HHS Public Access

Author manuscript

*Exp Hematol*. Author manuscript; available in PMC 2024 April 26.

Published in final edited form as:

*Exp Hematol*. 2023 ; 125-126: 25–36.e1. doi:10.1016/j.exphem.2023.08.001.

# Myeloid *Hif2α* is not essential to maintain systemic iron homeostasis.

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## Abstract

Dietary consumption serves as the primary source of iron uptake, while erythropoiesis acts as a major regulator of systemic iron demand. In addition to intestinal iron absorption, macrophages play a crucial role in recycling iron from senescent red blood cells. The kidneys are responsible for the production of erythropoietin (*Epo*), which stimulates erythropoiesis, while the liver plays a central role in producing the iron-regulatory hormone hepcidin. The transcriptional regulator hypoxia-inducible factor (HIF)2α has a central role in the regulation of *Epo*, hepcidin, and intestinal iron absorption and therefore plays a crucial role in coordinating the tissue crosstalk to maintain systemic iron demands. However, the precise involvement of *Hif2α* in macrophages in terms of iron homeostasis remains uncertain. Our study demonstrates that deleting *Hif2α* in macrophages does not disrupt the expression of iron transporters or basal erythropoiesis. Mice lacking *Hif2α* in myeloid cells exhibited no discernible differences in hemodynamic parameters, including hemoglobin levels and erythrocyte count, when compared to littermate controls. This similarity was observed under conditions of both dietary iron deficiency and acute erythropoietic demand. Notably, we observed a significant increase in the expression of iron transporters in the duodenum during iron deficiency, indicating heightened iron absorption. Therefore, our findings suggest that the disruption of *Hif2α* in myeloid cells does not significantly impact systemic iron homeostasis under normal physiological conditions. However, its disruption induces adaptive

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Contribution: C.J. and Y.M.S. conceived and designed the study; C.J., S.P., W.H., S.H., R.S., and K.E.L., developed the methodologies; C.J., S.P., W.H., S.H., R.S., and N.K.D. acquired the data; C.J., S.P., W.H., S.H., R.S., N.K.D., and K.E.L. analyzed and interpreted the data; K.E.L. provided the key reagents; C.J. and Y.M.S. supervised the study and wrote the manuscript; and all authors edited and provided inputs to the manuscript.

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

physiological changes in response to elevated iron demand, potentially serving as a mechanism to sustain increased erythropoietic demand.

**Keywords**

macrophage; hypoxia; HIF; iron; anemia; hemochromatosis

## Introduction

Systemic iron homeostasis is tightly regulated as both excess and deficiency of iron have deleterious effects. Despite tight systemic regulation, more than one billion people worldwide suffer from iron related disorders [1; 2]. Systemic iron homeostasis requires hetero-tissue communication. Diet is the sole source of iron, and it is actively absorbed in the small intestine through the transporter divalent metal transporter-1 (*Dmt1*; gene name *Slc11a2*) [3; 4]. Iron is stored within enterocyte in an iron storage protein, ferritin (FTN) or exported out of the cell and into circulation through the only known mammalian iron exporter ferroportin (*Fpn*; gene name *Slc40a1*)[5; 6]. Expression of *Fpn*, on the basolateral membrane is regulated at post translational level by a hepatic hormone called hepcidin (gene name *Hamp*)[7]. Under high systemic iron levels or iron overload conditions expression of hepcidin is induced in hepatocytes. Increased hepcidin expression degrades or blocks *Fpn*-mediated iron export and limits iron absorption in the intestine [8; 9; 10; 11]. Perturbations in this intestinal *Fpn*/hepatic hepcidin axis induce systemic iron overload or iron deficiency [12].

The vast majority of iron is used for synthesis of red blood cells (RBC) as an essential component of oxygen carrying protein hemoglobin. Iron and oxygen coordination are central to maintain the production of RBCs. Hypoxia-inducible factor (HIF)2α is an iron and oxygen regulatable transcription factor and a critical regulator of iron regulatory genes [13]. Intestinal disruption of *Hif2α* abrogates dietary iron absorption [14]. More, recently a heterocellular crosstalk between hepatic hepcidin and intestinal *Hif2α* was identified to be instrumental in maintaining iron flux through the duodenal epithelium [15]. *Hif2α* also plays an essential role in regulating the expression of erythropoietin (*Epo*)[16]. Liver is the primary source of *Epo* embryonically, whereas the kidney is major tissue to maintain *Epo* levels in adults [17]. Both hepatic and renal *Epo* expression is regulated by *Hif2α* [18]. Mice lacking *Hif2α* in the kidney show lower circulating levels of *Epo* and are highly anemic [19]. Lastly, *Hif2α* activation of *Epo* is a major repressor of hepatic hepcidin further increasing iron absorption to maintain RBC synthesis [20; 21]. Activation of *Hif2α* by pharmacological drugs reduced hepcidin production and increase iron absorption in the intestine [22]. Moreover, the untranslated region of *Hamp* contains *Hif2α* binding HRE elements and deletion of these HREs lead to loss to regulation of hepcidin by systemic iron cues [23].

Apart from the intestine, liver and kidney, macrophages are central to maintain systemic iron by recycling iron from senesced RBCs. Macrophage iron stores are mobilized in response to systemic iron levels through the *Fpn*-hepcidin axis very similar to that in the duodenum [24; 25]. However, the role of *Hif2α* in regulating iron recycling is not well understood. Moreover, with clinically relevant agents that can increase *Hif2α* activity[26.27] or inhibit its function[28],

it is critical to understand which cell types are essential to maintain cellular and systemic iron homeostasis following modulation of HIF2α.

In this study we identify that macrophage *Hif2a* does not play a role in regulating systemic iron homeostasis. We report no changes in systemic iron levels, erythrocytes count as well as hemoglobin levels under basal or iron deficient conditions. Moreover, *Hif2a* in macrophages was not essential in maintaining RBC levels following an erythropoietic stimuli. However, under iron deficiency, mice disrupted for *Hif2a* in the myeloid cells showed significantly increased expression of iron transporters involved in the intestinal iron absorption. We speculate this could be a compensatory adaptation to maintain erythropoiesis under potentially impaired iron recycling through the macrophages.

