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Fumarate hydratase is a critical metabolic regulator of hematopoietic stem cell functions


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Strict regulation of stem cell metabolism is essential for tissue functions and tumor suppression. In this study, we investigated the role of fumarate hydratase (Fh1), a key component of the mitochondrial tricarboxylic acid (TCA) cycle and cytosolic fumarate metabolism, in normal and leukemic hematopoiesis. Hematopoiesis-specific Fh1 deletion (resulting in endogenous fumarate accumulation and a genetic TCA cycle block reflected by decreased maximal mitochondrial respiration) caused lethal fetal liver hematopoietic defects and hematopoietic stem cell (HSC) failure. Reexpression of extramitochondrial Fh1 (which normalized fumarate levels but not maximal mitochondrial respiration) rescued these phenotypes, indicating the causal role of cellular fumarate accumulation. However, HSCs lacking mitochondrial Fh1 (which had normal fumarate levels but defective maximal mitochondrial respiration) failed to self-renew and displayed lymphoid differentiation defects. In contrast, leukemia-initiating cells lacking mitochondrial Fh1 efficiently propagated Meis1/Hoxa9-driven leukemia. Thus, we identify novel roles for fumarate metabolism in HSC maintenance and hematopoietic differentiation and reveal a differential requirement for mitochondrial Fh1 in normal hematopoiesis and leukemia propagation.

INTRODUCTION
Successful clinical application of hematopoietic stem cells (HSCs) is critically dependent on their ability to give long-term multilineage hematopoietic reconstitution (Weissman and Shizuru, 2008). Multiple studies have revealed the paradigmatic transcription factors driving HSC self-renewal and differentiation to sustain multilineage hematopoiesis (Göttgens, 2015). Emerging evidence indicates that strict control of HSC metabolism is also essential for their life-long functions (Suda et al., 2011; Manesia et al., 2015), but the key metabolic regulators that ensure stem cell integrity remain elusive. Although highly proliferative fetal liver (FL) HSCs use oxygen-dependent pathways for energy generation (Suda et al., 2011; Manesia et al., 2015), adult HSCs are known to suppress the flux of glycolytic metabolites into the mitochondrial tricarboxylic acid (TCA) cycle and heavily rely on glycolysis to maintain their quiescent state (Simsek et al., 2010; Takubo et al., 2013). Whereas pharmacological inhibition of glycolytic flux into the TCA cycle enhances HSC activity upon transplantation (Takubo et al., 2013), severe block of glycolysis (i.e., Ldha deletion) and a consequent elevated mitochondrial respiration abolishes HSC maintenance (Wang et al., 2014). The switch from glycolysis to mitochondrial oxidative metabolism is essential for adult HSC differentiation rather than maintenance of their self-renewing pool (Yu et al., 2013). Leukemia-initiating cells (LICs) are even more...
dependent on glycolysis than normal HSCs (Wang et al., 2014). Partial or severe block in glycolysis (elicited by deletion of Pkm2 or Ldha, respectively) and a metabolic shift to mitochondrial respiration efficiently suppress the development and maintenance of LICs (Lagadinou et al., 2013; Wang et al., 2014). Thus, the maintenance of adult self-renewing HSCs and LICs appears to depend critically on glycolysis rather than the mitochondrial TCA, which is thought to be less important for this process. However, thus far, the requirement for any of the TCA enzymes in FL and adult HSC and LIC maintenance has not been investigated.

Genetic evidence in humans indicates that rare recessive mutations in the FH gene encoding a TCA enzyme fumarate hydratase (Fh1) result in severe developmental abnormalities, including hematopoietic defects (Bourgeron et al., 1994). Consistent with this, we also found that monozygous twins with recessive FH mutations (Tregoning et al., 2013) display leukopenia and neutropenia (Table S1), thus suggesting a role for FH in the regulation of hematopoiesis. Mitochondrial and cytosolic fumarate hydratase enzyme isoforms, both encoded by the same gene (called FH in humans and Fh1 in mice; Stein et al., 1994; Sass et al., 2001), catalyze hydration of fumarate to malate. Whereas mitochondrial Fh1 is an integral part of the TCA cycle, cytosolic Fh1 metabolizes fumarate generated during arginine synthesis, the urea cycle, and the purine nucleotide cycle in the cytoplasm (Yang et al., 2013). Autosomal dominant mutations in FH are associated with hereditary leiomyomatosis and renal cell cancer, indicating that FH functions as a tumor suppressor (Launonen et al., 2001; Tomlison et al., 2002). Given that FH mutations have been associated with hematopoietic abnormalities and tumor formation, here, we investigated the role of Fh1 in normal and malignant hematopoiesis.

RESULTS
Fh1 is required for FL hematopoiesis
Fh1 is uniformly expressed in mouse Lin− Sca-1+ c-Kit+ (LSK) CD48− CD150+ HSCs, LSKCD48− CD150+ multipotent progenitors, primitive hematopoietic progenitor cells (HPCs; i.e., LSKCD48− CD150− HPC-1 and LSKCD48− CD150− HPC-2 populations), and Lin− Sca-1− c-Kit+ (LK) myeloid progenitors sorted both from the FL (the major site of definitive hematopoiesis during development) of 14.5− days post-coitum (dpc) embryos and adult BM (Fig. 1 A). To determine the requirement for Fh1 in HSC maintenance and multilineage hematopoiesis, we conditionally deleted Fh1 specifically within the hematopoietic system shortly after the emergence of definitive HSCs using the Vav-iCre deleter strain (de Boer et al., 2003). We bred Fh1fl/fl mice (Pollard et al., 2007) with Vav-iCre mice and found no viable Fh1fl/fl;Vav-iCre offspring (Table S2). Fh1fl/fl;Vav-iCre embryos were recovered at 14.5 dpc at normal Mendelian ratios, suggesting fetal or perinatal lethality. FLs isolated from Fh1fl/fl;Vav-iCre embryos appeared abnormally small and pale indicating severe impairment in FL hematopoiesis (Fig. 1 B). Fh1 loss from the hematopoietic system was confirmed by the absence of Fh1 transcripts (Fig. 1 C) in CD45+ and c-Kit+ hematopoietic cells from Fh1fl/fl;Vav-iCre FLs and absence of Fh1 protein in FL c-Kit+ cells from Fh1fl/fl;Vav-iCre embryos (Fig. 1 D). Whereas Fh1fl/fl;Vav-iCre FLs had decreased numbers of hematopoietic cells because of reduced numbers of differentiated lineage− (Lin−) cells, the numbers of primitive FL Lin− cells remained unchanged (Fig. 1 E). Colony-forming cell (CFC) assays indicated the failure of Fh1-deficient FL cells to differentiate (Fig. 1 F). Analyses of erythroid differentiation revealed a block of erythropoiesis resulting in severe anemia (Fig. 1 G).

