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MYELOID NEOPLASIA

# HIF-1α can act as a tumor suppressor gene in murine Acute Myeloid Leukemia

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**Running title:** Tumor suppressor role of HIF-1α in AML

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**Key points**

- Disease initiation and maintenance in murine AML models occurs via HIF-1α independent mechanisms.
- HIF-1α deficiency in mice accelerates leukemogenesis induced by certain oncogenes.

**Abstract**

Self-renewal of hematopoietic stem cells (HSCs) and leukemia-initiating cells (LICs) has been proposed to be influenced by low oxygen tension (hypoxia). This signaling, related to the cellular localization inside the bone marrow niche and/or influenced by extrinsic factors, promotes the stabilization of hypoxia inducible factors (HIFs). Whether HIF-1α can be used as a therapeutic target in the treatment of myeloid malignancies remains unknown. We have used three different murine models to investigate the role of HIF-1α in acute myeloid leukemia (AML) **initiation/progression** and self-renewal of LICs. Unexpectedly, we failed to observe a delay or prevention of disease development from hematopoietic cells lacking *Hif-1α*. In contrast, deletion of *Hif-1α* resulted in faster development of the disease and an enhanced leukemia phenotype in some of the investigated models. Our results therefore warrant a reconsideration of the role of HIF-1α and, as a consequence, question its generic therapeutic usefulness in AML.

**Introduction**

Contrary to chronic myeloid leukemia (CML), acute myeloid leukemia (AML) presents no common genetic "Achilles heel" that can be targeted, due to the high level of genetic heterogeneity of this disease. Whole exome/genome sequencing (WES/WGS) has recently identified over 40 different driver mutations in AML, of which 5-7 normally occur in any given patient with AML[1]. These mutations can be grouped by function (kinases, transcription factors, epigenetic modifiers, etc) into different classes, of which many cooperate in leukemogenesis. The genetic heterogeneity of AML unfortunately poses a great challenge for the development of molecular targeted therapies. Even common genetic alterations that are supposedly easy drug-targets, like the *FLT3*-ITD mutation, have proven problematic with regards to drug development. Loss-of function mutations of transcription factors or epigenetic modifiers will also be extremely difficult to use as drug targets since the function of these proteins has to be restored or lethality-based therapies be developed. Therefore, more "lumping" instead of "splitting" based on the biology of AML would facilitate the identification of common pathways that the AML-initiating cells (AML-ICs) rely on, and that can be used for therapeutic targeting. One of these shared factors that could affect all genetic subtypes of AML could be the extrinsic signaling that the cells receive from their microenvironment.

It has been proposed that hematopoietic stem cells (HSCs) as well as leukemia initiating cells (LICs) reside in a hypoxic bone marrow niche[2]. This hypoxic microenvironment is thought to contribute to quiescence and self-renewal and, in the case of leukemia, also to chemotherapy resistance[3]. The major molecular response to hypoxia is the stabilization of Hypoxia Inducible Factors (HIFs), a family of transcription factors that activates over hundred target genes involved in the adaption to hypoxia by regulating numerous

processes such as angiogenesis, metabolism and proliferation[4,5]. HIF proteins are composed of two different subunits: an oxygen-labile α subunit and a constitutively expressed β subunit. There are three different α subunits, of which HIF-1α and HIF-2α mainly differ in their expression pattern with regards to kinetics and tissue specificity. Their target genes are widely overlapping with few of them selectively expressed by only one member of the HIF family[6]. HIF-3α, the third member of the family, has by contrast been proposed to be a negative regulator of other HIF complexes[7].

Deletion of *Hif-1α* in HSCs leads to a loss of self-renewal when HSCs are challenged by serial transplantation, while there seems to be no effect of *Hif-1α* under steady state conditions[8]. This weak HSC phenotype of the *Hif-1α* conditional knockout mice might be explained by functional redundancy of other HIF family members, likely HIF-2α. However, analysis of the *Hif-1/2α* double knockout mice did not reveal a more severe defect in HSCs function than observed in the *Hif-1α* single knockout mice, arguing against a compensation of *Hif-2α* for the loss of *Hif-1α* function[9].

Recent work indicates that HIFs might also be activated, apart from hypoxia, by other signals in the bone marrow (BM)[10]; for instance growth factors such as TPO and SCF, but also by the transcription factor MEIS1. High levels of TPO and SCF are supposedly present in the BM HSC niche and result in HIF-1α stabilization in the absence of hypoxia[11,12]. MEIS1 overexpression leads to activation of the HIF-1α pathway while the deletion of *Meis1* results in HSCs depletion[13,14]. Interestingly, *Meis1* is upregulated in approximately 50% of AMLs[15-17]. While only a small fraction (10%) of these appears to be caused by the expression of MLL (mixed-lineage leukemia) fusion proteins, of which

MEIS1 is a direct target[18], the mechanism of MEIS1 activation in the remaining cases of AML remains unclear.

The role of HIF-1α in leukemia development has been studied in different murine and human models, which suggests that HIF-1α has an important role in proliferation and self-renewal of leukemic cells. HIF-1α is required for LICs self-renewal in a mouse model of chronic myeloid leukemia (CML)[19]. Similar observations have been made for the requirement of HIF-1/2α in human AML using Echinomycin inhibition[20] or shRNA down-regulation[21] of the HIF complexes. However, whether HIF-1/2α is required for all genetic subgroups of AML and could be used as a molecular target without affecting normal HSCs remains still elusive.

In this study, we tested the requirement of HIF-1α in the initiation and maintenance of AML using three different experimentally well-defined AML models: two leukemic oncogenes that are known to signal directly towards HIF-1α (*Meis*1 and *MLL*)[22] and one with no known relation to HIF-1α (*AML1-ETO9a*)[23]. Using a genetic approach where oncogenes were retrovirally expressed in HSPC from *Hif-1*α-conditional knockout mice followed by gene deletion after engraftment we could not observe differences in latency and disease phenotype dependent on the HIF-1α status. Additionally, we demonstrate that self-renewal of LICs persists even after *Hif-1*α deletion. Collectively, these data strongly argue against HIF-1α as a required signaling pathway in LICs and a general therapeutic target for AML.

## Materials and methods

### Transgenic mice

*Hif-1α*$^{fl/fl}$ mice[24] were crossed with the interferon inducible *Mx1-Cre* mice[25] to generate conditional knockout *Hif-1α*$^{fl/fl}$; *Mx1-Cre* mice. A detailed description of inducible transgenic MLL-ENL mice will be described elsewhere (Ugale et al, manuscript submitted). Briefly, a human MLL-ENL fusion gene was targeted into KH2 ES cells[26], injected into blastocysts, and a germline colony was established from primary chimeric animals. KH2 ES cells carry the reverse Tetracycline transactivator (rtTA) driven constitutively from the Rosa26 promoter, making MLL-ENL expression strictly Tetracycline-inducible. Cells used carried two copies of rtTA and two copies of MLL-ENL. All animals were bred and maintained in accordance with Lund University's ethical regulations.

### Retroviral vectors and virus production

The following retroviral vectors were used for retrovirus production: MIGR1, AML1-ETO9a (AE9a) (MIGR1-AML1-ETO9a-GFP, Addgene), HoxA9-Meis1 (MSCV-HoxA9-Meis1) and MLL-AF9 (MIGR1-MLL-AF9-GFP). The HRE-GFP lentiviral hypoxia reporter was constructed from the HRE-GFP vector[27] as described in supplemental methods. Retroviral supernatants were obtained by transient transfection of amphotropic Phoenix cells (Nolan laboratory, Stanford University) and supernatants were harvested after 48h. Lentiviral supernatants were produced as previously described[28].

**Retroviral transduction**

BM cells from femurs, tibiae and hip bones from 8-12 weeks old mice were harvested for retroviral transduction/transplantation experiments. c-kit$^+$ cells were isolated using a magnetic separation system (MACS®) and anti-c-kit magnetic beads (Miltenyi Biotec). Cells were cultured in SFEM media (Stem Cell Technologies) supplemented with 20 ng/mL mIL3, 50 ng/mL hIL6, 50 ng/mL hTPO and 50 ng/mL mSCF for 24h. Two rounds of transduction were performed the following two days with MOIs ranging between 0.5-3, depending on the vector. Cells were transplanted on the fourth day after harvesting.