## Methods

### Animals and treatments.

For myeloid-specific disruption of *Hif2a*, mice floxed for *Hif2a* (*Hif2a*$^{fl/fl}$) on a C57BL/6J background were crossed with C57BL/6J mice harboring Cre recombinase under the control of lysozyme 2 gene (*Lyz2*) (LysM$^{Cre}$) to generate *LysM*$^{Cre}$ *Hif2a* $^{fl/fl}$ mice. WT littermates were used as controls for all animal studies (*Hif2a*$^{fl/f}$), and analysis began on mice that were between 2 and 2.5 months of age for each of the respective experiments. Phz (Sigma-Aldrich) was administered via i.p. injection at a dose of 60 mg/kg BW, as described previously [34]. All mice were fed ad libitum and maintained under a 12-hour light/12-hour dark cycle in a specific pathogen free environment. All mice were fed either a standard chow diet (Research Diets) or a purified AIN-93G iron-replete (350 ppm) or low-iron (<5 ppm) diet (Diets). All mice were housed in the Unit for Laboratory Animal Management (ULAM) at the University of Michigan.

### Hematological and iron analysis.

The Unit for Laboratory Animal Medicine Pathology Core at The University of Michigan performed the complete blood count analysis. Serum iron was measured as described in Das et.al 2015 [35].

### Bone Marrow-derived macrophage (BMDM) isolation and culture

Male and female *LysM*$^{Cre}$ *Hif2a* $^{fl/fl}$ mice or wild type littermates were euthanized by cervical dislocation and the femur bones were dissected. The extra muscles were removed, and the bone marrow was extracting by cutting the end of the bones and flushing with 10mL sterile PBS using a 25-gauge needle in a sterile petri-dish. The extracted bone marrow was passed through a 70μm cell strainer. The suspension was centrifuged at 250g for 5 minutes to pellet the cells. The pelleted cells were resuspended in 9mL of water to lyse all the erythroid progenitor cells, followed by addition of 1mL of 10X PBS immediately after. The remaining cells were counted and resuspended and plated at a cell density of $2x10^6$ cells/10 mL in complete RPMI media containing 10%FBS, 1% antibiotic-antimycotic and 20ng/mL GM-CSF in a 10cm petri dish. The cells were cultured in 37°C incubator with 5% $CO_2$ with media change every 3 days.

### BMDMs culture and treatment

The BMDMs maintained in an $M_0$ stage were plated on day 10 after the isolation and were treated with LPS 20ng/mL (Sigma L4391-1mg) or IL4 20ng/mL (Gibco 2141420UG) for 48 hours to polarize to M1 and M2, respectively.

### Quantitative reverse transcription PCR.

Total RNA was extracted from the BMDMs, liver, kidney and small intestine using the Trizol reagent. 1ug of the total RNA was reverse transcribed to cDNA using SuperScriptTM III First-Strand Synthesis System (Invitrogen). mRNA was measured by quantitative reverse transcription PCR (qPCR) (Life Technologies, Thermo Fisher Scientific). The primers used are listed in Supplemental Table 1. Quantification cycle (Cq) values were normalized to β-actin and expressed as the fold change.

**Inductively coupled plasma mass spectrometry (ICP-MS).—**Total cellular iron was quantification was performed as previously described in Das et. 2022[36]. BMDMs were isolated and cultured from the Male and female *LysM*$^{Cre}$ *Hif2α* $^{fl/fl}$ mice or wild type littermates as described above and the BMDMs were digested with 2 ml/g total cell pellet weight nitric acid (BDH Aristar Ultra) for 24 h and then digested with 1 ml/g total weight hydrogen peroxide (BDH Aristar Ultra) for 24 h at room temperature. Specimens were preserved at 4 °C until quantification of metals. Ultrapure water was used for the final sample dilution. Samples were analyzed using a PerkinElmer Nexion 2000 ICP-MS. For iron-57Fe measurements, 57Fe was dissolved in 0.4 mol/l H2SO4 to achieve a total concentration of 22.85 g/l overnight at a 37 °C shaking incubator.

### Flow Cytometry.

Blood, spleen, and bone-marrow were isolated from LysM$^{Cre}$ Hif2α$^{fl/fl}$ mice and their WT littermates. The blood was lysed with RBC lysis buffer (Gibco), centrifuged, and washed twice with FACS buffer (PBS +2%FBS). Spleen was homogenized and filtered with 70μM filter after which the solution was centrifuged and washed with the FACS buffer. Bone-marrow cells were differentiated to BMDMs as described above under '*Bone Marrow-derived macrophage (BMDM) isolation and culture*'. BMDMs were harvested using PBS with 10mM EDTA. Blood cells, splenic cells and BMDMs were stained for 45 minutes on ice with CD45 Alexa-eFluor 780, 1:200 (eBioscience) , CD11b APC 1:250 (eBioscience), Ly6C V450 1:200 (BD Bioscience), F4/80 BV510 1:100 (BD Bioscience), 7AAD Percp Cy 5.5 1:300 (BD Bioscience) and ferroOrange dye 1 μmol/l (Invitrogen). The cells were washed twice with FACS buffer, resuspended in PBS and 50,000 events was acquired on Cytek Aurora Spectral Analyzer (Cytek Biosciences). FSC-SSC gating was done to remove debris after which live single cells (negative 7AAD staining) were gated, and then leukocytes were identified using CD45 positivity. From the leukocyte population, monocytes (Ly6C and CD11b positive) and macrophages (F4/80 and CD11b positive) cells were gated. The mean fluorescence intensity of FerroOrange on monocyte/macrophage gate was used to evaluate the level of labile iron.

### Statistics.

Results are expressed as the mean ± SEM. Significance between 2 groups was tested using a 2-tailed, unpaired *t* test. Significance among multiple groups was tested using a 1-way or 2-way ANOVA followed by Tukey's post hoc test for multiple comparisons. A *P* value of less than 0.05 was considered statistically significant. GraphPad Prism 9.0 was used to conduct the statistical analyses.

### Study approval.

All animal procedures were approved by the IACUC of the University of Michigan.

## Results

### *Hif2α* deletion in macrophages does not alter expression of iron transporters.

To understand the role of *Hif2α* in maintaining macrophage and systemic iron levels, mice that express a constitutive Cre recombinase protein under the control of the lysozyme M promoter (*LysM*) were crossed with *Hif2α* floxed mice (*Hif2α*$^{\Delta m\varphi}$) (Figure 1A). The *Hif2α*$^{\Delta m\varphi}$ mice are healthy, viable and indistinguishable from wild-type littermates (*Hif2α*$^{fl/fl}$). *Hif2α* transcript was significantly decreased in BMDMs isolated from *Hif2α*$^{\Delta m\varphi}$ mice (Figure 1B). Consistent with prior work, *Hif2α* modulates macrophages cell states [37] and our data suggests that *Hif2α* regulates the anti-inflammatory response in the macrophages but not the pro-inflammatory response (Figure 1C). *Hif2α* is also established as a transcriptional regulator of intracellular iron uptake and subcellular distribution. However, BMDMs from *Hif2α*$^{\Delta m\varphi}$ mice did not show any significant alterations in the total cellular iron content measured by ICP-MS (Figure1D). Furthermore, macrophage expression of transferrin receptor (*Tfrc*), *Slc11a2* and *Slc40a1* were not altered in *Hif2α* $^{\Delta m\varphi}$ compared to wild-type littermates (Figure 1E). This data indicates that *Hif2α* does not play a significant role in regulating basal iron transporters in macrophages.

