Ndfip1-deficient mice have impaired DMT1 regulation and iron homeostasis

*Natalie J. Foot,[1] *Yew Ann Leong,[1] Loretta E. Dorstyn,[1] Hazel E. Dalton,[1] Kristen Ho,[1] Lin Zhao,[2] Michael D. Garrick,[2] Baoli Yang,[3] Devendra Hiwase,[1] and Sharad Kumar[1]

[1]Department of Hematology, Centre for Cancer Biology, SA Pathology, Adelaide, Australia; [2]Department of Biochemistry, State University of New York at Buffalo, Buffalo, NY; and [3]Department of Obstetrics and Gynecology, Carver College of Medicine, University of Iowa, Iowa City, IA

The divalent metal ion transporter DMT1 is critical for nonheme iron import. We have previously shown that DMT1 is regulated in vitro by ubiquitination that is facilitated by the adaptor proteins Ndfip1 and Ndfip2. Here we report that in *Ndfip1*$^{-/-}$ mice fed a low-iron diet, DMT1 expression and activity in duodenal enterocytes are significant higher than in the wild-type animals. This correlates with an increase in serum iron levels and transferrin saturation. Liver and spleen iron stores were also increased in *Ndfip1*$^{-/-}$ mice fed a normal diet. Counterintuitive to the increase in iron uptake, *Ndfip1*$^{-/-}$ mice fed a low iron diet develop severe microcytic, hypochromic anemia. We demonstrate that this is due to a combination of iron deficiency and inflammatory disease in *Ndfip1*$^{-/-}$ mice, because *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ immunodeficient mice fed a low iron diet did not develop anemia and showed an iron overload phenotype. These data demonstrate that Ndfip1 is a critical mediator of DMT1 regulation in vivo, particularly under iron restricted conditions. (*Blood*. 2011;117(2):638-646)

Introduction

Iron is essential for many biological processes. Iron homeostasis needs to be tightly controlled, because iron deficiency results in anemia, while iron overload leads to tissue damage and fibrosis.[1] Iron uptake and metabolism are complex and highly regulated, involving a number of enzymes and transport proteins.[2,3] The divalent metal ion transporter DMT1 plays a major role in both iron uptake into the body and iron release within cells.[4] DMT1 is expressed at the apical membrane of duodenal enterocytes where it transports non-heme iron into the cell[5] and in the endocytic compartment where it releases iron, internalized via the transferrin system, from the endosomes to the cytoplasm.[6] Little is known about the posttranslational regulation of DMT1.

Ubiquitination plays an important role in the trafficking and degradation of proteins.[7] The Nedd4 family of HECT ubiquitin ligases (E3s) is known to ubiquitinate a number of channels and transporters via a direct interaction between the WW domains of the E3s and PPxY (PY) motifs of the substrate. However, many Nedd4 family targets do not contain PY motifs, suggesting a need for adaptor or accessory proteins.[8] We identified 2 proteins, Ndfip1 and Ndfip2,[9-11] which regulate DMT1 in vitro via ubiquitination by the Nedd4 family members WWP2 and Nedd4-2.[12,13] Both Ndfips bind to DMT1 and act as adapters for WWP2 and Nedd4-2, mediating the ubiquitination and subsequent degradation of DMT1.[12,13]

In this paper, we show that DMT1 regulation is impaired in *Ndfip1*$^{-/-}$ mice, especially when challenged with low iron availability. *Ndfip1*$^{-/-}$ mice fed a low-iron diet show increased duodenal DMT1 levels and DMT1 activity in isolated *Ndfip1*$^{-/-}$ enterocytes, leading to higher serum iron levels and greater transferrin saturation. The hepatic and splenic iron stores were significantly increased in *Ndfip1*$^{-/-}$ mice fed a normal iron diet. Together, this suggests an increase in iron uptake in *Ndfip1*$^{-/-}$ mice. Due to their known inflammatory phenotype,[14] these mice develop anemia. By removing the inflammation and subsequent anemia using *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice, we further uncovered increased iron uptake in these mice, demonstrating a critical role for Ndfip1 in the regulation of DMT1 in vivo.

Methods

Animals

Ndfip1$^{-/-}$ mice have been described previously.[14] *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice were generated by crossing *Ndfip1*$^{+/-}$ mice (backcrossed for 3 generations) with mouse strain B6.129S7-*Rag1*^{tm1Mom}/J (*Rag1*$^{-/-}$ mice, Jackson Laboratory) to generate *Ndfip1*$^{+/-}$/*Rag1*$^{+/-}$ mice. These mice were then backcrossed once with *Rag1*$^{-/-}$ mice to generate *Ndfip1*$^{+/-}$/*Rag1*$^{-/-}$ mice, which were then intercrossed to generate *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice. On average, the genetic background of the *Ndfip1*$^{-/-}$ and the *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice used in this study would have approximately 95% and 97% contribution from C57Bl/6J, respectively. Animals were fed ad libitum on a standard (164 mg/kg iron), high iron (20 g/kg), or low iron (15 mg/kg; Specialty Feeds) rodent diet for 3 weeks. All studies were performed on 6-week-old mice. All animal studies were approved by the institutional animal ethics committee at the Center for Cancer Biology.