**Transplantations and monitoring of mice**

8-12 weeks old B6SJL (CD45.1) recipient mice were lethally irradiated with 900 cGy 4-15 hours previous to transplantation. 4 x $10^5$ cells were intravenously injected into the tail vein of recipient mice accompanied by 2 x $10^5$ freshly isolated total BM supporting cells from B6SJLxC57BL/6J (CD45.1-CD45.2) mice. Donor chimerism and leukemia development were analyzed by peripheral blood (PB) analysis every 4 weeks. Deletion of *Hif-1α* was induced by intraperitoneal injection of 400 μg of pIpC (Sigma) on 3 alternate days 4 weeks after transplantation. *Hif-1α* deletion was verified by PCR analysis of PB-derived colonies 4 weeks later. Total white blood cells (WBC) counts were determined by a cell counter (KX-21N, Sysmex). For secondary transplantations, equal number (4 x $10^5$) leukemia-derived BM cells were injected into lethally irradiated recipients in combination with 2 x $10^5$ supporting cells (CD45.1-CD45.2).

**FACS analysis**

Engraftment and expansion of transduced cells were monitored by flow cytometry analysis of PB, BM and spleen cells. PB samples were lysed with ammonium chloride (Stem Cell Technologies) prior to staining. DAPI (Sigma) was used to exclude dead cells. For chimerism and lineage analysis the following antibodies were used: Gr1 (RB6-8C5), Mac1 (M1/70), B220 (RA3-6B2), CD3 (145-2C11), CD45.1 (A20) (BioLegend) and CD45.2 (104) (eBiosciences).

**Colony-forming unit (CFU) assay**

Colony-forming units were assayed plating 10,000 BM cells/plate in methylcellulose supplemented with cytokines (Methocult GM-3434; Stem Cell Technologies) in triplicates. Colonies were counted and/or picked after 7-10 days of culture. Inducible MLL-ENL derived colonies were grown in presence of 1 μg/mL of Doxycycline (Sigma).

**Statistical analysis**

All data are expressed as the mean ± SEM. Differences between groups were assessed by unpaired Student's t-test. Differences at different time points were assessed using Two-way ANOVA followed by Sidak's/Bonferroni multiple comparison test. Statistical analysis of survival curves was performed using Mantel-Cox log-rank test. All analyses were performed with Prism software version 6.0 (GraphPad Software).

## Results

### HIF-1α is not essential for initiation/progression of AE9a-induced leukemia

Few leukemic fusion proteins can induce AML as a single genetic element in mice. AE9a, which is a truncated version of the t(8;21) product AML1-ETO, has been shown to induce AML in a murine transduction/transplantation model[23]. To test whether AE9a-induced-AML **initiation/progression** depends on HIF-1α signaling, hematopoietic stem and progenitor cells (HSPCs) (c-kit$^+$ cells) from *Hif-1α*$^{fl/fl}$; *Mx1-Cre* or *Hif-1α*$^{fl/fl}$ mice were transduced with a retroviral vector expressing AE9a and transplanted into recipient mice (referred here on to as *Hif-1α*$^{\Delta/\Delta}$ and control mice respectively). We induced the deletion of *Hif-1α* four weeks after transplantation and verified deletion efficiency by genotyping single colonies derived from BM cells (supplemental Figure S1). **We observed that the *Mx1-Cre* model (widely used in studies investigating gene function in HSCs and LICs) is prone to "spontaneous" deletion of the floxed gene, in this case *Hif-1α* when the retroviral transduction/transplantation model is used. In our hands, the "spontaneous" deletion frequency was around 30-40% depending on the experiment (data not shown), which is far higher than the described deletion rate in steady state conditions that is around 2-3%[29,30]. This indicates that we were working with cells with different *Hif-1α* genotypes even before the pIpC injection at week 4.** Blood analysis was performed periodically to study chimerism and contribution of the transduced cells in transplanted mice until they got moribund (mean time of 35 weeks after transplantation), after which they were sacrificed for analysis. Latency of the disease was similar in *Hif-1α*$^{\Delta/\Delta}$ and control animals ($P$=0.3941) (Figure 1A). The percentage of GFP$^+$ cells in PB was higher in *Hif-1α*$^{\Delta/\Delta}$ mice 20 weeks post-transplantation (mean: 14.5% versus 5.5% in controls)

(Figure 1B), which was accompanied by a slight decrease in myeloid cells (Figure 1C) and in agreement with the fact that AE9a-derived leukemia is lineage-negative ($Lin^-$), as previously described[23].

PB, BM and spleen cells of diseased animals were analyzed by flow cytometry for lineage markers and GFP expression. We failed to observe statistically significant differences between the two genetic groups, although there was a trend towards lower numbers of $Gr1^+/Mac1^+$ cells and a higher fraction of $Lin^-$ cells ($Gr1^-$, $Mac1^-$, $B220^-$, $CD3^-$ and also $CD45^-$) in PB from the *Hif-1*$\alpha^{\Delta/\Delta}$ group (Figure 1D-F and data not shown). The percentage and morphology of $Lin^-$ cells in BM did not differ between the two genotypes (Figure 1G-H). To test whether the number of LICs in BM was affected by the absence of *Hif-1*α, we performed CFU-assays. No difference between the two groups was observed (Figure 1I).

We next decided to investigate the ability of cells to induce AE9a driven AML when cells, either expressing *Hif-1*α or not, directly competed against each other. To achieve this, cells with different *Hif-1*α status were mixed in a 1:1 ratio, transduced with AE9a and transplanted into recipients. We assumed that if *Hif-1*α would play a prominent negative or positive role in AML initiation or maintenance, LICs of one genetic background should be dominant in developing AMLs. CFU-assays were performed with BM cells from moribund mice and PCR from single colonies was performed to detect the presence of *Hif-1*α or GFP. Colonies with both genotypes (*Hif-1*$\alpha^{\Delta/\Delta}$ and *Hif-1*$\alpha^{+/+}$) were found in the same proportion, indicating that none of the genotypes has an advantage in direct competition in the same environment (Figure 1J). These data support our previous finding that *Hif-1*α status does not influence AE9a-induced AML **initiation/progression**. Taken together, these data strongly suggest that the status of

*Hif-1α* in LICs in AE9a-induced leukemia is not affecting the **initiation/progression** of the disease in this murine AML model.

**Loss of *Hif-1α* does not affect self-renewal of AE9a-expressing LICs but can increase proliferation**

The requirement of specific genes for the self-renewal of LICs in murine leukemia models has mainly been shown by secondary transplantation[19,31]. Therefore, to test whether *Hif-1α* has a role in self-renewal capacity of LICs, we performed secondary transplantations with BM cells from AE9a driven leukemia. We transplanted equal numbers of BM GFP$^+$ cells from both groups. Ten weeks after transplantation, *Hif-1α*$^{\Delta/\Delta}$-transplanted animals displayed overt signs of disease, while control animals appeared healthy. Survival assessments indicated a significant shorter latency of *Hif-1α*$^{\Delta/\Delta}$ mice compared to controls (mean survival of 10 weeks compared to 18 weeks), an observation in accordance with the percentage of GFP$^+$ cells present in PB and the increment of the malignant Lin$^-$ population (Figure 2A-E). When animals became moribund (mean time of 10 weeks after transplantation), they were sacrificed and analyzed. We observed a statistically significant increase in spleen and liver size in *Hif-1α*$^{\Delta/\Delta}$ mice, indicating a more advanced stage of the disease (Figure 2F). This was accompanied by a higher percentage of GFP$^+$ ($P$=0.0031) and Lin$^-$ cells ($P$=0.0044) in spleen and Lin$^-$ cells ($P$=0.0047) in BM (Figure 2G-H). Taken together, these data suggests that loss of HIF-1α can accelerate the progression of the disease rather than negatively impacting on the LICs self-renewal in the AE9a-induced leukemia model.

**HIF-1α status does not influence the initiation/progression of MEIS1-induced leukemia**

Approximately half of all AML cases show an activation of *Meis1* expression[15-17], a transcription factor demonstrated to signal directly to *Hif-1α*[14]. Therefore, we wanted to investigate whether characteristics of MEIS1-induced AML are influenced by the status of *Hif-1α*.

Similar to the AE9a model, we transduced BM c-kit$^+$ cells from *Hif-1α*$^{fl/fl}$; *Mx1-Cre* or *Hif-1α*$^{fl/fl}$ mice with *HoxA9-Meis1* expressing retrovirus. Forced expression of *HoxA9* and *Meis1* is sufficient to transform murine HSPCs[32]. To study disease latency and progression of myeloid cells over time, transplanted mice were bled sequentially. No difference in survival was observed between the experimental groups ($P$=0.7281) (Figure 3A). Disease burden was higher 8 weeks after transplantation in *Hif-1α*$^{\Delta/\Delta}$ mice but reaching similar levels, according to percentage of myeloid cells in PB, at week 12 (Figure 3B). Next, moribund animals were sacrificed (mean time of 12 weeks after transplantation) and PB, BM and spleen cells were analyzed. *Hif-1α* deletion did not affect the development of the disease in this model as indicated by equal numbers of total WBC and myeloid cells in PB, BM and spleen from mice from both cohorts (Figure 3C-E). No gross difference in spleen or liver sizes between groups was observed (Figure 3F). According to CFU assays, there was no difference in the number of LICs present in BM of these mice (Figure 3G).