### *M*onocyte differentiation and intracellular labile iron are not altered following HIF2α in myeloid cells.

Macrophages are tissue resident immune cells and are integral to innate immune system. In the systemic circulation macrophages exist in the non-differentiated monocyte state and differentiate into activated macrophages within the tissues. To test whether *Hif2α*$^{\Delta m\varphi}$ mice exhibit any changes in the overall circulating/splenic monocyte population or BMDMs a flow cytometry-based approach was utilized.*Hif2α*$^{\Delta m\varphi}$ mice did not show any significant changes in the circulating monocytes, BMDMs, splenic monocytes and macrophages (Figure 2A–C). To assess the intracellular labile iron status (which is considered as accessible iron pool unlike total cellular iron which could be complexed with various macromolecules) a cell permeable labile iron dye specific to ferrous iron was utilized. No difference in the intracellular labile iron pool was observed in the *Hif2α*$^{\Delta m\varphi}$ mice as compared to wild-type littermate controls (Figure 2D–F). This data indicates that myeloid *Hif2α* is not essential for monocyte/macrophage differentiation and maintenance and does not contribute to intracellular iron homeostasis.

### *Hif2α* deletion in macrophages is not required for basal erythropoiesis.

Alterations in the systemic iron levels either due to iron deficiency, malabsorption of dietary iron or inflammation often manifest as anemia. We did not observe any obvious phenotypic differences in the *Hif2α* $^{\Delta m\varphi}$ mice as compared to wild-type littermates in 10-12 week old mice. To understand if *Hif2α* deletion in macrophages alters the systemic iron levels and cause anemia in older mice, 8 month old *Hif2α* $^{\Delta m\varphi}$ mice were utilized. The total serum iron levels remain unchanged in *Hif2α* $^{\Delta m\varphi}$ mice as compared to the wild-type littermate controls (Figure 3A) Complete blood count (CBC) results revealed no statistically significant changes in any RBC parameters such as RBC count, mean corpuscular volume (MCV), Hemoglobin (HB) and Hematocrit (HCT) (Figure 3B). The *Hif2α* $^{\Delta m\varphi}$ mice showed normal total white blood, monocytes, neutrophils, and non-myeloid lymphocytes (Figure 3C). This data indicated that *Hif2α* is not essential in mobilizing iron stores from the macrophages under normal physiological conditions. Moreover, *Hif2α* deletion does not dysregulate hematopoiesis and maintenance of RBCs.

### Deletion of *Hif2α* does not alter the systemic response to acute iron and erythropoietic demand.

Erythropoiesis is an essential physiological process and is highly dependent on systemic iron availability. An increase in erythropoietic demand leads to an adaptive response inducing iron mobilization from hepatocytes and macrophages and increase in intestinal iron absorption [34; 38; 39; 40] . Several studies have shown the role of *Hif2α* in coordinating the intestinal and hepatic response to iron deficiency or erythropoietic demand [16; 18; 41; 42; 43]. To determine whether *Hif2α* plays a role in mobilization of iron stores in the macrophages under acute iron demand we utilized a phenyl hydrazine-induced hemolytic anemia model (Figure 4A). Phenyl hydrazine induce hemolytic anemia causes systemic inflammation due high levels of free hemoglobin and degraded products of hemoglobin from the lysed red corpuscles. *Hif2α* $^{\Delta m\varphi}$ and littermate controls showed signs of anemia, with reduced RBC numbers, HB, and HCT as compared to the mice treated with vehicle control (Figure 4B). Upon phenyl hydrazine treatment both groups showed increased MCV suggesting accumulation of circulating unhealthy erythrocytes (Figure 4B). However, no significant differences were observed within the *Hif2α* $^{\Delta m\varphi}$ and littermate controls. This data demonstrates that *Hif2α* is not essential for the mobilization of iron stores from macrophages under acute iron demand following hemolysis. The circulating immune cell levels upon Phenyl Hydrazine treatment also remain unchanged in both *Hif2α* $^{\Delta m\varphi}$ and the littermate controls (Figure 4C). Therefore, indicating that *Hif2α* in macrophages does not attenuate inflammatory response in hemolytic model.

### The systemic response to iron deficiency is not altered under *Hif2α* deletion in the myeloid cells.

Macrophages play a crucial role in clearing senescent RBCs and releasing the erythrocytic iron back into circulation. To determine whether *Hif2α* is essential for the mobilization of macrophage iron stores under iron stress, we used a diet-induced iron deficiency model. This model causes mild to moderate iron deficiency in 2 weeks, with mice showing signs of anemia after 4 weeks. We placed 12-week old *Hif2α*$^{\Delta m\varphi}$ mice and littermate controls on

standard (350ppm) or iron-deficient (5ppm) diet (Figure 5A). We regularly monitored the food intake of the mice to ensure that any observed differences were not due to variations in food consumption. At the end of the 2 weeks, the mice were euthanized and blood was collected to perform CBC and to assess serum iron levels. The serum iron did not show any significant differences in the *Hif2α*$^{\Delta m\varphi}$ mice and littermate controls or within the groups given standard (350ppm) or iron-deficient (5ppm) diet (Figure 5B) There were no significant differences in any of the hematological parameters measured such as RBC count, MCV, HB and HCT (Figure 5C). As mentioned previously, anemia is often a late symptom of chronic systemic iron deficiency. To account for any late stage effects of *Hif2α* deletion, we repeated the experiment in 8 month old mice. In the older mice, which had *Hif2α* deletion for a longer period of time, there were no statistically significant changes in the serum iron levels (Figure 5D). A similar trend was observed in RBC number, MCV, HB and HCT levels (Figure 5E). These results indicate that *Hif2α* in macrophages does not regulate the mobilization of iron stores iron deficiency and does not alter the physiological response to iron deficiency. However, long term effects of iron deficiency would need to be assessed with a long term dietary intervention.