Fh1 is therefore essential for multilineage differentiation of FL stem and/or progenitor cells.

Fh1 is essential for HSC maintenance
Next, we asked whether Fh1 is required for the maintenance of the stem and progenitor cell compartments in FLs. FLs from Fh1fl/fl;Vav-iCre embryos had normal absolute numbers of LK myeloid progenitors (Fig. 1 H) and LSK stem and primitive progenitor cells (Fig. 1 I) but displayed an increase in total numbers of HSCs compared with control FLs (Fig. 1 J). To test the repopulation capacity of Fh1-deficient HSCs, we transplanted 100 CD45.2+ HSCs sorted from 14.5− dpc FLs into lethally irradiated syngeneic CD45.1+ CD45.2− recipients and found that Fh1-deficient HSCs failed to reconstitute short−term and long−term hematopoiesis (Fig. 1, K and L). To test the possibility that Fh1− deficient FLs contain stem cell activity outside the immunophenotypically defined LSKCD48− CD150+ HSC compartment, we transplanted unfractionated FL cells from Fh1fl/fl;Vav-iCre and control embryos into lethally irradiated recipient mice (together with support BM cells) and found that Fh1− deficient FL cells failed to repopulate the recipients (Fig. 1 M). Thus, Fh1 is dispensable for HSC survival and expansion in the FL but is critically required for HSC maintenance upon transplantation.

To establish the requirement for Fh1 in adult HSC maintenance, we generated Fh1fl/fl;Mx1-Cre mice in which efficient recombination is induced by treatment with polyinosinic:polycytidylic acid (pIpC; Kühn et al., 1995). We mixed CD45.2+ BM cells from untreated Fh1fl/fl;Mx1-Cre, Fh1+/−;Mx1-Cre, or control mice with CD45.1+ BM cells, transplanted them into recipient mice, and allowed for efficient reconstitution (Fig. 1 N). pIpC administration to the recipients of Fh1fl/fl;Mx1-Cre BM cells resulted in a progressive decline of donor-derived CD45.2+ cell chimerism in PB (Fig. 1 N) and a complete failure of Fh1-deficient cells to contribute to primitive and mature hematopoietic compartments of the recipients (Fig. 1 O). Therefore, Fh1 is critical for the maintenance of both FL and adult HSCs.

Fh1 deficiency results in cellular fumarate accumulation and decreased maximal mitochondrial respiration
We next investigated the biochemical consequences of Fh1 deletion in primitive hematopoietic cells. To investigate how Fh1 loss affects the oxidative phosphorylation capacity of
Figure 1. Hematopoiesis-specific Fh1 deletion results in severe hematopoietic defects and loss of HSC activity. (A) Relative levels of Fh1 mRNA (normalized to Actb) in HSCs, multipotent progenitors (MPP), HPC-1 and HPC-2 populations, and LSK and LK cells sorted from 14.5-dpc FLs and BM of C57BL/6 adult (8–10 wk old) mice. n = 3. (B) FLs from 14.5-dpc Fh1fl/fl;Vav-iCre embryos are smaller and paler compared with Fh1+/fl;Vav-iCre and control
primitive FL c-Kit+ hematopoietic cells, we measured oxygen consumption rate (OCR) under basal conditions and in response to sequential treatment with oligomycin (ATPase inhibitor), carbonyl cyanide-4-(trifluoromethoxy)phenyldrazone (FCCP; mitochondrial uncoupler), and a concomitant treatment with rotenone and antimycin A (complex I and III inhibitors, respectively). The basal OCR was not affected in Fh1-deficient FL c-Kit+ cells, suggesting that the majority of mitochondrial NADH (nicotinamide adenine dinucleotide) required for oxygen consumption in these cells originates from TCA-independent sources. However, the maximal OCR (after treatment with FCCP), which reflects maximal mitochondrial respiration, was profoundly decreased in Fh1-deficient cells (Fig. 2 A; but not in Fh1+/fl;Vav-iCre cells; not depicted), indicating that Fh1 deficiency may result in a compromised capacity to meet increased energy demands associated with metabolic stress or long-term survival (Yadava and Nicholls, 2007; Ferrick et al., 2008; Choi et al., 2009; van der Windt et al., 2012; Keuper et al., 2014). We also found that primitive Fh1-deficient FL hematopoietic cells failed to maintain ATP synthesis upon galactose-mediated inhibition of glycolysis (Fig. 2 B, left), consistent with an increased reliance on glycolysis for ATP production. Furthermore, Fh1-deficient c-Kit+ cells had increased expression of glucose transporters (Glut1 and Glut3) and key glycolytic enzymes Hk2 and Pfkp and displayed increased extracellular acidification rate (ECAR), indicative of enhanced glycolysis (Fig. 2, C–D). Thus, Fh1 deficiency results in an increase in glycolytic flux and impaired maximal mitochondrial respiration.

To determine the impact of Fh1 deletion on fumarate levels, we performed mass spectrometry analyses. Fh1-deficient FL c-Kit+ cells accumulated high levels of endogenous cellular fumarate (Fig. 2 E), consistent with previous observations in nonhematopoietic tissues harboring Fh1 mutation (Adam et al., 2013). Under the conditions of elevated fumarate, argininosuccinate is generated from arginine and fumarate by the reversed activity of the urea cycle enzyme argininosuccinate lyase (Zheng et al., 2013). We found that argininosuccinate is produced from high levels in Fh1-deficient c-Kit+ cells (Fig. 2 F). Furthermore, when accumulated at high levels, fumarate modifies cysteine residues in many proteins, forming S-(2-succinyl)-cysteine (2SC; Alderson et al., 2006; Adam et al., 2011; Bardella et al., 2011; Ternette et al., 2013). Fh1-deficient c-Kit+ cells exhibited high immunoreactivity for 2SC (Fig. 2 G). Thus, primitive hematopoietic cells lacking Fh1 have compromised maximal mitochondrial respiration, display increased glycolysis, fail to maintain normal ATP production upon inhibition of glycolysis, and accumulate high levels of fumarate resulting in excessive protein succination.