To test whether *Hif-1α* might be involved in LICs self-renewal and progression of the disease, we performed secondary transplantations. These experiments failed to reveal

differences in overall survival between *Hif-1α*$^{\Delta/\Delta}$ and control mice ($P$=0.0616) (Figure 4A). The phenotype of the disease with different HIF-1α status was similar with regards to myeloid cells in the peripheral blood and spleen, while a few differences were observed in total WBC counts and myeloid cells in the BM (Figure 4B-E). Taken together, these results indicate that the absence of HIF-1α does not substantially impair *HoxA9-Meis1*-induced leukemia development, even though this oncogene has been suggested to signal directly to the HIF-1α pathway.

**MLL-fusions can stabilize HIF proteins but HIF-1α status does not influence AML phenotype**

Translocations involving *MLL* give rise to the most potent leukemic oncogenes described, and therefore have often been used to model human and murine AML. *MLL* fusion genes do not only activate *MEIS1* and *HOX* cluster genes, but also require expression of these genes for maintenance of leukemia[22]. The fact that MEIS1 directly signals to *Hif-1α* lead us to hypothesize that *Hif-1α* might be directly stabilized by MLL fusion proteins.

To investigate whether *MLL* fusion genes activate *Hif-1α*, we took BM cells from mice with Doxycycline-inducible expression of *MLL-ENL* (Ugale et al, manuscript submitted) and transduced them with a lentiviral hypoxia reporter[27]. Stabilization of both HIF-1/2α results in binding to the HRE sites and activation of *EGFP* transcription. The induction of *MLL-ENL* by doxycycline lead to an increase in EGFP median fluorescence intensity (MFI) in normoxic (20% oxygen) and hypoxic (1% oxygen) conditions (Figure 5A; supplemental Figure S2), indicating a direct effect of the MLL fusion protein on HIF-1/2α stabilization.

To test whether HIF-1α stabilization was required for MLL-induced AML, we performed transplantation experiments using the *MLL-AF9* oncogene. Similar to previous results on *AE9a* and *HoxA9-Meis1*, the latency of developed disease in these animals was not significantly different in the *Hif-1α*$^{\Delta/\Delta}$ group compared to controls ($P$=0.6780) (Figure 5B). Analysis of GFP$^+$ and myeloid cells in PB indicated a similar development of the leukemia in both groups, independently of the presence of HIF-1α (Figure 5C-D). Analyses of hematopoietic organs 12 weeks post-transplantation, when both groups showed signs of disease, revealed no significant differences in leukemia parameters with respect to *Hif-1α* status (Figure 5E-H).

To test the role of HIF-1α in self-renewal of MLL-AF9-induced AML, we transplanted equal numbers of GFP$^+$ cells into secondary recipients. Survival curves showed a slight acceleration of AML in animals transplanted with *Hif-1α*$^{\Delta/\Delta}$ cells (Figure 6A), also indicated by their reduced total weight compared to controls (Figure 6B). Analyses of these mice 3 weeks post-transplantation showed a similar phenotype of the disease by multiple criteria in both groups (Figure 6 C-G). Interestingly, macroscopic analyses revealed a hepatic pathology in *Hif-1α*$^{\Delta/\Delta}$ mice (Figure 6H). This could indicate differences in homing of *Hif-1α*$^{\Delta/\Delta}$ cells in MLL-AF9-driven leukemia. These results show that even in AMLs that originate from leukemic oncogenes that signal directly towards HIF-1α (*HoxA9-Meis1* and *MLL*), HIF-1α does not appear to be a crucial regulator of LICs self-renewal.

To test whether these two oncogenes stabilize HIF-1α and if the deletion of HIF-1α leads to a compensatory expression of HIF-2α, we analyzed the levels of these two proteins in BM cells from diseased mice of the three different leukemia models (supplemental

Figure S3 and S4). We found a compensatory overexpression of HIF-2α after deletion of HIF-1α only in the AE9a model. In this leukemia model, deletion of HIF-1α resulted in a dramatic upregulation of HIF-2α.

**Metabolic analysis showed no divergent energetic conditions of *Hif-1α*$^{\Delta/\Delta}$ and control leukemic cells**

HIF-1α has been postulated to maintain the quiescence of HSCs, thereby protecting them from agents that can cause genotoxic damage. For instance, HIF-1α contributes to the reduction of reactive oxygen species (ROS) levels by switching from oxidative respiration to glycolytic metabolism in hypoxic cells[33].

To investigate whether the absence of HIF-1α in the three leukemia models is affecting the quiescence and proliferation of LICs, we studied their cell cycle profile in secondary recipients (Figure 7A). In AE9a-induced leukemia, the proportion of cells in the three different phases of the cell cycle was identical in the *Hif-1α*$^{\Delta/\Delta}$ and control mice despite the faster progression of the disease observed in the *Hif-1α*$^{\Delta/\Delta}$ mice. In the HOXA9-MEIS1 model, we found a significant increment of cycling cells (S/G2/M phase) in BM ($P$<0.0001) and spleen ($P$=0.0217) from *Hif-1α*$^{\Delta/\Delta}$ mice, consistent with the idea that HIF-1α could be acting as a negative cell cycle regulator, at least in some cases of AML. In the case of MLL-AF9-derived leukemia, malignant cells presented a similar cell cycle profile in both groups indicating a similar kinetics of the disease independently of the HIF-1α status of the cells. Finally, we observed that in the two models that did not show differences in cycling in these organs, more cycling cells were present in the blood (supplemental Figure S5).

We next wanted to explore whether the differences of *Hif-1α*$^{\Delta/\Delta}$ status were reflected in their viability status and energetic conditions by studying levels of apoptosis, ROS and mitochondrial activity. To this end, we analyzed GFP$^+$ cells from AE9a and MLL-AF9 secondary recipients and the myeloid population (Gr1$^+$/Mac1$^+$) from the HOXA9-MEIS1 model. We observed an increment in Annexin V$^+$ cells indicating higher proportion of cells undergoing apoptosis in the *Hif-1α*$^{\Delta/\Delta}$ cells (Figure 7B and supplemental Figure S5). This could indicate a higher sensitivity of these cells to stress or a higher accumulation of damage. We failed to observe a general increment of mitochondrial activity and mitochondrial or total ROS in the *Hif-1α*$^{\Delta/\Delta}$ cells from the three different AML models (Figure 7B and supplemental Figure S5). Only some of the samples showed significant differences with no consistency among the investigated models. In summary, we observed that AE9a and MLL-AF9 are increasing the mitochondrial activity of the cells in absence of HIF-1α but not HOXA9-MEIS1 (supplemental Table S1). Thus, metabolic analyses of the investigated models also indicated similar phenotypes independently of their HIF-1α status.

**Discussion**

Molecular mechanisms of self-renewal in normal and malignant HSCs remain largely elusive. Hypoxia and hypoxia signaling, through the transcription factors HIF-1/2α, have recently been implicated in these processes in normal HSCs, AML and CML cells[8,19,20].

There have been several studies suggesting a role of *HIF-1α* as an oncogene in AML, although for the most part, such studies did not take into account the nature of the particular originating alterations. In this way, it has previously been suggested that human AML cells require both HIF-1α and HIF-2α. This dependence has been demonstrated using shRNA and inhibitors to block HIF-1/2α function, followed by transplantation into NOD/SCID/IL2Rg$^{null}$ (NSG) mice[20,21]. However, genetic alterations of the AML samples used were not investigated, so it remains unclear whether the requirement for HIFs is dependent on signaling directly to HIF-1α by the respective genetic alterations of each particular AML sample. Additionally, shRNAs and inhibitors against HIF-1α can be quite unspecific with off-target effects that might lead to unspecific toxicities.

In the case of BCR-ABL in CML, it has been demonstrated that the fusion oncogene stabilizes HIF-1α directly with CML initiating cells requiring HIF-1α for proliferation[19,34]. Differences in leukemia latency upon secondary transplantations demonstrated a role for HIF-1α in the self-renewal of CML-IC, with an activating/oncogenic role in tumorigenesis of HIF-1α in this hematological malignancy.

In the case of solid tumors, evidence has emerged that *Hif-1/2α* can act as a tumor suppressor gene[35,36]. In clear renal cell carcinoma (RCC), where VHL loss of function leads to an accumulation of HIF-1/2α, *HIF-1α* is lost during tumor progression, indicating its tumor suppressor role in the later stages of tumorigenesis[37,38].