### *Hif2α* deletion in the macrophages induce dietary iron absorption under iron deficiency.

Mice with a myeloid specific *Hif2α* deletion do not show any differences in terms of systemic iron homeostasis under normal physiology as well as iron deficiency and erythropoietic demand. Under systemic iron deficiency as described previously several physiological adaptive changes occur to maintain erythropoietic output. These physiological changes include drop in hepatic hepcidin to ramp up intestinal iron absorption, increased renal erythropoietin expression to stimulate maturation of erythroid progenitor cells in the bone marrow and finally increased expression of luminal iron transporters. To determine whether there are other compensatory adaptive responses which maintain the erythropoietic output under *Hif2α* deletion in the macrophages, we measured mRNA expression of hepatic hepcidin *(Hamp)*, renal *Epo* and intestinal iron transporters. Dcytb (duodenal cytochrome b) is a luminal reductase expressed in the brush border cells of the duodenal epithelium which reduces ferric iron to ferrous form for efficient transport via *Dmt1 (Slc11a2)*. Both *Hif2α*$^{\Delta m\varphi}$ mice and littermate controls showed a decrease in the hepcidin transcript under iron deficiency, consistent with the previous reports regarding the role of hepcidin is regulating systemic iron homeostasis (Figure 6A) [7]. The levels of *Epo* in the kidney showed a moderate increase between the groups that received standard (350ppm) or iron-deficient (5ppm) diet however which was not statistically significant (Figure 6B). The expression of iron transporters *Slc11a2*, *Slc40a1* and *Tfrc* were significantly induced in the *Hif2α*$^{\Delta m\varphi}$ mice under iron deficiency as compared littermate controls (Figure 6C–F). This data suggests that the transcriptional changes seen in the intestine correspond to increased intestinal absorption of the dietary iron which then leads to maintenance of RBC counts under iron deficiency.

## Discussion

Systemic iron homeostasis is regulated through a multi-organ, heterocellular communication network between the intestine, liver, and kidney, in which *Hif2α* is thought to be a key

transcriptional regulator [44; 45]. In this study, we generated mice that were disrupted for *Hif2a* in macrophages via a myeloid specific Cre recombinase and assessed systemic iron homeostasis under various physiological conditions. We found that myeloid *Hif2a* does not significantly regulate systemic iron homeostasis in mice. Mice lacking *Hif2a* in macrophages did not develop symptoms associated with dysregulated iron homeostasis, such as anemia, and had normal red and white blood cell counts. These mice also responded to dietary iron deficiency similarly to wild type controls and did not have increased susceptibility to iron deficiency anemia. The disruption of *Hif2a* in macrophages during iron demand did not alter the systemic response, and there were no differences in RBC counts or hemoglobin levels observed. However, under iron deficiency mice lacking *Hif2a* in macrophages showed a significant increase in the expression of various iron transporters in the duodenum, associated with increased iron absorption.

Previous studies from our lab and other groups have shown that *Hif2a* is a major transcriptional regulator of iron homeostasis and induces expression of various iron transporters [13; 14; 34; 43; 46]. However, we show that deletion of *Hif2a* did not alter the expression of the major cellular iron transporters. The expression of these transporters is also under the control of other iron sensing pathways through iron regulatory protein 1 and 2 which could be the dominant regulator in macrophages [47; 48].

*Fpn* is the only known mammalian iron transporter and is essential in mobilization of iron stores from macrophages and contribute to systemic homeostasis by regulating iron recycling from the senescent erythrocytes. Deletion of *Fpn* in the macrophages leads to mild iron deficiency anemia [49]. *Fpn* deficient mice developed severe anemia upon PHZ treatment to induce acute hemolytic as well as under dietary iron deficiency, suggesting that iron recycling is essential for maintaining an adequate RBC count. This study also showed iron retention in liver and spleen suggesting impaired mobilization of iron stores which is previously shown to be dependent on *Fpn*. Similarly, under iron deficiency mice lacking *Hif2a* in macrophages showed a significant increase in the expression of various iron transporters in the duodenum, associated with increased iron absorption. This could be an adaptive response to maintain erythroid pool during iron deficiency when iron recycling is potentially disrupted upon *Hif2a* deletion.

*Fpn* is shown to be transcriptionally regulated by *Hif2a* in the intestine where iron deficiency in the enterocytes located on the intestinal epithelium stabilizes and activates *Hif2a* which then induces *Fpn* expression thereby ramping up iron absorption [15]. Intracellular iron deficiency in the enterocytes indicate systemic iron deficiency and therefore increase in *Fpn* would be an ideal response to increasing iron absorption. However, in the macrophages, induction of *Fpn* under cellular iron deficiency via *Hif2a* signaling would lead to increase iron export and further depletion of cellular iron which can be detrimental for the macrophages. Interestingly, apart from the transcriptional regulation, *Fpn* protein stability and export function are also regulated by the hepatic hepcidin [7; 50]. It is possible that iron export from macrophages through the *Fpn* pathway is primarily regulated by serum hepcidin rather than cellular *Hif2a*. This would ensure that iron mobilization from macrophages is better synchronized with systemic iron demands, rather than being influenced solely by cellular iron levels. Consequently, this could provide an explanation as

to why we observe no alteration in the *Fpn* transcript when *Hif2a* is deleted in macrophages, along with other iron transporters.

In conclusion, our study demonstrates that myeloid-specific deletion of *Hif2a* in macrophages does not significantly impact systemic iron homeostasis in mice. Despite the established role of *Hif2a* as a key transcriptional regulator in the multi-organ communication network involved in systemic iron regulation, the absence of *Hif2a* in macrophages does not result in anemia or abnormal blood cell counts. Interestingly, while the disruption of *Hif2a* in macrophages does not affect the systemic response to iron deficiency, it leads to increased expression of various iron transporters in the duodenum, potentially promoting enhanced iron absorption. This response may serve as an adaptive mechanism to maintain the erythroid pool during iron deficiency when iron recycling is compromised due to *Hif2a* deletion.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The schematic diagram (Figure 1A, Figure 3A, Figure 4A) were created with BioRender.com.

This work was supported by National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grants R01DK095201, the Center for Gastrointestinal Research (DK034933) (Y.M.S.), University of Michigan, Rackham International student fellowship (C.J.). NIH/NCI grant R01CA269025 (K.E.L.), Crohn's and Colitis Foundation Research Fellowship Award (1003279) (R.S.)