**Efficient fumarate metabolism is essential for HSC maintenance and multilineage hematopoiesis**

Mechanistically, the phenotypes observed upon Fh1 deletion could result from the genetic block in the TCA cycle or the accumulation of cellular fumarate (Pollard et al., 2007; Adam et al., 2011). To differentiate between these two mechanisms, we used mice ubiquitously expressing a human cytoplasmic isoform of FH (FHcyt), which lacks the mitochondrial targeting sequence and therefore is excluded from the mitochondria; Adam et al., 2013). Although primitive hematopoietic cells from FLs of Fh1+/fl;FHcyt;Vav-iCre embryos had normal mitochondrial membrane potential (not depicted), they displayed defective maximal respiration (Fig. 2 A) and impaired compensatory mitochondrial ATP production upon inhibition of glycolysis (Fig. 2 B, right), as well as an increase in ECAR (Fig. 2 D) similar to Fh1+/fl;Vav-iCre FL cells. Furthermore, primitive Fh1+/fl;FHcyt;Vav-iCre FL cells had significantly reduced levels of cellular fumarate (Fig. 2 E) and argininosuccinate (Fig. 2 F) and undetectable immunoreactivity to 2SC (Fig. 2 G), indicating that the biochemical consequences of fumarate accumulation were largely abolished by the FHcyt transgene expression. Although FHcyt transgene decreased overall cellular levels of fumarate, argininosuccinate, and succinated proteins, we cannot exclude the possibility that fumarate is elevated in mitochondria and contributes to the impairment of mitochondrial function in the absence of mitochondrial Fh1. Collectively, although cells from Fh1+/fl;FHcyt;Vav-iCre FLs displayed impaired maximal respiration, they had cellular fumarate levels comparable with control cells.
Figure 2. Cytosolic isoform of Fh1 restores normal steady-state hematopoiesis in Fh1<sup>fl/fl</sup>;Vav-iCre mice. (A) OCR in FL c-Kit<sup>+</sup> cells under basal conditions and after the sequential addition of oligomycin, FCCP, and rotenone and antimycin A. Control, n = 5; Fh1<sup>fl/fl</sup>;Vav-iCre, n = 3; control; Fh1<sup>fl/fl</sup>;Cyt, n = 10; Fh1<sup>fl/fl</sup>;FHCyt;Vav-iCre, n = 5. (B) Oxidative phosphorylation–dependent ATP production in galactose (Gal)-treated FL c-Kit<sup>+</sup> Fh1<sup>fl/fl</sup>;Vav-iCre and Fh1<sup>fl/fl</sup>;FHCyt;Vav-iCre cells. FL c-Kit<sup>+</sup> cells were cultured in DMEM supplemented with either 25 mM glucose (Glu) or 25 mM Gal. The graph shows the ratio of ATP produced in the presence of Gal (permissive for oxidative phosphorylation only) to ATP generated in the presence of Glu (permissive for both oxidative phosphorylation and glycolysis). Control, n = 9; Fh1<sup>fl/fl</sup>;Vav-iCre, n = 9; control; Fh1<sup>fl/fl</sup>;Cyt, n = 6; Fh1<sup>fl/fl</sup>;FHCyt;Vav-iCre, n = 10. (C) Relative expression (normalized
Notably, Fh1£/£;FH5/5;Vav-iCre mice were born at normal Mendelian ratios (Table S3) and matured to adulthood without any obvious defects. BM cells from adult Fh1£/£;FH5/5;Vav-iCre mice efficiently generated myeloid colonies (Fig. 2 H), had normal BM cellularity (Fig. 2 I), and displayed multilineage hematopoiesis (Fig. 2 J and K), despite reduced numbers of B cells (Fig. 2 K). Furthermore, they had unaffected numbers of LK myeloid progenitor cells (Fig. 2 L) and increased numbers of LSK cells (Fig. 2 M) and HSCs (Fig. 2 N). Therefore, it is critical that HSCs and/or primitive progenitor cells efficiently metabolize fumarate to sustain hematopoietic differentiation. Finally, HSCs that acquire mitochondrial Fh1 deficiency (which abolishes maximal mitochondrial respiration) shortly after their emergence manage to survive, expand in the FL, colonize the BM, and sustain steady-state multilineage hematopoiesis, implying that mitochondrial Fh1 is largely dispensable for these processes.

Mitochondrial Fh1 deficiency compromises HSC self-renewal
To stringently test the long-term self-renewal capacity of HSCs lacking mitochondrial Fh1, we performed serial transplantation assays. We transplanted 100 HSCs from FLs of Fh1£/£;FH5/5;Vav-iCre, Fh1£/£;Vav-iCre, and control 14.5-dpc embryos together with 200,000 support BM cells. Whereas Fh1£/£;Vav-iCre HSCs failed to repopulate the recipients, Fh1£/£;FH5/5;Vav-iCre HSCs contributed to primitive and more mature hematopoietic compartments of the recipient mice (Fig. 3 A). Primary recipients of Fh1£/£;FH5/5;Vav-iCre, Fh1£/£;Vav-iCre, and control 14.5-dpc embryos displayed efficient myeloid lineage reconstitution, whereas B and T lymphoid–lineage reconstitution was less robust (Fig. 3 B), suggesting that mitochondrial Fh1 is required for lymphoid cell differentiation or survival. Next, we sorted BM LSK cells from the primary recipients and retransplanted them into secondary recipients. Fh1£/£;FH5/5;Vav-iCre HSCs failed to contribute to the BM hematopoietic compartments of the recipients 20 wk after transplantation (Fig. 3 C). Therefore, HSCs lacking mitochondrial Fh1 display progressive loss of self-renewal potential upon serial transplantation.