Whether LICs reside in a hypoxic environment and depend on HIF-signaling has been a matter of debate. Several different scenarios for the stabilization of HIFs in LICs can be envisioned. First, LICs might reside in a hypoxic BM environment leading to the activation of HIFs signaling by inhibition of HIFs degradation. In a second scenario, high levels of cytokines (like SCF and TPO) in a non-hypoxic niche might result in increased HIFs expression levels by upregulating their transcription. Thirdly, genetic changes in myeloid malignancies may lead to activation of HIFs in a cell intrinsic manner to mimic hypoxic signaling in normoxia. This would allow LICs to become hypoxia- and thereby niche-independent, which is a hallmark of leukemia.

We present here the first evidence of the HIF-1α independence of LICs in AML and a new role of *Hif-1α* as a potential tumor suppressor gene in hematological diseases. To test whether there might be a difference in the requirement for HIF-1α in murine AML depending on the identity of the oncogene, we expressed different leukemic oncogenes that either lead to HIF-1α activation (*MLL-AF9*, *HOXA9-MEIS1*) or have no known connection to hypoxic signaling (*AE9a*), followed by investigations of disease latency and phenotype of the resulting AMLs (see schematic summary in supplemental Table S1). Somewhat surprisingly, none of the three AML models studied by us was dependent on HIF-1α with regards to leukemia **initiation/progression** and LICs self-renewal.

Rather, we found that loss of HIF-1α resulted in increased proliferation in some of the AML models during the preleukemic/leukemic stage. Importantly, however, we failed to find a higher dependence on HIF-1α in the malignancies that were driven by HIF-1α activating oncogenes. **Whether this phenomenon is due to a direct effect of HIF-1α deletion or by an indirect compensatory effect of overexpressing HIF-2α has to be studied in more detail. We only found the increased expression of HIF-2α in the AE9a model but not in the other two AML models that also did not show an impairment of the initiation/progression of leukemia. Interestingly, shRNA mediated downregulation of either HIF-1α or HIF-2α was sufficient to negatively affect engraftment of human AML cells[20,21].**

Our data challenge the role of HIF-1α in AML initiation/maintenance and LICs self-renewal and quiescence. Unlike the observations made in normal HSCs[8], we found no loss of self-renewal in the *Hif-1α*$^{\Delta/\Delta}$ cells, which repopulated the BM niche after secondary transplantation as efficiently as *Hif-1α*$^{+/+}$ cells and even gave rise to faster leukemia development in the AE9a model. In our models we also failed to observe a consistent loss of quiescence during stress conditions, perhaps with the exception of the HOXA9-MEIS1 model. Our studies investigating the role of HIF-1α in disease latency, phenotype and LICs self-renewal might indicate major differences in human and murine AML and suggest a fundamentally different role for HIF-1α in leukemia to what has been described to date. While *Hif-1α* could act as an oncogene in particular tumor types, the results presented here together with other observations in certain solid tumors, indicate that HIF-1α can also act as a tumor-suppressor. Most likely, its function as one or the other is dependent on the particular genetic alteration that initiates the malignancy. Our results therefore have implications for therapeutic strategies based on HIF-1α targeting,

and reveal once more the enormous heterogeneity of AML. Further work is needed to investigate whether HIF-1α can be used as a therapeutic target in different molecularly defined subtypes of AML.

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**Authorship**

Contribution: T.V.H designed the research, performed experiments, analyzed data, made figures and wrote the manuscript; A.H.W. and M.R. performed experiments; D.B. provided access to novel transgenic mice, analyzed data and provided advice and discussion; J.C. conceived and supervised the project, designed the research and wrote the manuscript. All authors read and approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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**Figure legends**

**Figure 1. HIF-1α is not required for the initiation/progression of AE9a induced-leukemia.**

(A) Kaplan-Meier survival curves of mice transplanted with AE9a expressing cells or MIGR1 control (*Hif-1*$\alpha^{\Delta/\Delta}$ + AE9a, n=7; *Hif-1*$\alpha^{fl/fl}$ + AE9a, n=5; *Hif-1*$\alpha^{\Delta/\Delta}$ + MIGR1, n=4; *Hif-1*$\alpha^{fl/fl}$ + MIGR1, n=5). Log-rank (Mantel-Cox) test was used to assess statistical significance. (B) Percentage of transduced cells ($GFP^+$) in PB 3 and 20 weeks after transplantation (*Hif-1*$\alpha^{\Delta/\Delta}$, n=17; *Hif-1*$\alpha^{fl/fl}$, n=10). (C) Percentage of myeloid cells ($Gr1^+$ and/or $Mac1^+$) in PB 3, 7, 10 and 20 weeks after transplantation (*Hif-1*$\alpha^{\Delta/\Delta}$, n=17; *Hif-1*$\alpha^{fl/fl}$, n=10). (D) Representative FACS plots of BM cells from diseased mice showing that the $GFP^+$ cells are all included in the $Lin^-$ malignant population. The differentiated populations are stained with the following antibodies: CD3 for T-cells (T), B220 for B-cells (B) and Gr1/Mac1 for myeloid cells (M). (E-G) Analysis of different parameters of sacrificed mice at an advanced stage of the disease: myeloid cells in PB (E), $Lin^-$ cells in PB (F) and BM (G) (*Hif-1*$\alpha^{\Delta/\Delta}$, n=9; *Hif-1*$\alpha^{fl/fl}$, n=5). (H) BM cell cytospins from leukemic mice showing similar morphology of the main population in both genotypes. Scale bar = 20 μm. (I) CFU-assay derived from BM cells from both genotypes (*Hif-1*$\alpha^{\Delta/\Delta}$, n=8; *Hif-1*$\alpha^{fl/fl}$, n=3). (J) Percentage of colonies derived from BM cells from each genotype in a competitive transplantation 1:1 (n=4). Plots and columns represent mean ± SEM and boxes represent mean ± min to max values. Unless otherwise stated, two-tailed student's *t*-test was used to assess statistical significance. * $P<0.05$, *** $P<0.001$.

**Figure 2. *Hif-1α* deletion accelerates the AE9a malignant phenotype in secondary recipients.**

(A) Kaplan-Meier survival curve of secondary recipients from two independent experiments transplanted with cells derived from leukemic mice of both genotypes (*Hif-1α*$^{\Delta/\Delta}$, n=7; *Hif-1α*$^{fl/fl}$, n=9). Log-rank (Mantel-Cox) test was used to assess statistical significance. The development of the disease was measured by the percentage of transduced cells (GFP$^+$) (B), myeloid cells (Gr1$^+$ and/or Mac1$^+$) (C) and Lin$^-$ cells (D) in PB 3, 7 and 10 weeks after transplantation (*Hif-1α*$^{\Delta/\Delta}$, n=11; *Hif-1α*$^{fl/fl}$, n=18). Eleven weeks after transplantation, mice were sacrificed and disease analyzed by measuring several parameters: total WBC (E), spleen and liver weight (F) and GFP$^+$ and Lin$^-$ cells in BM and spleen (G) (*Hif-1α*$^{\Delta/\Delta}$, n=7; *Hif-1α*$^{fl/fl}$, n=9). A two-way ANOVA test was used to assess statistical significance along the different time points. (H) Representative FACS plots of spleen cells from diseased mice showing that the GFP$^+$ cells are all included in the Lin$^-$ malignant population. The differentiated populations are stained with the following antibodies: CD3 for T-cells (T), B220 for B-cells (B) and Gr1/Mac1 for myeloid cells (M). Plots represent mean ± SEM. Unless otherwise stated, two-tailed student's *t*-test was used to assess statistical significance. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

**Figure 3. *Hif-1α* deletion accelerates early development of HOXA9-MEIS1-induced AML.**

(A) Kaplan-Meier survival curve of mice transplanted with *HoxA9-Meis1* expressing cells (n=10). Log-rank (Mantel-Cox) test was used to assess statistical significance. (B) Percentage of myeloid cells in PB of transplanted animals at different time points after

transplantation (week 3: n=10; week 8: *Hif-1α*$^{\Delta/\Delta}$, n=10; *Hif-1α*$^{fl/fl}$, n=9; week 11: *Hif-1α*$^{\Delta/\Delta}$, n=6; *Hif-1α*$^{fl/fl}$, n=7). Two-way ANOVA was used to test statistical significance. (C) Representative FACS plots of PB cells at week 8 after transplantation showing an increment in the myeloid population of *Hif-1α*$^{\Delta/\Delta}$ samples. The differentiated populations are stained with the following antibodies: CD3 for T-cells (T), B220 for B-cells (B) and Gr1/Mac1 for myeloid cells (M). Diseased mice were sacrificed at an advanced stage of disease and several parameters were analyzed: percentage of myeloid cells in PB, BM and spleen (D), WBC (E) and spleen and liver weight (F) (*Hif-1α*$^{\Delta/\Delta}$, n=9; *Hif-1α*$^{fl/fl}$, n=6). (G) CFU-assay derived from BM cells from both genotypes (n=5). Plots and columns represent mean ± SEM. Unless otherwise stated, two-tailed student's *t*-test was used to assess statistical significance. * $P<0.05$.

