochemical staining of Ki67 in the liver (left image) as well as a representative image of the morphometric quantification of Ki67 positive (green) and Ki68 negative (blue) liver cells by Visiopharm (right image). Further shown is the percentage of Ki67 positive liver cells in TF and TS mice (n=4). Data are shown as box plot with min to max whiskers. A Mann-Whitney test was performed. *p<0.05
Supplemental Figure 4. Iron homeostasis in Trp53\textsuperscript{flox}WapCre mice. Tissues of tumor bearing Trp53\textsuperscript{flox}WapCre mice (grey boxes) were isolated immediately after tumor onset (0d), 7 days after tumor onset (7d) and when the tumor reached maximal permitted size (defined as terminal stage; TS). Age-matched tumor free (TF) Trp53\textsuperscript{flox}WapCre mice (white boxes) served as control. (A) Shown are plasma iron, unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), and transferrin saturation (n=9-22) analyzed by a bathophenanthroline assay as well as plasma ferritin analyzed by ELISA (n=6) and relative liver gene expression levels of ferritin heavy chain 1 (\textit{Fth1}) quantified by qPCR and normalized.
to β-actin (Actb) (n=5-11). (B) Shown is the tissue iron concentration per dry tissue weight determined by bathophenanthroline assay in healthy mammary tissue of tumor free mice (TF Mamma) and in tumor tissue of terminal stage (TS) mice (n=3-24) as well as in spleen and liver of TF and TS mice (n=8-9). (C) Shown are representative images of bone marrow, spleen, liver and kidney of TS and TF mice as well as tumor tissue of terminal stage TS) Trp53^flx/WapCre mice. Tissue sections were stained with DAB-enhanced Perls’ to visualize iron (dark brown color). (D) Shown are the absolute hepcidin (Hamp1) mRNA copy number per ng RNA as well as Hamp1 mRNA copy number normalized to β-actin (Actb) copy number determined by digital droplet PCR. Further shown are the relative liver gene expression levels of (Hamp1), bone morphogenetic protein 6 (Bmp6), transferrin (Trf), homeostatic iron regulator (Hfe), as well as transferrin receptor 1 (Tfrc) and 2 (Tfr2), divalent metal transporter 1 (Dmt1), solute carrier family 39 member 14/metal cation symporter ZIP14 (Zip14), bone morphogenetic protein 2 (Bmp2), hemojuvelin (Hjv), transmembrane serine protease 6 (Tmprss6), suppressor of cytokine signaling 3 (Socs3), and interleukin-6 receptor (Il-6r) quantified by qPCR and normalized to Actb (n=5-11). (E) Shown are representative liver sections (upper images) and spleen sections (lower images) of immunohistological analyzed ferroportin (FPN1) protein expression in tumor free (left panel) and terminal stage mice (right panel) (n=3) as well as relative liver (left graph) and spleen (right graph) gene expression levels of ferroportin (Fpn1) quantified by qPCR and normalized to Actb (n=6-9). Data are shown as box plot with min to max whiskers. Data were analyzed by a Student’s t-test (black symbols, p-values) or a Mann-Whitney test (red symbols) for single comparison or by a one-way ANOVA with a Bonferroni’s multiple comparison test (black symbols) or a Kruskal-Wallis test with a Dunn’s multiple comparison test (red symbols) (***p<0.001; **p<0.01; *p<0.05). Scale bar 100 µm.
Supplemental Figure 5. Expression levels of hypoxia inducible transcription factors downstream targets and platelet derived growth factor BB in anemic Trp53<sup>flox</sup>WapCre mice. Liver, spleen, and kidney of tumor bearing Trp53<sup>flox</sup>WapCre mice (grey boxes) were isolated from mice when the tumor reached maximal permitted size (defined as terminal stage; TS) and from tumor free (TF, white boxes) age-matched controls. Shown are relative mRNA expression levels of hypoxia target genes Egl-9 family hypoxia inducible factor 1 (<i>Egln1</i>) and adrenomedullin (<i>Adm</i>) as well as platelet derived growth factor subunit b (<i>Pdgfb</i>) and PDGF-BB downstream target genes Camp-responsive element binding protein (<i>Creb1</i>) and Camp-responsive element binding protein 3 like 1 (<i>Creb3ll</i>) in liver (upper left panel), spleen (upper right panel), and kidney (lower left panel) of tumor free (TF, white boxes) and terminal stage (TS, grey boxes) Trp53<sup>flox</sup>WapCre mice. Data are shown as box plot with min to max whiskers and were analyzed by a Student's t-test (**p<0.001; *p<0.01; *p<0.05).