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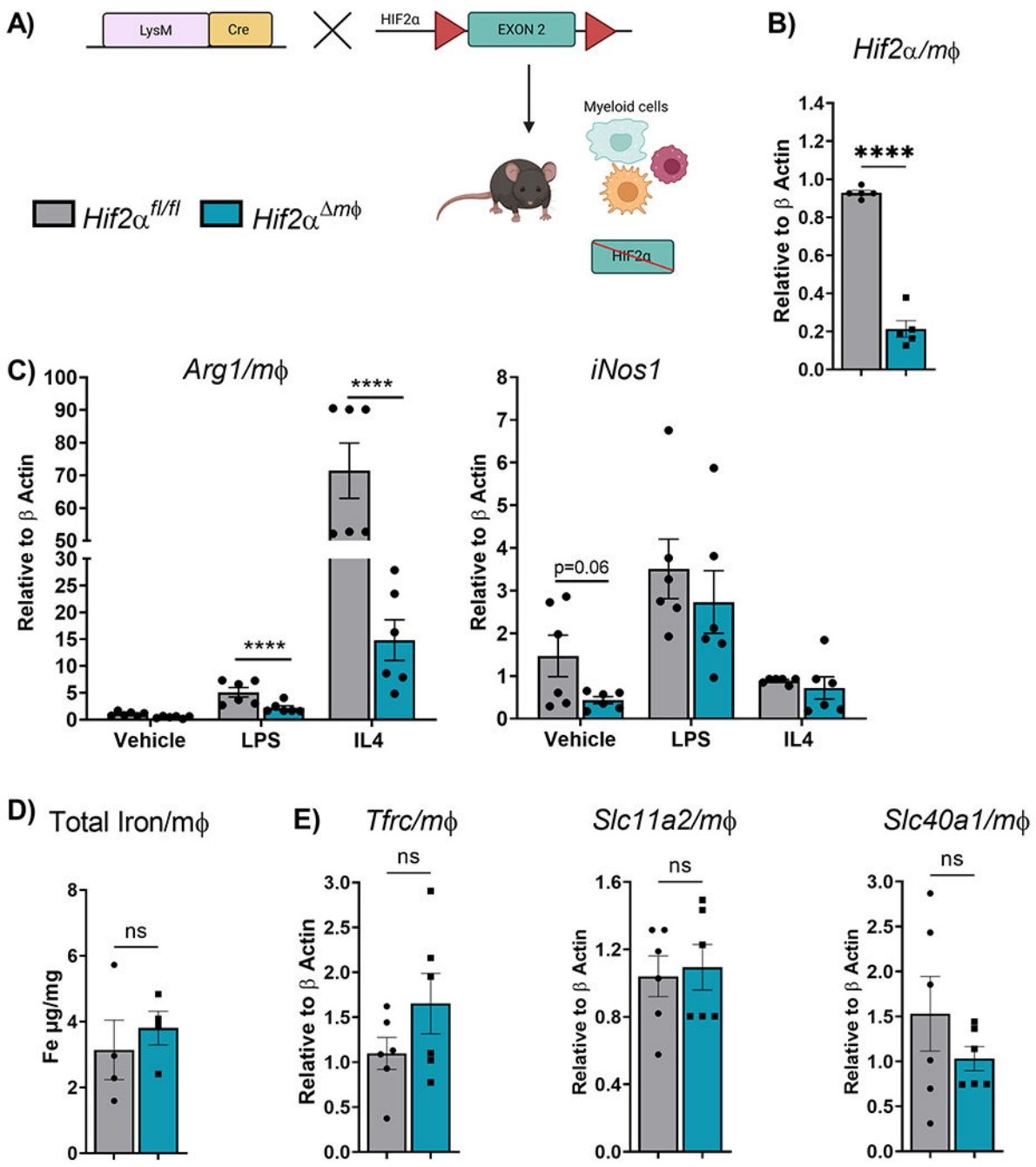


**Figure 1. *Hif2α* deletion in macrophages does not alter expression of iron transporter.**

**(A)** Schematic representation of mice with disruption of *Hif2α* in the myeloid lineage cells. **(B)** qPCR analysis of macrophage *Hif2α* transcript expression levels (n=5-7 per group). **(C)** qPCR analysis of *Arg 1* and *iNos1* from Bone marrow derived macrophages (BMDMs) maintained in GMCSF (10ng/mL) for 5 days followed by treatment with IL4 (20ng/mL) and LPS (20ng/mL) for 48 hours (n=4-5 per group). **(D)** Total intracellular iron measured by Inductively coupled plasma mass spectrometry (ICP-MS). **(E)** qPCR analysis of macrophage *Tfrc* (Transferrin receptor), *Slc11a2 (Dmt1)*, *Slc40a* (*Fpn*) transcript expression levels (n=5–

7 per group). Data represent the mean ± SEM. Significance was determined by unpaired *t* test. *$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$ versus the *Hif2a*$^{fl/fl}$ group.