**Figure 4. HOXA9-MEIS1 induced AML shows no difference in LICs self-renewal or phenotype depending on HIF-1α.**

(A) Kaplan-Meier survival curves of mice transplanted with *HoxA9-Meis1* expressing-BM cells derived from primary recipients. Cells derived from two different donors were used to transplant 4 x $10^5$ cells into secondary recipients (n=12). Log-rank (Mantel-Cox) test was used to assess statistical significance. Leukemic animals derived from two independent experiments were sacrificed at week 3 after transplantation and WBC (n=20) (B), percentage of myeloid cells in PB (C), BM and spleen (D) and spleen and liver weight (E) analyzed for evaluating the stage of the disease (n=11). Plots represent mean ± SEM. Two-tailed student's *t*-test was used to assess statistical significance. ** $P<0.01$, *** $P<0.001$.

**Figure 5. MLL-induced leukemia initiation/progression is independent of HIF-1α status although MLL signals directly towards HIF.**

(A) GFP expression of a clone derived from Doxycycline-inducible MLL-ENL BM cells transduced with a GFP-hypoxic reporter. Cells from this clone were grown in methylcellulose for one week under normoxic (20% oxygen) or hypoxic (1% oxygen) conditions in media ± Doxycycline (Dox) and GFP expression analyzed by flow cytometry. (B) Kaplan-Meier survival curve of mice transplanted with *MLL-AF9* expressing cells (*Hif-1*$\alpha^{\Delta/\Delta}$, n=6; *Hif-1*$\alpha^{fl/fl}$, n=7). Log-rank (Mantel-Cox) test was used to assess statistical significance. The development of the disease was measured by the percentage of transduced cells ($GFP^+$) (C) and myeloid cells ($Gr1^+$ and/or $Mac1^+$) (D) in PB 3, 7 and 10 weeks after transplantation (n=10). At 12 weeks after transplantation, a set of mice was sacrificed and the phenotype of the disease analyzed by measuring several parameters: WBC (E), $GFP^+$ cells (F) and myeloid cells ($Gr1^+$ and/or $Mac1^+$) (G) in BM and spleen and spleen and liver weight (H) (n=3). Plots and columns represent mean ± SEM. Two-tailed student's *t*-test was used to assess statistical significance.

**Figure 6. HIF-1α status does not affect the self-renewal of MLL-AF9 LICs but alters the extramedullary location of infiltrating leukemic cells.**

(A) Kaplan-Meier survival curve of secondary recipients transplanted with BM cells derived from leukemic mice of both genotypes (n=6). Log-rank (Mantel-Cox) test was used to assess statistical significance. (B) Total weight of sex and age-matched mice 3 weeks after transplantation (n=4). The development of the disease was measured 3 weeks after transplantation in animals derived from two independent experiments by

numbers of WBC (n=16) (C), percentage of transduced cells ($GFP^+$) (D) and myeloid cells ($Gr1^+$ and/or $Mac1^+$) (E) in PB (n=16). A set of mice was sacrificed and stage of the disease analyzed by: spleen and liver weight (F), macroscopic examination of the livers (G), $GFP^+$ cells and myeloid cells ($Gr1^+$ and/or $Mac1^+$) in BM and spleen (H) (n=11). Plots represent mean ± SEM and boxes represent mean ± min to max values. Two-tailed student's *t*-test was used to assess statistical significance. * $P<0.05$, *** $P<0.001$.

**Figure 7. HIF-1α status does not generally influence cell cycle or metabolism of leukemic cells, but its deletion increases apoptosis.**

(A) Cell cycle analysis of the leukemic cells ($GFP^+$ or myeloid, depending on the model) from the different indicated organs of diseased mice from two independent experiments (AE9a: n=7; HoxA9-Meis1: n=11; MLL-AF9: n=11). (B) Apoptosis analysis of the leukemic cells ($GFP^+$ or myeloid, depending on the model) from the different indicated organs of diseased mice (HoxA9-Meis1: n=8; MLL-AF9: n=7). (C) Analysis of mitochondrial activity, mitochondrial ROS and total ROS. Median fluorescence intensity (MFI) of MitoTracker-stained (n=7-11), MitoSOX-stained (n=7-8) or CellROX-stained (n=7-11) leukemic cells ($GFP^+$ cells in AE9a and MLL-AF9 models and myeloid cells in HOXA9-MEIS1 model) from diseased secondary recipients in BM and spleen was normalized to the mean control values. Plots represent mean ± SEM. Two-tailed student's *t*-test was used to assess statistical significance. * $P<0.05$, ** $P<0.01$.

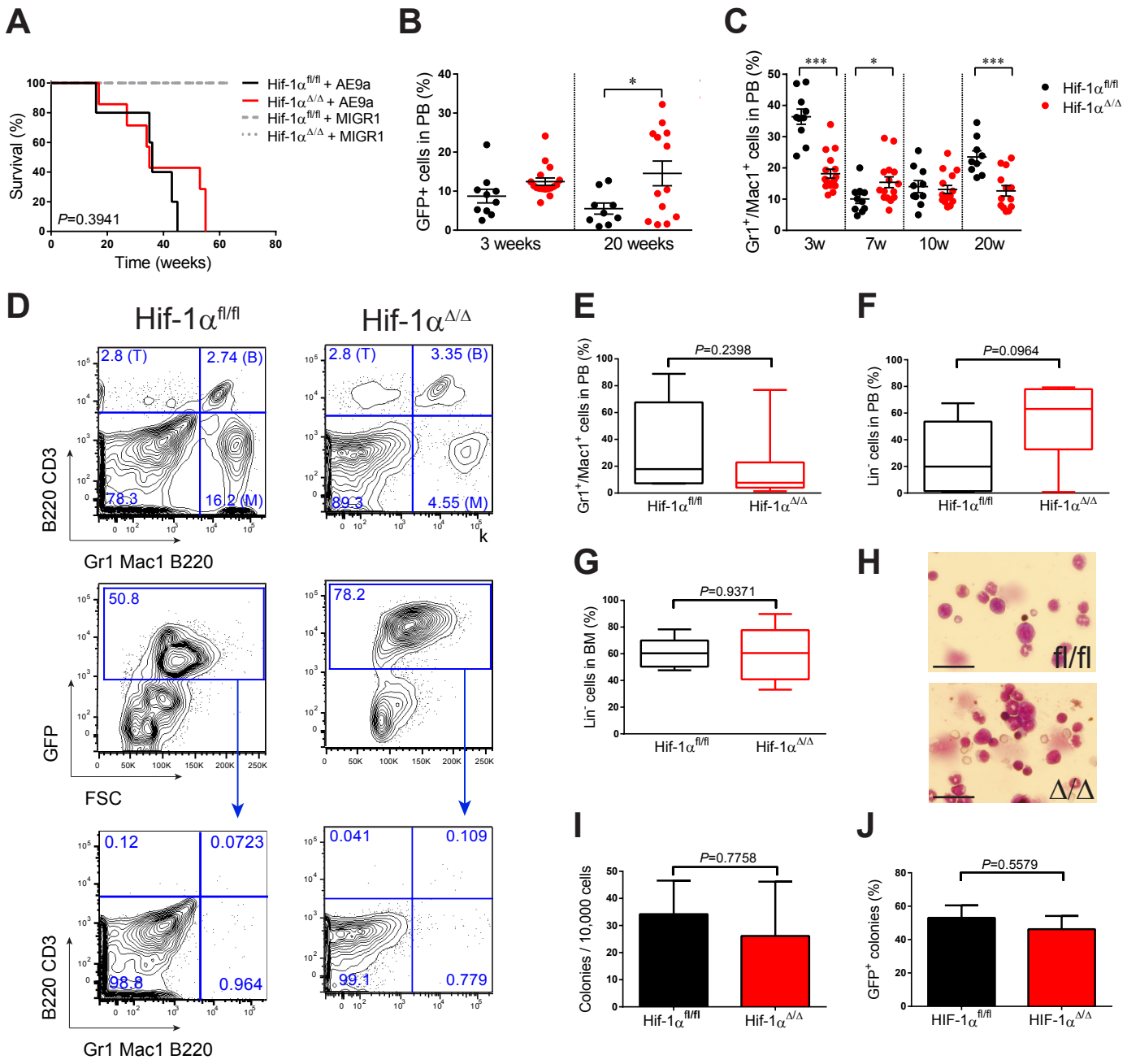
A
Survival (%)
Time (weeks)
P=0.3941
Hif-1α fl/fl + AE9a
Hif-1α Δ/Δ + AE9a
Hif-1α fl/fl + MIGR1
Hif-1α Δ/Δ + MIGR1
B
GFP+ cells in PB (%)
3 weeks
20 weeks
C
Gr1+/Mac1+ cells in PB (%)
3w
7w
10w
20w
Hif-1α fl/fl
Hif-1α Δ/Δ
D
Hif-1α fl/fl
Hif-1α Δ/Δ
B220 CD3
Gr1 Mac1 B220
GFP
FSC
E
Gr1+/Mac1+ cells in PB (%)
P=0.2398
F
Lin- cells in PB (%)
P=0.0964
G
Lin- cells in BM (%)
P=0.9371
H
fl/fl
Δ/Δ
I
Colonies / 10,000 cells
P=0.7758
J
GFP+ colonies (%)
P=0.5579
HIF-1α fl/fl
HIF-1α Δ/Δ