Hematopoietic defects upon Fh1 deletion
are not caused by oxidative stress or the activation of Nrf2–dependent pathways
Because elevated cellular fumarate is a major cause of hematopoietic defects in Fh1£/£;Vav-iCre FLs, we next explored potential mechanisms through which fumarate impairs FL hematopoiesis. In nonhematopoietic tissues, fumarate succinates cysteine residues of biologically active molecules (including glutathione [GSH]; Sullivan et al., 2013; Zheng et al., 2015) and numerous proteins (Adam et al., 2011; Ter-Nette et al., 2013). Elevated fumarate causes oxidative stress by succinizing GSH and, thus, generating succinic GSH and depleting the GSH pool (Sullivan et al., 2013; Zheng et al., 2015). We found that reactive oxygen species (ROS) were modestly increased in Fh1£/£;Vav-iCre FL c-Kit+ cells compared with control cells (Fig. 4 A). The quantity of succinic GSH was elevated in Fh1£/£;Vav-iCre FL c-Kit+ cells (Fig. 4 B), but succinic GSH constituted only ~1.5% of the total pool of GSH species (Fig. 4 C). Furthermore, we found that administration of the antioxidant N-acetylcysteine (NAC) to timed-mated pregnant females did not rescue the reduced FL cellularity (not depicted) and failed to reverse decreased numbers of Lin− FL cells (Fig. 4 D). Finally, HSCs sorted from NAC-treated Fh1£/£;Vav-iCre FLs failed to reconstitute hematopoiesis in NAC-treated recipient mice (Fig. 4 E). Therefore, oxidative stress caused by GSH depletion does not cause hematopoietic defects resulting from fumarate accumulation.

Loss of Fh1 in renal cysts is associated with up-regulation of the Nrf2-mediated antioxidant response pathway because of fumarate-mediated succination of Keap1, which normally promotes Nrf2 degradation (Adam et al., 2011). However, the analyses of global gene expression profiling of FL Lin− c-Kit+ primitive hematopoietic cells from 14.5-dpc Fh1£/£;Vav-iCre and control embryos revealed no significant enrichment for Nrf2 signature (Fig. 4 F). Thus, the activation of the Nrf2-dependent pathways is not responsible for defective hematopoiesis in Fh1£/£;Vav-iCre FLs.

Fh1 deficiency in primitive hematopoietic cells has no impact on the Hif-1–dependent pathways and does not affect global 5-hydroxymethylcytosine levels
Fumarate is known to competitively inhibit 2-oxoglutarate (2OG)–dependent oxygenases including Hif prolyl hydroxylase Phd2 resulting in stabilization of Hif-1α (Adam et al., 2011). Given that Phd2 deletion and stabilization of Hif-1α results in HSC defects (Takubo et al., 2010; Singh et al., 2013), we asked whether elevated fumarate increases...
the Hif-1α protein levels upon Fh1 deletion in primitive hematopoietic cells. We found that Hif-1α protein was undetectable in Fh1<sup>+/fl</sup>;Vav-iCre and control FL c-Kit<sup>+</sup> cells (Fig. 4 G), and additional deletion of Hif-1α in Fh1-deficient embryos failed to rescue embryonic lethality (not depicted), restore total FL cellularity, reverse decreased FL Lin<sup>−</sup> cell numbers, or normalize elevated numbers of FL HSCs (Fig. 4 H). Thus, Hif-1α is not involved in generating hematopoietic defects in Fh1<sup>+/fl</sup>;Vav-iCre FLs.

**Figure 3.** Mitochondrial Fh1 is essential for HSC self-renewal. (A–C) 100 FL HSCs were transplanted into lethally irradiated 8–10-wk-old C57BL/6 CD45.1<sup>−</sup>/CD45.2<sup>−</sup> recipient mice together with 2 × 10<sup>5</sup> CD45.1<sup>+</sup> syngeneic competitor BM cells. The primary recipients were analyzed 20 wk after transplantation. 2,000 CD45.2<sup>+</sup>LSK cells were sorted from their BM and transplanted into secondary recipients together with competitor BM cells. Secondary recipients were analyzed 20 wk after transplantation. (A) Percentage of CD45.2<sup>+</sup> cells in the Lin<sup>+</sup>, Lin<sup>−</sup>, LK, LSK, and HSC compartments in the BM of primary recipients. n = 4–5 recipients per donor. (B) Percentage of CD45.2<sup>+</sup> cells in the monocyte, neutrophil, B cell, and T cell compartments in the PB of primary recipients. n = 4–5 recipients per donor. Number of donors used in A and B: control, n = 6; Fh1<sup>+/fl</sup>;Vav-iCre, n = 2; Fh1<sup>+/fl</sup>;Vav-iCre, n = 2; control;FHCyt, n = 6; Fh1<sup>+/fl</sup>;FHCyt;Vav-iCre, n = 3; Fh1<sup>+/fl</sup>;FHCyt;Vav-iCre, n = 3. (C) Percentage of CD45.2<sup>+</sup> cells in the Lin<sup>+</sup>, Lin<sup>−</sup>, LK, LSK, and HSC compartments in the BM of the secondary recipients. n = 4–5 recipients per donor. Number of donors: control, n = 3; control;FHCyt, n = 4; Fh1<sup>+/fl</sup>;FHCyt;Vav-iCre, n = 2; Fh1<sup>+/fl</sup>;FHCyt;Vav-iCre, n = 3. Data are mean ± SEM. *, P < 0.05; **, P < 0.01 (Mann-Whitney U test).

**Fh1 deletion results in increased histone H3 trimethylation in primitive hematopoietic cells**

Emerging evidence indicates that 2OG-dependent JmjC domain–containing histone demethylases (KDMs) play important roles in HSC biology and hematopoiesis (Stewart et al., 2015; Andricovich et al., 2016). Given that fumarate inhibits enzymatic activity of KDMs (Xiao et al., 2012), we examined the abundance of H3K4me3, H3K9me3, H3K27me3, and H3K36me3 in nuclear extracts from FL c-Kit<sup>+</sup> cells isolated from 14.5-dpc Fh1<sup>+/fl</sup>;Vav-iCre and control embryos. Western blot analyses revealed an increase in levels of H3K4me3, H3K27me3, and H3K36me3 in nuclear extracts from FL c-Kit<sup>+</sup> cells isolated from 14.5-dpc Fh1<sup>+/fl</sup>;Vav-iCre and control embryos. Western blot analyses revealed an increase in levels of H3K4me3, H3K27me3, and H3K36me3 but not H3K4me3 in Fh1-deficient cells (Fig. 4, I and J). Although these data suggest that Fh1 deficiency results in enhanced trimethylation of H3, the identity of KDMs that are inhibited by fumarate and the causal roles for increased H3 trimethylation in me-
Figure 4. Molecular consequences of Fh1 deletion in primitive hematopoietic cells. (A) Intracellular ROS in FL c-Kit+ cells. The mean of mean fluorescence intensities ± SEM is shown. Control, n = 6; Fh1fl/fl;Vav-iCre, n = 3. (B and C) GSH species in 14.5-dpc FL c-Kit+ cells measured using LC-MS. Succinic GSH levels (arbitrary units; B) and percentage of Succinic GSH within the total GSH species (C) are shown. Control, n = 6; Fh1fl/fl;Vav-iCre, n = 3.
diating HSC and hematopoietic defects upon \( Fh1 \) deletion remain to be elucidated.