Supplemental Figure 6. Effect of low iron diet on anemia in Trp53^{fox}WapCre mice. In panel (A-D), Trp53^{fox}WapCre mice were kept on a standard diet with high iron content (iron 250 mg/kg). When the mice were 15 weeks of age, they either continued on the high iron diet (dark blue boxes) or they were placed on a low iron diet (50 mg/kg) (light blue boxes) until the end of the experiment. Tissues and blood of terminal stage mice (TS) were isolated when the tumor reached maximal permitted size. Age-matched tumor free Trp53^{fox}WapCre mice (TF) served as control. (A) Shown are modified Kaplan-Meier curves that show percentage of Trp53^{fox}WapCre mice on a low (light blue) or high (dark blue) iron diet, which developed tumors (incidence) (n=11-60) and, which reached the terminal stage (n=7-32). Data were
Iron- and erythropoietin-resistant mice with AoC

Supplemental Information

analyzed with a Log-rank (Mantel-Cox) test. Further shown is hematocrit and hemoglobin analyzed by microcentrifugation and by ABL800, respectively (n=6-37). (B) Shown is liver gene expression of Hepcidin 1 (Hamp1) quantified by qPCR and normalized to β-actin (Actb) mRNA expression (n=4-10). (C) Shown are plasma iron levels, unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), and transferrin saturation quantified by a bathophenanthroline assay (n=4-22). Further shown is the tissue iron concentration quantified by a bathophenanthroline assay in liver and spleen with the corresponding total iron amount in liver and spleen (n=5-9) as well as the iron concentration quantified by bathophenanthroline in tumor tissue (n=6-24). (D) Shown are representative images of liver, spleen bone marrow, kidney and tumor tissue sections stained with DAB-enhanced Perl’s of tumor free (upper panel) and terminal stage (lower panel) Trp53^{flox}WapCre mice kept on a low iron diet.

In (E), Trp53^{flox}WapCre mice were kept either on a high (dark blue) or on a low iron diet (50 mg/kg) (light blue) directly after weaning until the end of the experiment. Blood of tumor mice was isolated when the tumor reached maximal permitted size (defined as terminal stage; TS). Age-matched tumor free Trp53^{flox}WapCre mice (TF) served as control. Shown are modified Kaplan-Meier curves that show percentage of Trp53^{flox}WapCre mice on a low or high iron diet, which developed tumors (incidence) (n=12-60) and, which reached the terminal stage (n=12-32). Data were analyzed with a Log-rank (Mantel-Cox) test. Further shown are hematocrit and hemoglobin analyzed by microcentrifugation and by ABL800, respectively (n=9-38). Plasma interleukin-6 (IL-6) levels of Trp53^{flox}WapCre mice on a low iron diet were analyzed by ELISA, in TF mice, immediately (0 d) and 15 days (15 d) after tumor onset as well as in terminal stage (TS mice) (n=5-7). Additionally, we compared plasma IL-6 levels in terminal stage mice on a high (dark blue) and low (light blue) iron diet (n=4-6). Data are shown as box plot with min to max whiskers and were analyzed by a Student’s t-test or a Mann-Whitney test (no significant changes) for single comparison, by a Kruskal-Wallis test with a Dunn’s multiple comparison test (red symbols) or by a two-way ANOVA with a Bonferroni’s multiple comparison test (black symbols) (**p<0.001; *p<0.01; *p<0.05). Scale bar 100 µm.