Figure 1

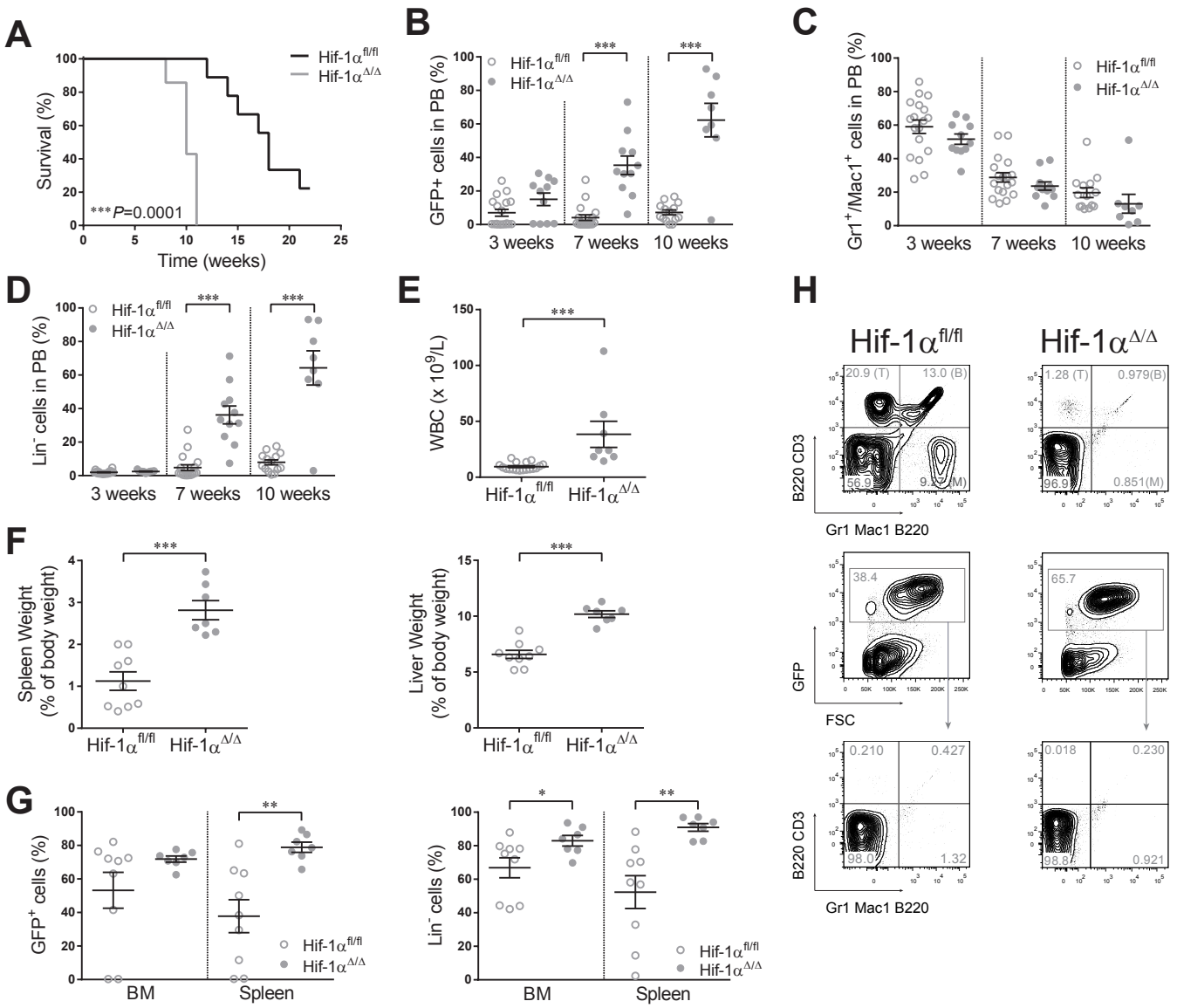
A
Survival (%)
Time (weeks)
***P=0.0001
Hif-1α fl/fl
Hif-1α Δ/Δ
B
GFP+ cells in PB (%)
3 weeks
7 weeks
10 weeks
***
C
Gr1+/Mac1+ cells in PB (%)
D
Lin- cells in PB (%)
E
WBC (x 10^9/L)
F
Spleen Weight (% of body weight)
Liver Weight (% of body weight)
G
GFP+ cells (%)
Lin- cells (%)
BM
Spleen
*
**
H
20.9 (T)
13.0 (B)
56.9
9.5 (M)
1.28 (T)
0.979(B)
96.9
0.851(M)
B220 CD3
Gr1 Mac1 B220
38.4
65.7
GFP
FSC
0.210
0.427
98.0
1.32
0.018
0.230
98.8
0.921

Figure 2

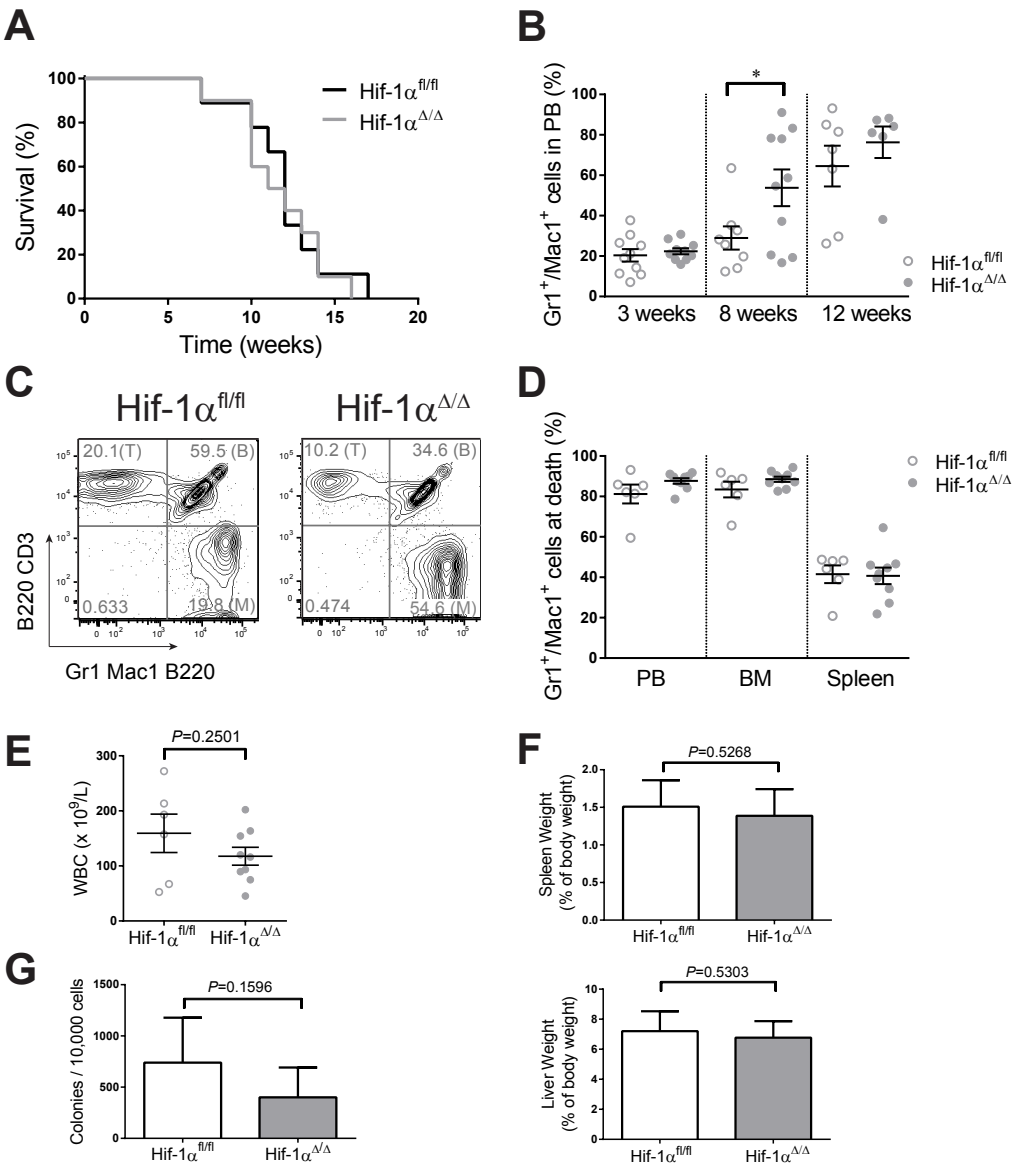
A
Survival (%)
Time (weeks)
Hif-1α fl/fl
Hif-1α Δ/Δ
B
Gr1+/Mac1+ cells in PB (%)
3 weeks
8 weeks
12 weeks
*
C
Hif-1α fl/fl
Hif-1α Δ/Δ
20.1(T)
59.5 (B)
0.633
19.8 (M)
10.2 (T)
34.6 (B)
0.474
54.6 (M)
B220 CD3
Gr1 Mac1 B220
D
Gr1+/Mac1+ cells at death (%)
PB
BM
Spleen
E
P=0.2501
WBC (x 10^9/L)
F
P=0.5268
Spleen Weight (% of body weight)
G
P=0.1596
Colonies / 10,000 cells
P=0.5303
Liver Weight (% of body weight)