**Fh1 deletion promotes a gene expression signature that facilitates hematopoietic defects**

To understand the molecular signatures associated with \( Fh1 \) deficiency, we performed gene expression profiling of FL Lin\(^{-}\)c-Kit\(^{+}\) primitive hematopoietic cells from \( Fh1^{+/+}, \text{Vav-iCre} \) and control embryos. We found that genes up-regulated in \( Fh1 \)-deficient cells are highly enriched in categories related to apoptosis in response to ER stress, protein metabolic process/protein translation, and unfolded protein response (Fig. 4, K and L). Down-regulated genes are enriched in pathways related to heme biosynthesis, erythroid and myeloid function, and the cell cycle (Fig. 4 K). Although further detailed work will be needed to experimentally verify this, we propose that enhanced ER stress, unfolded protein response, and increased protein translation (which are known to contribute to HSC depletion and hematopoietic failure; Miharada et al., 2014; Signer et al., 2014; van Galen et al., 2014) may be responsible for hematopoietic defects upon \( Fh1 \) deletion.

**Fh1 deficiency abolishes leukemic transformation and LIC functions**

Although FH is a tumor suppressor (i.e., \( FH \) mutations result in hereditary leiomyomatosis and renal-cell cancer) and fumarate is proposed to function as an oncometabolite (Yang et al., 2013), the role of FH in leukemic transformation remains unknown. Given that human acute myeloid leukemia (AML) cells express high levels of FH protein (López-Pedrera et al., 2006; Elo et al., 2014) and enzymatic activity of FH is increased in AML samples compared with cells from normal controls (Tanaka and Valentine, 1961), we next investigated the role for \( Fh1 \) in leukemic transformation. We used a mouse model of AML in which the development and maintenance of LICs is driven by \( Meis1 \) and \( Hoxa9 \) oncogenes (Wang et al., 2010; Vukovic et al., 2015). \( Meis1 \) and \( Hoxa9 \) are frequently overexpressed in several human AML subtypes (Lawrence et al., 1999; Drabkin et al., 2002), and their overexpression in mouse hematopoietic stem and progenitor cells generates self-renewing LICs (Kroon et al., 1998). In the \( Meis1/Hoxa9 \) model used here, the FL LSK or c-Kit\(^{+}\) cell populations are transduced with retroviruses expressing \( Meis1 \) and \( Hoxa9 \) and are serially replated, generating a preleukemic cell population, which, upon transplantation to primary recipients, develops into LICs causing AML. LICs are defined by their capacity to propagate AML with short latency in secondary recipients (Somerville and Cleary, 2006; Yeung et al., 2010; Vukovic et al., 2015). We found that \( Fh1^{+/+}, \text{Vav-iCre} \) FL stem and progenitor cells transduced with \( Meis1/Hoxa9 \) (Fig. 5 A) failed to generate colonies in methylcellulose (Fig. 5 B). To corroborate these findings, we used retroviruses expressing MLL fusions which are frequently found in acute monoblastic leukemia (AML M5) and are associated with an unfavorable prognosis in AML, namely MLL-ENL (fusion oncogene resulting from t[11;19]) and MLL-AF9 (resulting from t[9;11]; Krivtsov and Armstrong, 2007; Lavallée et al., 2015). We also used AML1-ETO9a, a splice variant of AML1-ETO that is frequently expressed in t(8;21) patients with AML M2, and its high expression correlates with poor AML prognosis (Jiao et al., 2009). MLL fusions and AML1-ETO9a drive leukemogenesis through distinct pathways and are frequently used to transform mouse hematopoietic cells (Zuber et al., 2009; Smith et al., 2011; Velasco-Hernandez et al., 2014). We found that \( Fh1 \)-deficient cells transduced with MLL-ENL, MLL-AF9, and AML1-ETO9a were unable to generate colonies (Fig. 5 B), indicating the requirement for \( Fh1 \) in vitro transformation. Next, we determined the impact of \( Fh1 \) deletion on the colony formation capacity of preleukemic cells (Fig. 5 C). We transduced \( Fh1^{+/+} \) and \( Fh1^{+/+} \) FL LSK cells with \( Meis1 \) and \( Hoxa9 \) retroviruses, and after three rounds of replating, we infected the transformed cells with Cre lentiviruses. \( Fh1^{+/+} \) cells expressing Cre failed to generate colonies in CFC assays (Fig. 5 D). Thus, \( Fh1 \) deletion inhibits the generation of preleukemic cells and abolishes their clonogenic capacity.

To determine the requirement for \( Fh1 \) in LICs, LSK cells from \( Fh1^{+/+} \) and \( Fh1^{+/+} \) FLs were transduced with \( Meis1 \) and \( Hoxa9 \) retroviruses and transplanted into pri-
Fh1 is essential for HSC functions | Guitart et al.

Mary recipients (Fig. 5 C). LICs isolated from leukemic primary recipient mice were infected with Cre lentivirus and plated into methylcellulose. We found that Fh1-deficient LICs were unable to generate colonies (Fig. 5 E). Next, we investigated the requirement for FH in human established leukemic cells by knocking down the expression of FH in AML (M5) THP-1 cells harboring MLL-AF9 translocation. We generated lentiviruses expressing three independent short hairpins (i.e., FH shRNA 1–3) targeting FH and β-actin in THP-1 cells described in Fig. 5 F. (H) Apoptosis assays performed with THP-1 cells transduced with lentiviruses expressing scrambled shRNA, FH shRNA1, and FH shRNA3. The graph depicts the percentage of annexin V+DAPI− cells in early apoptosis and annexin V+DAPI+ in late apoptosis. n = 4. (I) CFC assays with THP-1 cells expressing scrambled shRNA, FH shRNA1, and FH shRNA3. n = 5. Data are mean ± SEM. *, P < 0.05 (Mann-Whitney U test).