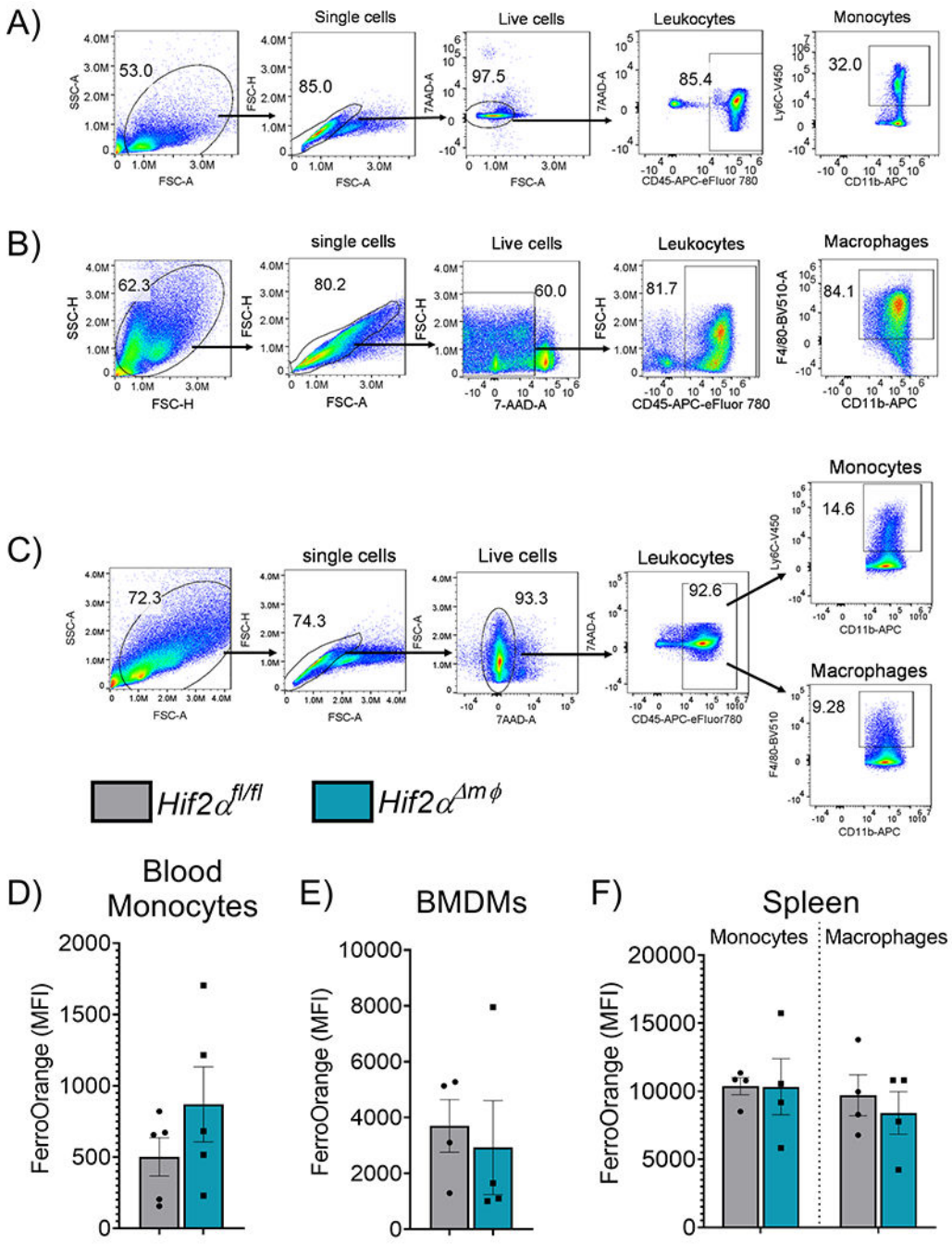


**Figure 2. *M*onocyte differentiation and intracellular labile iron are not altered following HIF2α in myeloid cells.**

Representative Gating strategy for **(A)** circulating blood monocytes (n=5,each group) **(B)** bone marrow derived macrophages (BMDMs) (n=4, each group) and **(C)** splenic monocytes (n=4, each group) or macrophages (n=4, each group). Mean fluorescence intensity of FerroOrange gated on (D) blood monocytes. **(E)** BMDMs or **(F)** splenic monocytes and macrophages. Data represent the mean ± SEM. Significance was determined by unpaired *t* test. $*P < 0.05$, $***P < 0.001$, and $****P < 0.0001$ versus the *Hif2α*$^{fl/fl}$ group.

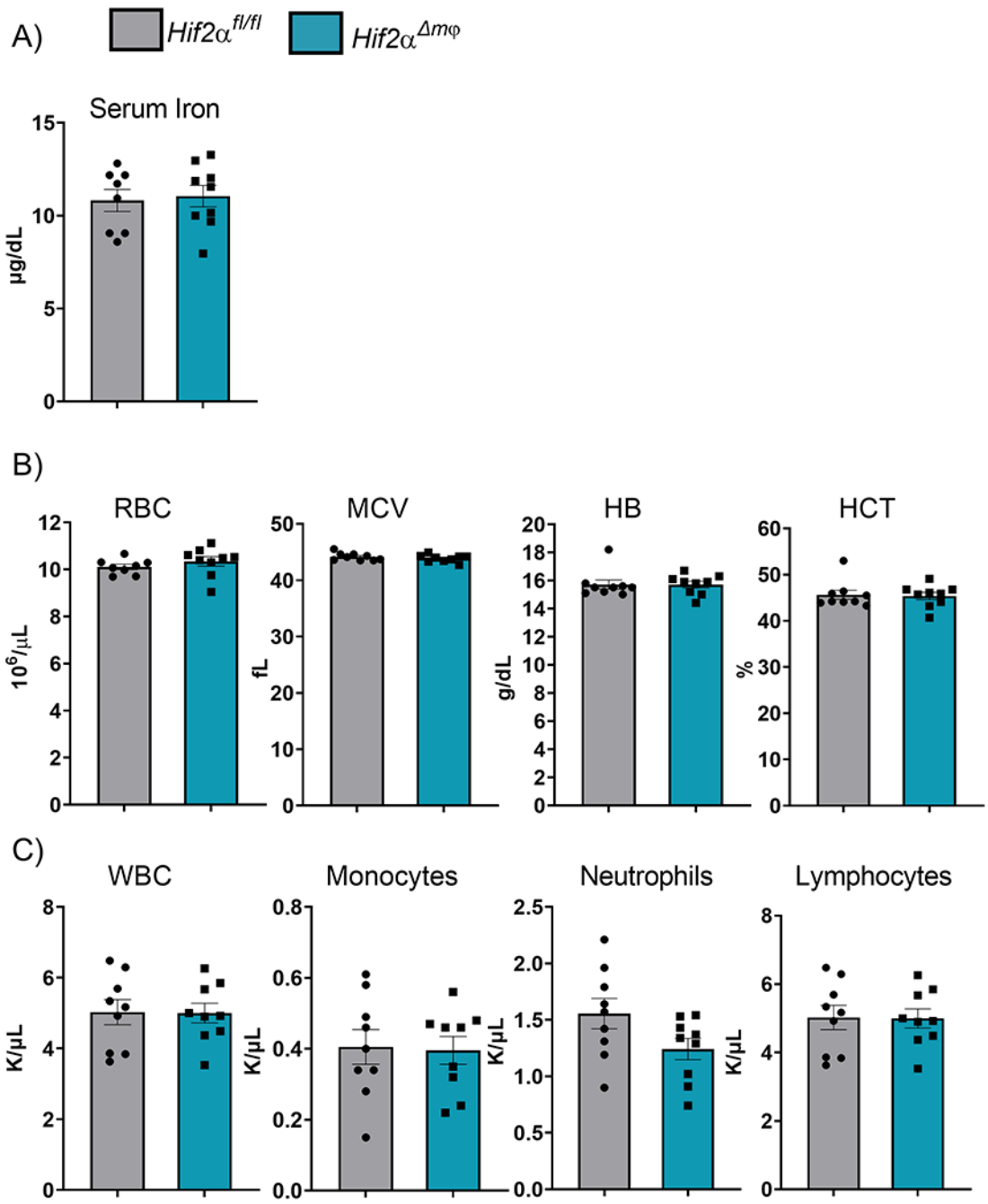


**Figure 3. *Hif2α* deletion in macrophages is not required for basal erythropoiesis.**

**(A)** Serum iron measured by colorimeteric iron assay. **(B)** Analysis of RBC, MCV, HB, and HCT from 48 month old *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ (n = 7-9 per group). **(C)** Analysis of WBC, Monocytes, Neutrophils, Lymphocytes from 8 month old *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ (n = 7–9 per group). Data represent the mean ± SEM. Significance was determined by unpaired *t* test. * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$ versus the *Hif2α*$^{fl/fl}$ group.