Figure 3

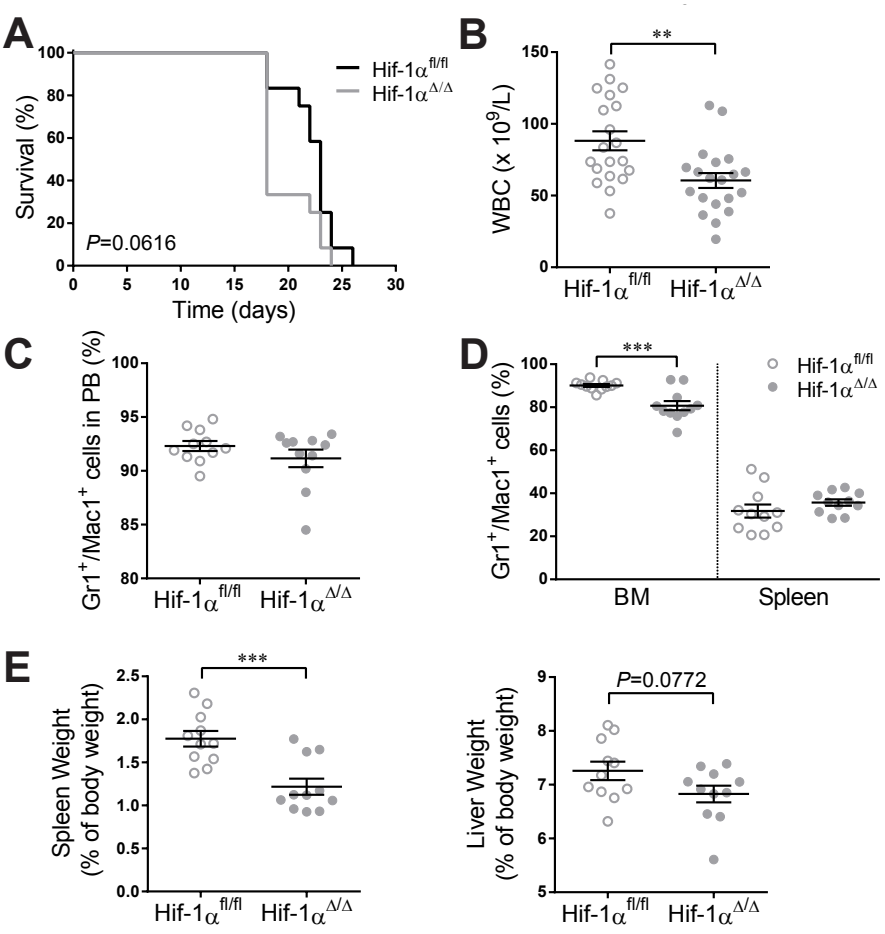
A
Survival (%)
Time (days)
P=0.0616
Hif-1α fl/fl
Hif-1α Δ/Δ
B
WBC (x 10^9/L)
**
C
Gr1+/Mac1+ cells in PB (%)
D
Gr1+/Mac1+ cells (%)
***
BM
Spleen
E
Spleen Weight (% of body weight)
***
Liver Weight (% of body weight)
P=0.0772

Figure 4

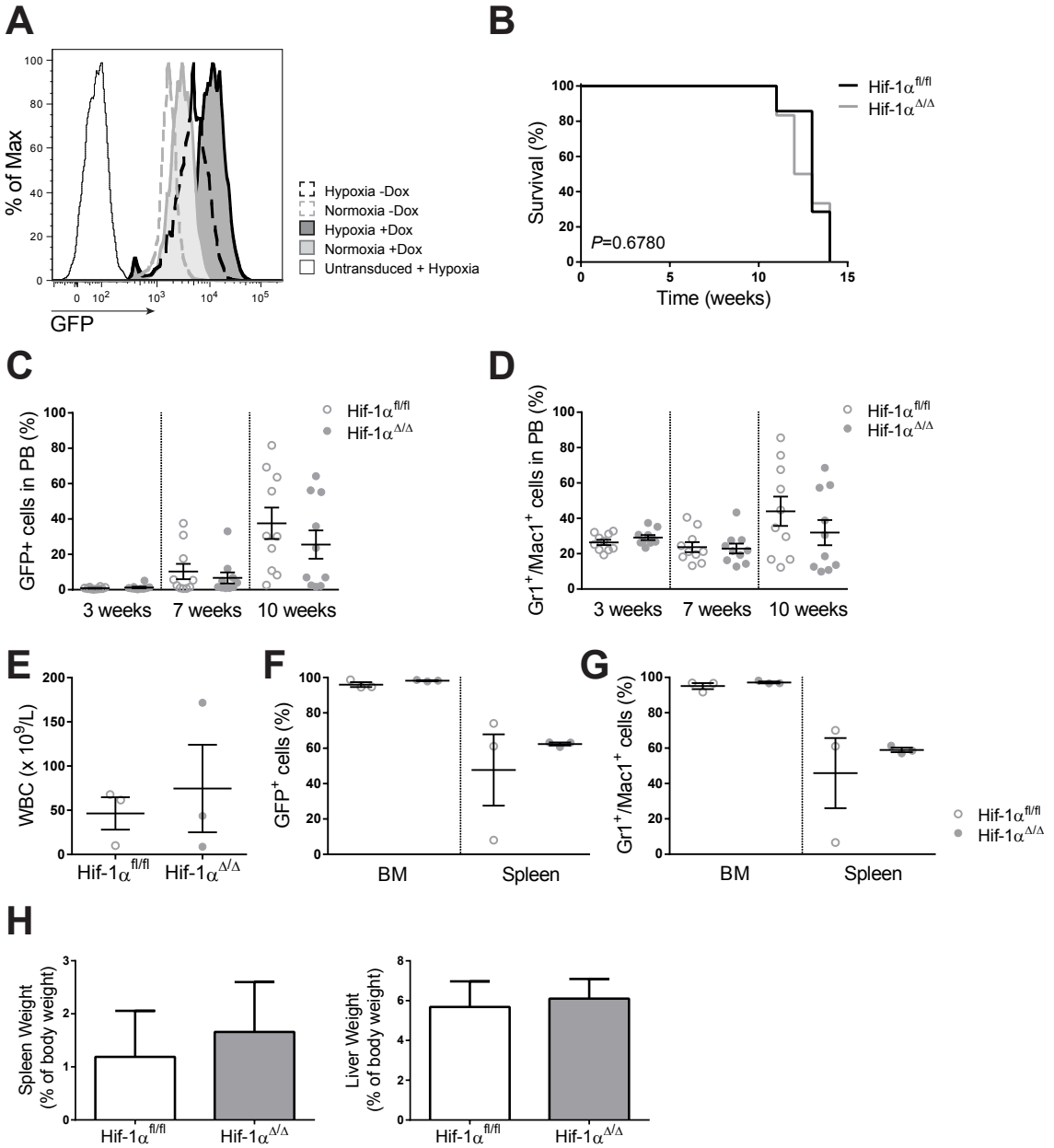
A
% of Max
GFP
Hypoxia -Dox
Normoxia -Dox
Hypoxia +Dox
Normoxia +Dox
Untransduced + Hypoxia
B
Survival (%)
Time (weeks)
P=0.6780
Hif-1α fl/fl
Hif-1α Δ/Δ
C
GFP+ cells in PB (%)
3 weeks
7 weeks
10 weeks
D
Gr1+/Mac1+ cells in PB (%)
E
WBC (x 10^9/L)
F
GFP+ cells (%)
BM
Spleen
G
Gr1+/Mac1+ cells (%)
H
Spleen Weight (% of body weight)
Liver Weight (% of body weight)