Mitochondrial Fh1 is necessary for AML development but is not required for disease maintenance

Next, we investigated the impact of mitochondrial Fh1 deficiency on in vitro transformation and development and maintenance of LICs (Fig. 6, A–C). FL Fh1fl/fl;FHCyt;Vav-iCre cells transduced with Meis1/Hoxa9 retroviruses had normal serial replating capacity (Fig. 6 B), and the established preleukemic cells had normal proliferative capacity and cell-cycle status (not depicted). Thus, elevated fumarate is largely responsible for the inability of Fh1fl/fl;Vav-iCre stem and progenitor cells to undergo in vitro transformation. To establish the requirement for mitochondrial Fh1 in AML development in vivo, we transplanted control (Fh1fl/fl), control; FHCyt, and Fh1fl/fl;FHCyt;Vav-iCre Meis1/Hoxa9-transduced preleukemic cells into sublethally irradiated recipient mice (Fig. 6 A). We found that the percentage of recipients of Fh1fl/fl;FHCyt;Vav-iCre cells that developed terminal AML...
was significantly reduced (and the disease latency was extended) compared with recipients of control and control;Fh1fl/fl cells (Fig. 6C). Finally, OCR measurements in LICs sorted from those recipients of Fh1fl/fl;FHCyt;Vav-iCre cells that succumbed to AML revealed that Fh1fl/fl;FHCyt;Vav-iCre LICs had defective maximal mitochondrial respiration compared with control and control;FHCyt LICs (not depicted). Thus, mitochondrial Fh1 is required for efficient generation of LICs and AML development in a Meis1/Hoxa9-driven model of leukemogenesis.

Given that mitochondrial Fh1 was important for leukemia initiation, we next asked whether inducible deletion of mitochondrial Fh1 from leukemic cells impacts on leukemia propagation and LIC maintenance (Fig. 6, D–J). We transduced control and Fh1fl/fl;FHCyt;Mx1-Cre FL LSK cells with Meis1/Hoxa9 retroviruses, and after serial replating, the resultant preleukemic cells were transplanted into sublethally irradiated recipients. Once leukemic CD45.2+ cells reached 20% in the PB of recipient mice, the recipients received eight doses of plpC. 10,000 LICs (CD45.2+c-Kit+) from primary recipients were transplanted into secondary recipients. Kaplan-Meier survival curve of secondary recipients transplanted with LICs sorted from leukemic primary recipients. n = 10 per genotype. (J) Representative gel showing PCR amplification of genomic DNA from the total BM of secondary recipients with terminal leukemia.
**DISCUSSION**

By performing genetic dissection of multifaceted functions of the key metabolic gene *Fh1*, we have uncovered a previously unknown requirement for fumarate metabolism in the hematopoietic system. We conclude that efficient utilization of intracellular fumarate is required to prevent its potentially toxic effects and is central to the integrity of HSCs and hematopoietic differentiation. Furthermore, although fumarate promotes oncogenesis in the kidney (Yang et al., 2013), it has the opposite effect in the hematopoietic system, i.e., it inhibits leukemic transformation. Our data, indicating a detrimental impact of fumarate on hematopoiesis, collectively with fumarate's functions as an oncometabolite in nonhematopoietic tumors (Yang et al., 2013), or a protective role within the myocardium (Ashrafian et al., 2012), highlight distinct functions of fumarate in different tissues.

Elevated fumarate within the hematopoietic system is likely to perturb multiple biochemical mechanisms. Fumarate is known to inhibit 2OG-dependent oxygenases, including HIF-hydroxylase Phd2 (Hewitson et al., 2007), the Tet enzymes, and KDMs (Xiao et al., 2012), and as a consequence, tumor cells with FH mutations have increased HIF-1α stability and display a hypermethylator phenotype (Isaacs et al., 2005; Pollard et al., 2007; Letouzé et al., 2013; Castro-Vega et al., 2014). We found that hematopoietic defects resulting from elevated fumarate are most likely generated through the Hif-1α–independent and Tet-independent mechanisms. However, consistent with the ability of fumarate to inhibit KDMs, we found that levels of H3K9me3, H3K27me3, and H3K36me3 were elevated in primitive hematopoietic cells lacking *Fh1*. Although detailed underlying mechanisms remain to be elucidated, we propose that elevated fumarate may cause the observed phenotypes by inhibiting these KDMs that are essential for normal hematopoiesis and HSC functions, including KDM5B (Stewart et al., 2015) and KDM2B (Andricovich et al., 2016).

Fumarate is also known to cause succination of cysteine residues of numerous proteins (e.g., Keap1, which normally promotes Nrf2 degradation; Adam et al., 2011; Ternette et al., 2013) or GSH (Sullivan et al., 2013; Zheng et al., 2015). However, our data indicate that hematopoietic defects upon *Fh1* deletion are unlikely to be mediated by Nrf2 activation or GSH depletion. Given our findings that *Fh1*-deficient cells have increased signatures of ER stress and unfolded protein response, it will be of high interest to determine whether increased global protein succination in *Fh1*-deficient cells results in protein misfolding in HSCs, leading to the activation of unfolded protein response, which is detrimental to HSC integrity (van Galen et al., 2014).

*Fh1* deletion with the simultaneous reexpression of cytosolic FH allowed us to investigate the genetic requirement for mitochondrial FH1 in long-term HSC functions. Our serial transplantation assays revealed that mitochondrial Fh1 was essential for HSC self-renewal, indicating a key role for an intact TCA cycle in HSC maintenance. Intriguingly, although mitochondrial Fh1 deficiency did not affect myeloid output under steady-state conditions and upon transplantation, the lack of mitochondrial Fh1 had an impact on the lymphoid output. These data imply differential requirements for the intact TCA cycle in lineage commitment and/or differentiation of primitive hematopoietic cells, meriting further investigations.