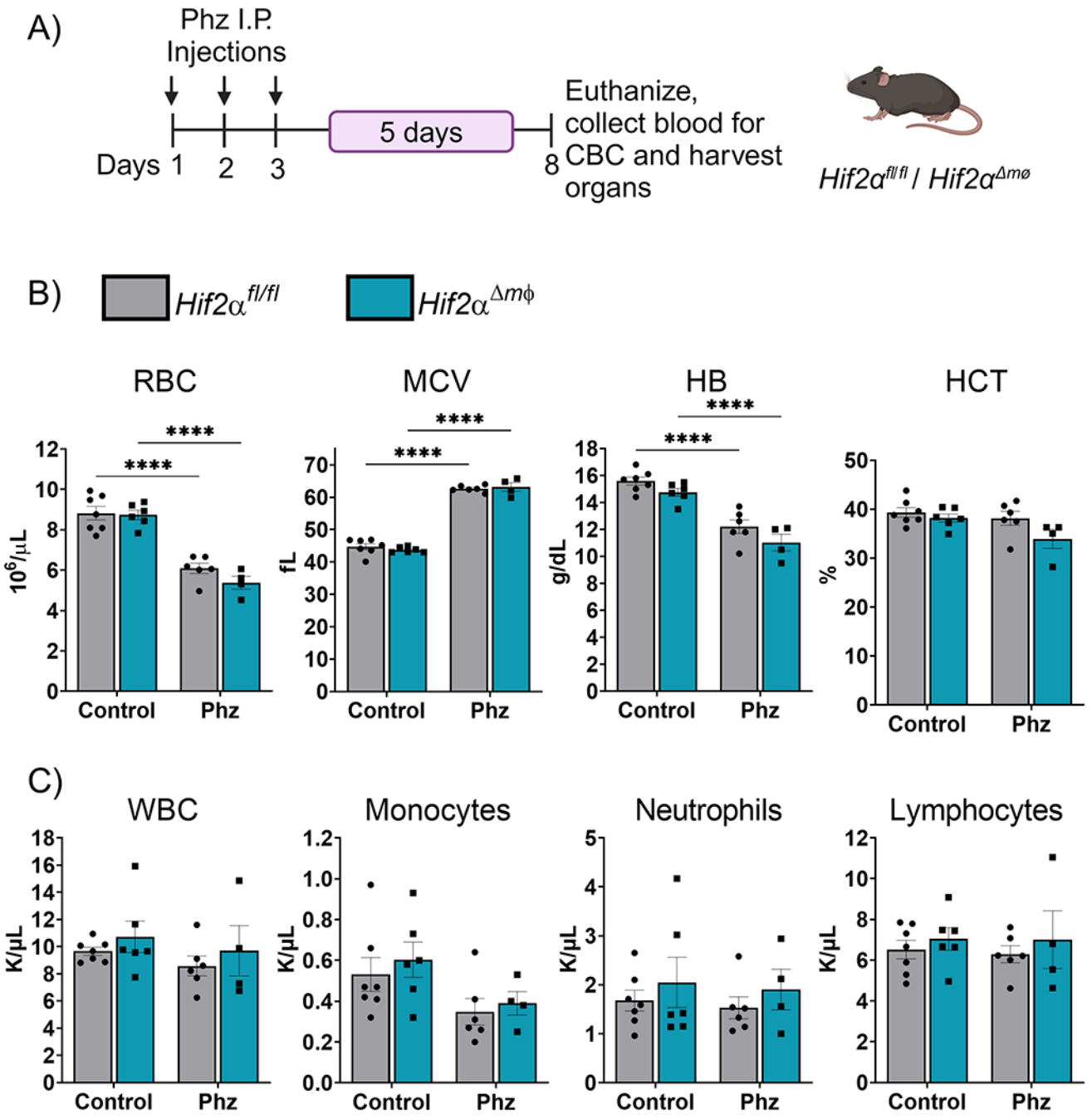


**Figure 4. Deletion of *Hif2α* does not alter the systemic response to acute iron and erythropoietic demand.**

**(A)** Experimental design for Phz-induced hemolytic anemia model. **(B)** Analysis of RBC, MCV, HB, and HCT from *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ (n = 7–9 per group). **(C)** Analysis of WBC, Monocytes, Neutrophils, Lymphocytes from *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ (n = 7–9 per group). Data represent the mean ± SEM. Significance was determined by unpaired *t* test. **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001 versus the *Hif2α*$^{fl/fl}$ group.