Figure 5

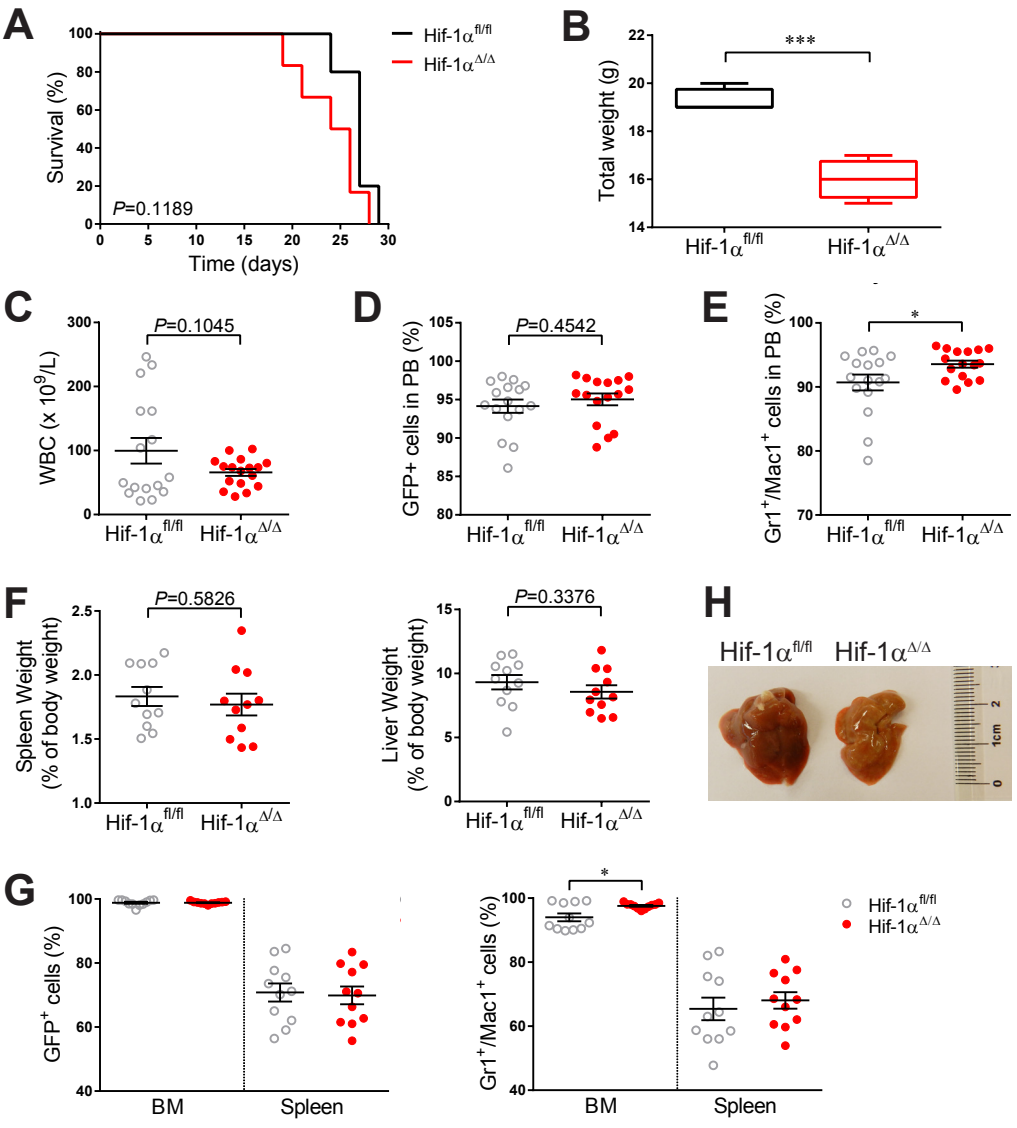
A
Survival (%)
Time (days)
P=0.1189
Hif-1α fl/fl
Hif-1α Δ/Δ
B
Total weight (g)
***
C
WBC (x 10^9/L)
P=0.1045
D
GFP+ cells in PB (%)
P=0.4542
E
Gr1+/Mac1+ cells in PB (%)
*
F
Spleen Weight (% of body weight)
P=0.5826
Liver Weight (% of body weight)
P=0.3376
H
G
GFP+ cells (%)
BM
Spleen
Gr1+/Mac1+ cells (%)

Figure 6

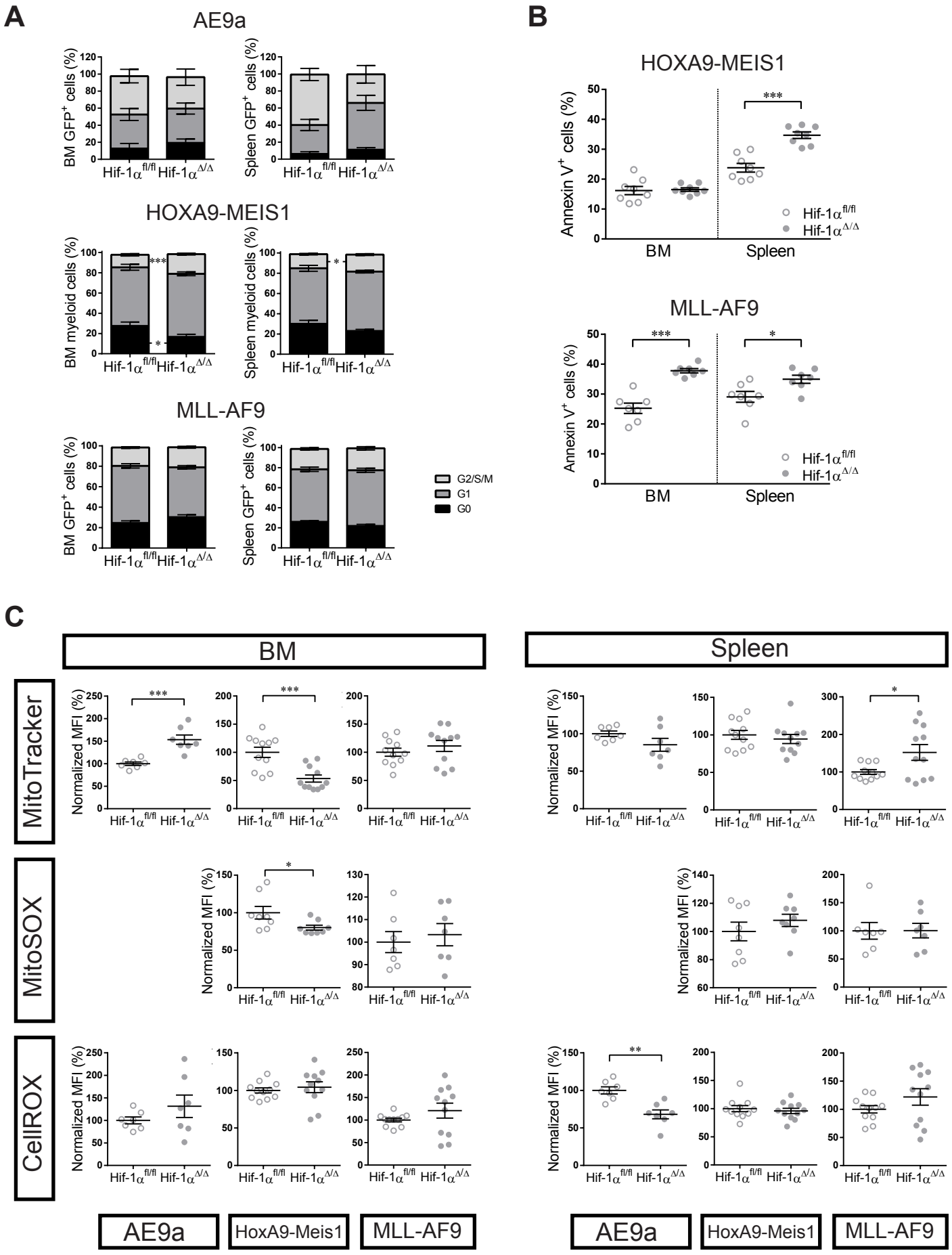
A
AE9a
BM GFP+ cells (%)
Spleen GFP+ cells (%)
Hif-1α fl/fl
Hif-1α Δ/Δ
HOXA9-MEIS1
BM myeloid cells (%)
Spleen myeloid cells (%)
MLL-AF9
G2/S/M
G1
G0
B
HOXA9-MEIS1
Annexin V+ cells (%)
BM
Spleen
MLL-AF9
C
BM
Spleen
MitoTracker
MitoSOX
CellROX
Normalized MFI (%)
AE9a
HoxA9-Meis1
MLL-AF9

Figure 7