Both self-renewing HSCs and LICs are thought to rely heavily on glycolysis while they suppress the TCA cycle (Simsek et al., 2010; Wang et al., 2014). We used the genetic mitochondrial Fh1 deficiency to examine the differential requirement for mitochondrial Fh1 in long-term HSC self-renewal and the development and maintenance of LICs. We conclude that self-renewing HSCs critically require intact mitochondrial Fh1 and the capacity for maximal mitochondrial respiration to maintain their pool. However, although mitochondrial Fh1 was necessary for LIC development, it had no impact on maintenance of LICs. Thus, we reveal a differential requirement for the mitochondrial TCA enzyme Fh1 in normal hematopoiesis and Meis1/Hoxa9-driven leukemia propagation. The discovery of mechanisms underlying different metabolic requirements in HSCs and LICs represents a key area for future investigations.

**MATERIALS AND METHODS**

**Mice**

All mice were on a C57BL/6 genetic background. *Fh1<ΔΔ>* (Pollard et al., 2007), *Hif-1α<ΔΔ>* (Ryan et al., 2000; Vukovic et al., 2016), and V5-FH<ΔΔ> (referred to as FH<ΔΔ>; Adam et al., 2013) were described previously. Vav-<c>Cre</c> and Mxl-<c>Cre</c> were purchased from The Jackson Laboratory. All transgenic and knockout mice were CD45.2<sup>+</sup>. Congenic recipient mice were CD45.1<sup>+</sup>/CD45.2<sup>+</sup>. All experiments on animals were performed under UK Home Office authorization.

**Flow cytometry**

All BM and FL samples were stained and analyzed as described previously (Kranc et al., 2009; Mortensen et al., 2011; Guitart et al., 2013; Vukovic et al., 2016). BM cells were obtained by crushing tibias and femurs with a pestle and mortar. FL cells were obtained by mashing the tissue through a 70-µm strainer. Single-cell suspensions from BM, FL, or PB were incubated with Fc block and then stained with antibodies. For HSC analyses, after incubation with Fc block, unfractionated FL or...
BM cell suspensions were stained with lineage markers containing biotin-conjugated anti-CD4, anti-CD5, anti-CD8a, anti-CD11b (not used in FL analyses), anti-B220, anti-Gr-1, and anti-Ter119 antibodies together with APC-conjugated anti-c-Kit, APC/Cy7-conjugated anti-Sca-1, PE-conjugated anti-CD48, and PE-Cy7-conjugated anti-CD150 antibodies. Then, biotin-conjugated antibodies were stained with Pacific blue–conjugated or PerCP-conjugated streptavidin. To distinguish CD45.2+ donor–derived HSCs in recipient mice, FITC-conjugated anti-CD45.1 and Pacific blue–conjugated anti-CD45.2 antibodies were included in the antibody cocktail. The multilineage reconstitution of recipient mice was determined by staining the BM or PB cell suspensions of the recipient mice with FITC-conjugated anti-CD45.1, Pacific blue–conjugated anti-CD45.2, PE-conjugated anti-CD4 and -CD8a, PE/Cy7-conjugated anti–Gr-1, APC-conjugated anti-CD11b, APC/Cy7–conjugated anti–CD19, and anti-B220. In all analyses, 7-AAD or DAPI was used for dead cell exclusion. Flow cytometry analyses were performed using an LSRFortessa flow cytometer (BD). Cell sorting was performed on a FACSAria Fusion cell sorter (BD).

CFC assays
CFC assays were performed using MethoCult (M3434; STEMCELL Technologies). Two replicates were used per group in each experiment. Colonies were tallied at day 10.

Leukemic transformation
LSK cells were sorted from FLs of 14.5-dpc embryos after c-Kit (CD117) enrichment using magnetic-activated cell-sorting columns (Miltenyi Biotec). 10,000 LSK cells were simultaneously transduced with mouse stem cell virus (MSCV)–Meis-1a-puro and MSCV–Hoxa9-neo retroviruses and subsequently subjected to three rounds of CFC assays in MethoCult (M3231) supplemented with 20 ng/ml stem cell factor, 10 ng/ml IL-3, 10 ng/ml IL-6, and 10 ng/ml granulocyte/macrophage stem cell factor. Colonies were counted 6–7 d after plating, and 2,500 cells were replated. Similarly, 200,000 FL c-Kit+ cells were transduced with MSCV–AMLL1-ETO9a-neo, MSCV–MLL–AF9-neo, or MSCV–MLL–ENL–neo and subsequently plated into methylcellulose.

Transplantation assays
Lethal irradiation of CD45.1+/CD45.2+ recipient mice was achieved using a split dose of 11 Gy (two doses of 5.5 Gy administered at least 4 h apart) at a mean rate of 0.58 Gy/min using a Cesium 137 irradiator (GammaCell 40; Best Theratronics). For sublethal irradiation, the recipient mice received a split dose of 7 Gy (two doses of 3.5 Gy at least 4 h apart). For primary transplantations, 100 HSCs (LSK CD48−CD150−CD45.2+) sorted from FLs of 14.5-dpc embryos or 200,000 unfractionated FL cells were mixed with 200,000 support CD45.1+ BM cells and injected into lethally irradiated (11 Gy delivered in a split dose) CD45.1+/CD45.2+ recipient mice. For secondary transplantations, 2,000 CD45.2+ LSK cells sorted from BM of primary recipients were mixed with 200,000 support CD45.1+ wild-type BM cells and retransplanted. For adult BM transplantations, 500,000 CD45.2+ BM cells were mixed with 500,000 support CD45.1+ wild-type BM cells and injected into lethally irradiated CD45.1+/CD45.2+ recipient mice. All recipient mice were analyzed 18–20 wk after transplantation, unless otherwise stated.

For leukemia induction, 100,000 Meis-1/Hoxa9-transduced c-Kit+ cells were transplanted into CD45.1+/CD45.2+ sublethally irradiated (7 Gy delivered in a split dose) recipient mice. The mice were monitored for AML development. For secondary transplantation, 10,000 LICs (CD45.2−c-Kit+ cells) were sorted from BM of primary recipients and transplanted into secondary CD45.1+/CD45.2+ sublethally irradiated recipient mice.

Inducible Mx1-Cre–mediated gene deletion
Mice were injected intraperitoneally six to eight times every alternate day with 300 µg pIpC (GE Healthcare) as previously described (Kruczn et al., 2009; Guitart et al., 2013).