Supplemental Figure 7. Iron treatment of anemic Trp53floxWapCre breast cancer mice. Trp53floxWapCre mice were intravenously injected with a single dose of iron (dark blue boxes) or saline (light blue boxes) immediately after tumor onset. In experiment 1, mice received 20 mg/kg Ferinject® and blood as well tissues were isolated 15 days after treatment (15 DAT). In experiment 2, mice received 13.28 mg/kg Ferinject® or saline and blood as well tissues were isolated when the tumor reached maximal permitted size (defined as terminal stage; TS). (A) Shown are hemoglobin levels in saline and iron treated mice (n=8-10). (B) Shown is plasma iron determined by a bathophenanthroline assay (n=5-10) (upper left panel) as well as tissue iron concentration per dry tissue weight determined by bathophenanthroline assay in spleen (upper middle panel), liver (upper right panel), and tumor (n=8-10) (lower left panel). Further shown are the corresponding total tissue iron content in spleen (lower middle panel) and liver (lower right panel) (n=8-10). Data are shown as box plot with min to max whiskers and were analyzed by a Student’s t-test (black symbols) or a Mann-Whitney test (red symbols, p-values) (**p<0.01; *p<0.05). Panels with black and red dotted lines indicate the average values of untreated tumor free (black dotted line) and terminal stage (red dotted line) mice. ## and # indicates differences (p<0.01 or 0.05) between saline treated TS and untreated tumor free (black dotted line) mice and iron treated TS mice and untreated TS mice (red line), respectively.
Supplemental Figure 8. Erythropoiesis in anemic Trp53\textsuperscript{flox}WapCre mice. Spleen and bone marrow cells of tumor bearing Trp53\textsuperscript{flox}WapCre mice (grey boxes) were isolated when the tumor reached maximal permitted size (defined as terminal stage; TS). Age-matched tumor free (TF) Trp53\textsuperscript{flox}WapCre mice (white boxes) served as control. (A) Shown are representative images gated plots to assess late erythroid maturation in bone marrow (BM) and spleen of tumor free and terminal stage Trp53\textsuperscript{flox}WapCre mice. After gating Terr119\textsuperscript{+} cells (middle images) clusters of differently maturated erythroid cells were identified in an FSC-Area vs. CD44 plot: (I) proerythroblasts, (II) basophilic erythroblasts, (III) polychromatic erythroblasts, (IV) orthochromatic erythroblasts (including reticulocytes) and (V) mature erythrocytes. (B) Shown is the proportion of Terr119\textsuperscript{+} in bone marrow (BM) and spleen as well as the proportion of I proerythroblasts, II basophilic erythroblasts, III polychromatic erythroblasts, IV orthochromatic erythroblasts (including reticulocytes) and V mature red blood cells in bone
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marrow (BM) and spleen analyzed by flow cytometry (n=4). (C) Shown are relative expression levels of erythropoietin receptor (Epor), glycophorin A (Gypa), hemoglobin subunit alpha 1 (Hba), hemoglobin subunit beta (Hbb), transferrin receptor 1 (Tfrc), transferrin receptor Tfr2, and the divalent metal transporter (Dmt1) in bone marrow (BM) and spleen quantified by qPCR and normalized to β-actin Actb (n=4-6). Data are shown as box plot with min to max whiskers and were analyzed by a Student's t-test (black symbols, p-values) or a Mann-Whitney test (red symbols, p-values) (*p<0.05, **p<0.05).

Supplemental Figure 9. Myelopoiesis in anemic Trp53\textsuperscript{lox}\textsuperscript{WapCre} mice. Spleen and bone marrow cells of tumor bearing Trp53\textsuperscript{lox}\textsuperscript{WapCre} mice (grey boxes) were isolated when the tumor reached maximal permitted size (defined as terminal stage; TS). Age-matched tumor free (TF) Trp53\textsuperscript{lox}\textsuperscript{WapCre} mice (white boxes) served as control. (A) Shown are representative bone marrow sections of tumor free (upper left image) and terminal stage (upper right image) stained with hematoxylin-eosin. The lower image shows a representative image of the morphometric quantification of bone marrow cells (green). Further shown are the cellularity in bone marrow tissue sections analyzed by Visiopharm (n=6) as well as cellularity in bone
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marrow (BM) and spleen analyzed by flow cytometry (n=5-8). (B) Shown is the proportion of immature and mature erythroid precursors, immature and mature myeloid precursors, lymphocytes, plasma cells, macrophages, eosinophils, and basophils in Giemsa-stained bone marrow smears (n=4). (C) Shown are relative expression levels of autophagy genes in bone marrow of tumor free (white boxes) and terminal stage Trp53\textsuperscript{flax}WapCre mice (grey boxes) analyzed by qPCR and normalized to β-actin (Actb) (n=4-6). Data are shown as box plot with min to max whiskers and were analyzed by a Student’s t-test (black symbols, p-values) or a Mann-Whitney test (red symbols, p-values) (*p<0.05, **p<0.05). Scale bar 250 µm
References