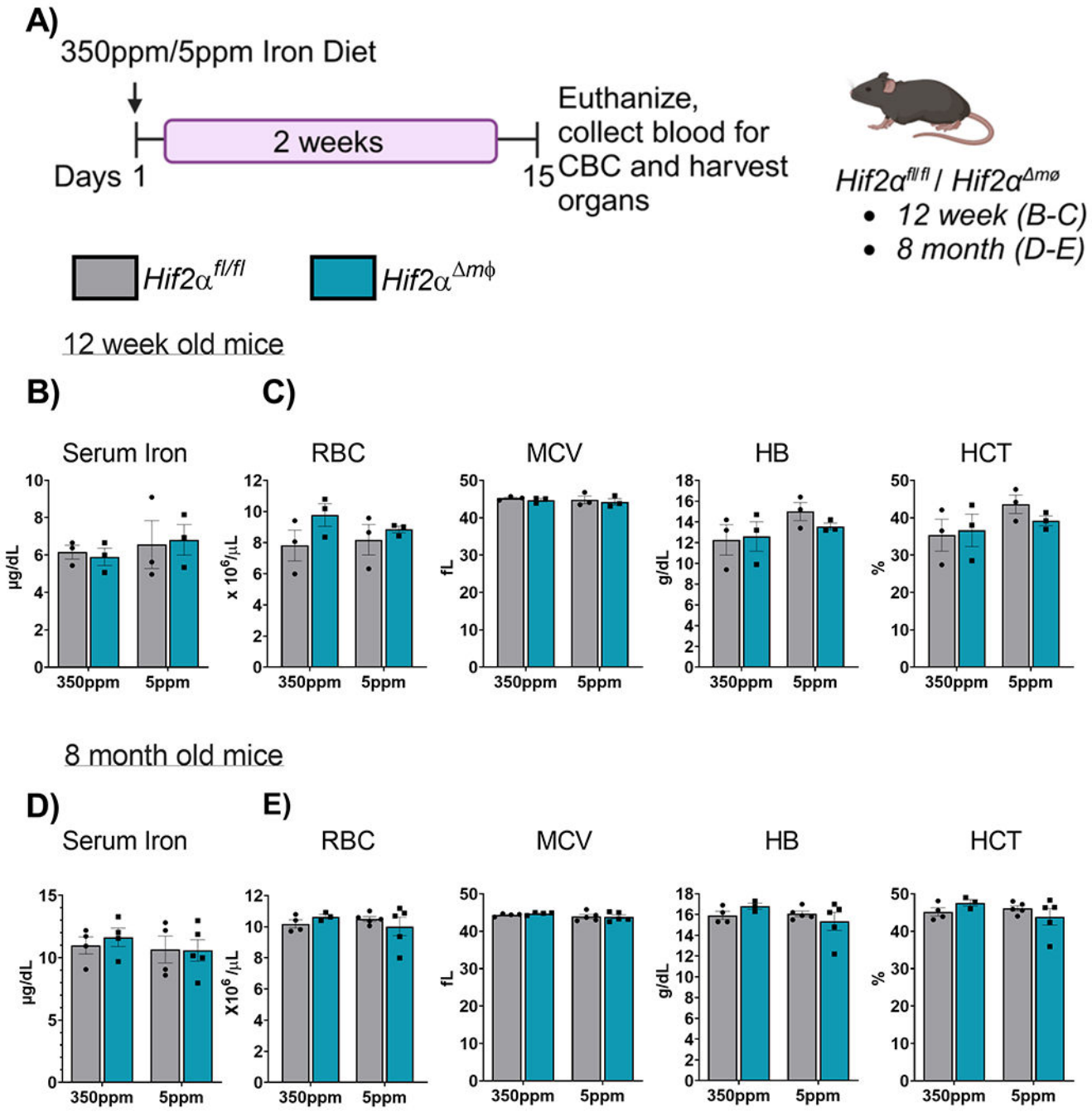


**Figure 5. The systemic response to iron deficiency is not altered under *Hif2α* deletion in the myeloid cells.**

**(A)** Schematic of iron diet treatment 350ppm and <5ppm in *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ mice to induce iron deficiency anemia. **(B)** Serum iron measured in 12 week old *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ mice 2 week post dietary intervention **(C)** Analysis of RBC, MCV, HB, and HCT from 12 week old *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ (n = 7–9 per group). **(D)** Serum iron measured in 8 month old *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ mice 2 week post dietary intervention. **(E)** Analysis of RBC, MCV, HB, and HCT from 8 month old *Hif2α*$^{fl/fl}$ and *Hif2α*$^{\Delta m\varphi}$ (n = 7–9 per

group). Data represent the mean ± SEM. Significance was determined by unpaired *t* test. **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001 versus the *Hif2a*$^{fl/fl}$ mice fed with 350ppm iron containing diet.

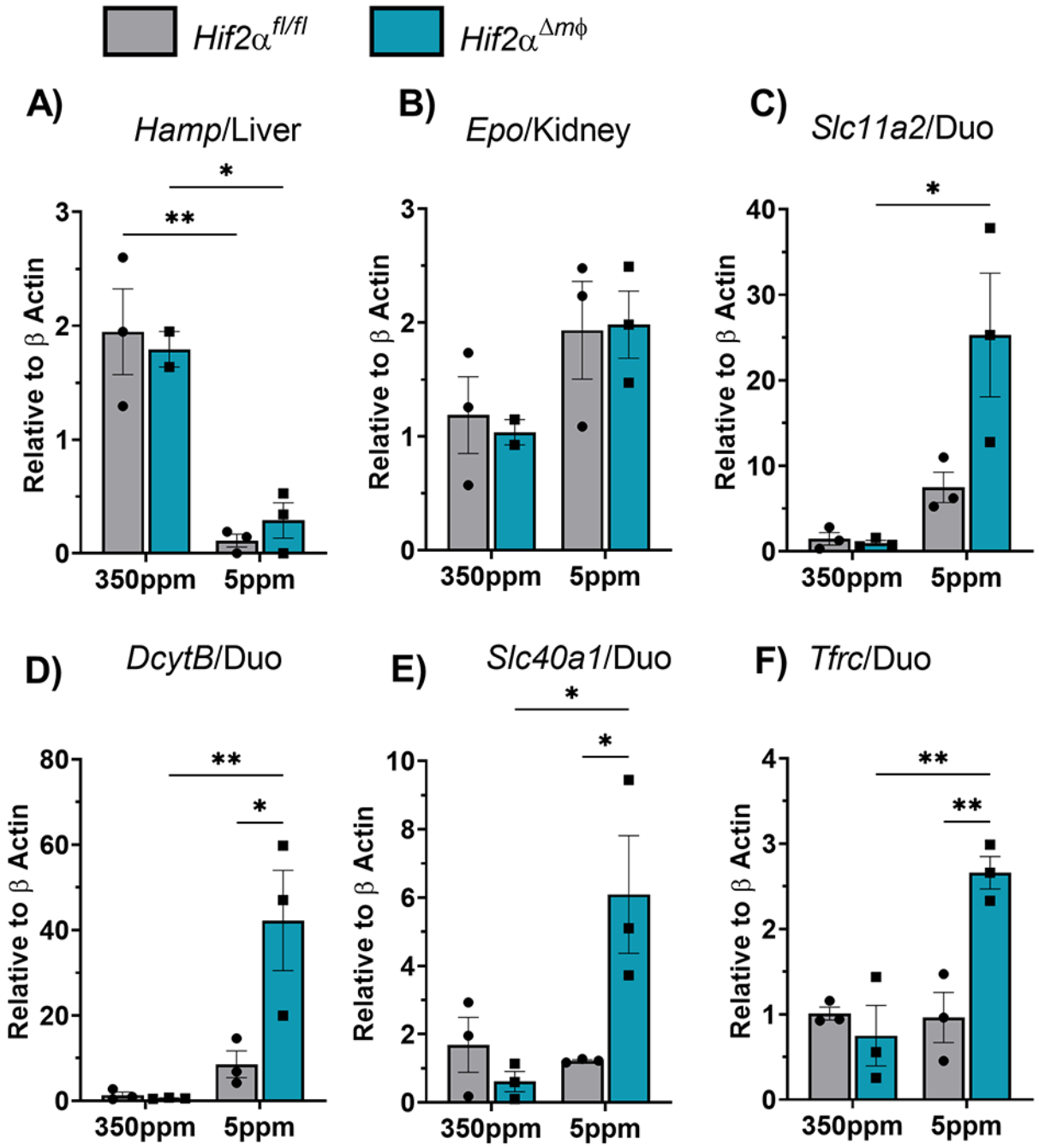


**Figure 6. *Hif2α* deletion in the macrophages induces dietary iron absorption under iron deficiency.**

qPCR analysis of **A)** Liver Hepcidin, **B)** Renal Erythropoietin and **C)** duodenal *Slc11a2* (*Dmt1*), **(D)** *DcytB*, **(E)** *Slc40a1 (Fpn)* and **(F)** *Tfrc* from mice fed 350ppm or 5ppm iron containing diet for 2 weeks (n=4-5 per group). Data represent the mean ± SEM. Significance was determined by unpaired *t* test. **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001 versus the *Hif2α*$^{fl/fl}$ mice fed with 350ppm iron containing diet.