Administration of NAC
Pregnant females received 30 mg/ml of NAC (Sigma-Aldrich) in drinking water (pH was adjusted to 7.2–7.4 with NaOH). For transplantation experiments, CD45.1+/CD45.2+ recipient mice were treated with 30 mg/ml NAC in drinking water 7 d before irradiation and remained under NAC treatment for the duration of the experiment. The water bottle containing NAC was changed twice per week.

Oxygen consumption assays
OCR measurements were made using a Seahorse XF-24 analyzer (Seahorse Bioscience) and the XF Cell Mito Stress Test kit as previously described (Wang et al., 2014). In brief, c-Kit+ cells from FLs of 14.5-dpc embryos were plated in XF-24 microplates precoated with cell-tak (BD) at 250,000 cells per well in XF Base medium supplemented with 2 mM pyruvate and 10 mM glucose, pH 7.4. OCR was measured three times every 6 min for basal value and after each sequential addition of oligomycin (1 µM), FCCP (1 µM), and finally concomitant rotenone and antimycin A (1 µM). Oxygen consumption measurements were normalized to cell counts performed before and after each assay.

Metabolite detection by liquid chromatography–mass spectrometry (LC-MS)
Metabolites from c-Kit+ cells from FLs of 14.5-dpc embryos were extracted into 50% methanol/30% acetonitrile and measured as previously described (Adam et al., 2013).

Western blotting
Protein extracted from FL c-Kit+ cells of 14.5-dpc embryos was subjected to a 10% SDS–PAGE and then transferred onto a polyvinylidene fluoride membrane and immunoblotted with
anti-Fh1, anti-2-SC, and anti–Hif-1α as previously described (Adam et al., 2011; Bardella et al., 2012). Anti-H3K4me3 (07-473; EMD Millipore), anti-H3K9me3 (ab8898; Abcam), anti-H3K27me3 (07-449; EMD Millipore), and anti-H3K36me3 (ab9050; Abcam) were used to determine levels of trimethylated H3. Anti-actin (A5316; Sigma-Aldrich), anti-tubulin (2146S; Cell Signaling Technology), and anti–H3 (ab1791; Abcam) immunobLOTS were used as loading controls.

RT–quantitative PCR
Gene expression analyses were performed as described previously (Kranc et al., 2009; Mortensen et al., 2011; Guitart et al., 2013). Differences in input cDNA were normalized with Actb (β-actin) expression.

ATP production
10,000 c-Kit+ cells from FLs of 14.5-dpc embryos were cultured in DMEM supplemented with either 25 mM glucose or 25 mM galactose. At 0 and 24 h after incubation, the cells were lysed, and ATP content was measured by luminescence or 25 mM galactose. At 0 and 24 h after incubation, the cells were lysed, and ATP content was measured by luminescence using CellTiterGlo Assay (Promega).

Measurement of mitochondrial membrane potential
50,000 FL c-Kit+ cells from 14.5-dpc embryos were incubated for 15 min at 37°C in 25 nM tetramethylrhodamine methyl ester (T-668; Thermo Fisher Scientific) and analyzed using the LSRFortessa flow cytometer.

Gene expression profiling and bioinformatics analyses
RNA from sorted FL Lin c-Kit+ cells was isolated by standard phenol/chloroform extraction. cDNA was synthesized from 50 ng of total RNA using the Ambion WT Expression kit (Thermo Fisher Scientific). Labeled, fragmented cDNA (GeneChip WT Terminal Labeling and Controls kit; Affymetrix) was hybridized to Mouse Gene 2.0 arrays for 16 h at 45°C and 60 rpm (GeneChip Hybridization, Wash, and Stain kit; Affymetrix). Arrays were washed and stained using the Fluidics Station 450 (Affymetrix) and scanned using a GeneArray Scanner (3000 7G; Hewlett-Packard). The microarray gene expression data have been deposited in the ArrayExpress database under accession no. E-MTAB-5425.

For bioinformatics analyses, a total of seven arrays (n = 3 Fh1+/Δn; n = 4 Fh1+/Δn;Vav-iCre) were quality control analyzed using the arrayQualityMetrics package in Bioconductor. Normalization of the 29,638 features across all arrays was achieved using the robust multarray average expression measure. Pairwise group comparisons were undertaken using linear modeling (LIMMA package in Bioconductor). Subsequently, empirical Bayesian analysis was applied, including vertical (within a given comparison) p-value adjustment for multiple testing, which controls for false discovery rate.

Gene set enrichment analysis (GSEA)
Gene expression differences were ranked by difference of log expression values, and this ranking was used to perform GSEA (Subramanian et al., 2005) on gene lists in the Molecular Signatures Database (MSigDB; version 5.2). The following datasets were used for analyses presented in Fig. 4: (a) hallmark, unfolded protein response; (b) gene ontology, intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress; (c) reactome, translation; and (d) NFE2L2.V2.

shRNA-mediated FH knockdown
THP-1 cells were transduced with lentiviruses expressing shRNAs (shRNA1, 5′-TAATCTGTGTTTCACTACGG-3′ [TRCN0000052463]; shRNA2, 5′-AAGGTATCATATGTCATCCGG-3′ [TRCN0000052464]; shRNA3, 5′-TTTATTAAACATGATCGTGGG-3′ [TRCN0000052465]; and shRNA Scr, 5′-TTCCTCCGAAGCTGTCACGTT-3′; RNAi Consortium; GE Healthcare). Transduced THP-1 cells were grown in the presence of 5 µg/ml puromycin.

ROS analysis
c-Kit+ cells were stained with 2.5 nM CellROX (C10491; Thermo Fisher Scientific) based on the manufacturer’s protocol and analyzed by FACS.

Statistical analyses
Statistical analyses were performed using Prism 6 (GraphPad Software). P-values were calculated using a two-tailed Mann-Whitney U test unless stated otherwise. Kaplan-Meier survival curve statistics were determined using the log-rank (Mantel-Cox) test.

Online supplemental material
Table S1 shows neutropenia in patients with recessive FH mutations. Table S2 shows hematopoiesis-specific Fh1 deletion results in embryonic lethality. Table S3 shows FF537 rescues embryonic lethality in Fh1−/Δn,Vav-iCre mice.

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