Blood and bone marrow analyses

Blood was collected via cardiac puncture and analyzed for total serum iron and transferrin saturation using the Ferene method (Thermo Electron). Serum transferrin (Alpha Diagnostics), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6; R&D Systems) were measured by enzyme-linked immunosorbent assay. Serum ferritin and complete

Submitted July 7, 2010; accepted October 5, 2010. Prepublished online as *Blood* First Edition paper, October 19, 2010; DOI 10.1182/blood-2010-07-295287.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

*N.J.F. and Y.A.L. contributed equally to this work

blood count (including blood smears) were performed by the Department of Clinical Pathology, SA Pathology. Parameters measured in the complete blood count included hemoglobin (Hb), hematocrit (Hct), red blood cell count (RBC), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration, and red cell distribution width.

Bone marrow was aspirated from femurs using a 23-gauge needle and 5-mL syringe. Bone marrow smears were stained with Perl Prussian Blue staining with 2% carbol fuchsin as the counterstain and May-Grünwald, Giemsa stain by the Department of Clinical Pathology, SA Pathology. Differential count was performed on 500 nucleated cells. Light images were captured at room temperature with an Olympus BX51 microscope, Olympus DP70 camera, and Olysia Bioreport software. The objectives used were 10× UPlanApo (NA 0.4), 20× UPlanApo (NA 0.7), and 40× UPlanApo (NA 0.85). Images were then compiled using Adobe Illustrator CS5 and Adobe Photoshop CS5 software. Statistical significance was determined using either 2-tailed unpaired *t* tests or 1-way analyses of variance with Tukey post-hoc tests (GraphPad Prism Version 4.03).

Cell isolation and fluorescence quenching assay

To isolate enterocytes, the proximal duodenum was removed and flushed through with solution A (1.5mM KCl, 96mM NaCl, 27mM sodium citrate, 8mM KH_2PO_4, 5.6mM Na_2HPO_4), followed by washing in solution A for 30 minutes at room temperature. The duodenum was minced in an enzyme cocktail (333 U/mL collagenase, 2.5 U/mL elastase, 10 μg/mL DNAse) in Krebs Ringer solution (120mM NaCl, 24mM $NaHCO_3$, 5mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 4.8mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 20mM glucose, 1mM $CaCl_2$) and incubated while shaking at 37°C for 30 minutes. Cells were filtered through a 40-μm filter and washed twice in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% β-mercaptoethanol, and 1% L-asparagine. Enterocytes were used immediately in a fluorescence quenching assay to measure DMT1 activity,[12] and statistical significance determined using a 2-tailed unpaired *t* test (GraphPad Prism Version 4.03).

Immunohistochemistry and immunoblotting

Tissues were fixed in Histochoice (ProSciTech), cryopreserved in 30% sucrose, and embedded in Tissue-Tek Optimal Cutting Temperature compound. Ten-micrometer frozen sections were mounted on polysine slides, fixed with cold acetone for 10 minutes, then air dried and rehydrated with phosphate-buffered saline (PBS). Sections were then blocked with 5% skim milk in PBS for 2 hours and stained with either rabbit anti-DMT1 (specific for exon 1A)[15] or rabbit anti-ferroportin (SLC40A1, Lifespan Biosciences) overnight at 4°C (1:200 in 5% skim milk in PBS), followed by rabbit AlexaFluor-488 (1:200; Molecular Probes) for 2 hours, and mounted in ProLong Gold antifade (Invitrogen). Confocal images were captured as previously described.[12]

For immunoblotting, tissues were snap frozen and protein extracted using lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 10% glycerol, 1% Triton X-100, 10mM EDTA [ethylenediaminetetraacetic acid]) containing a complete protease inhibitor mixture (Roche Applied Science). One hundred micrograms of protein were loaded per lane and immunoblotting performed as previously described.[12] Blots were probed using rabbit anti-ferroportin or rabbit anti-ferritin light chain (Abcam), using goat anti–rabbit Cy5 (GE Healthcare Life Sciences) as the secondary antibody. Ferroportin was detected mostly as an apparent dimer of approximately 120 kDa.

Real-time qPCR

Total RNA was extracted using Trizol reagent (Invitrogen) and used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was analyzed by real-time quantitative polymerase chain reaction (qPCR) in a Rotor-Gene 6000 system (QIAGEN) using RT2 Real-TimeTM SYBR Green PCR master mix (SABiosciences, QIAGEN) with primers shown in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Data were analyzed using Rotor-Gene 6000 Series Software 1.7 and normalized to β-actin.

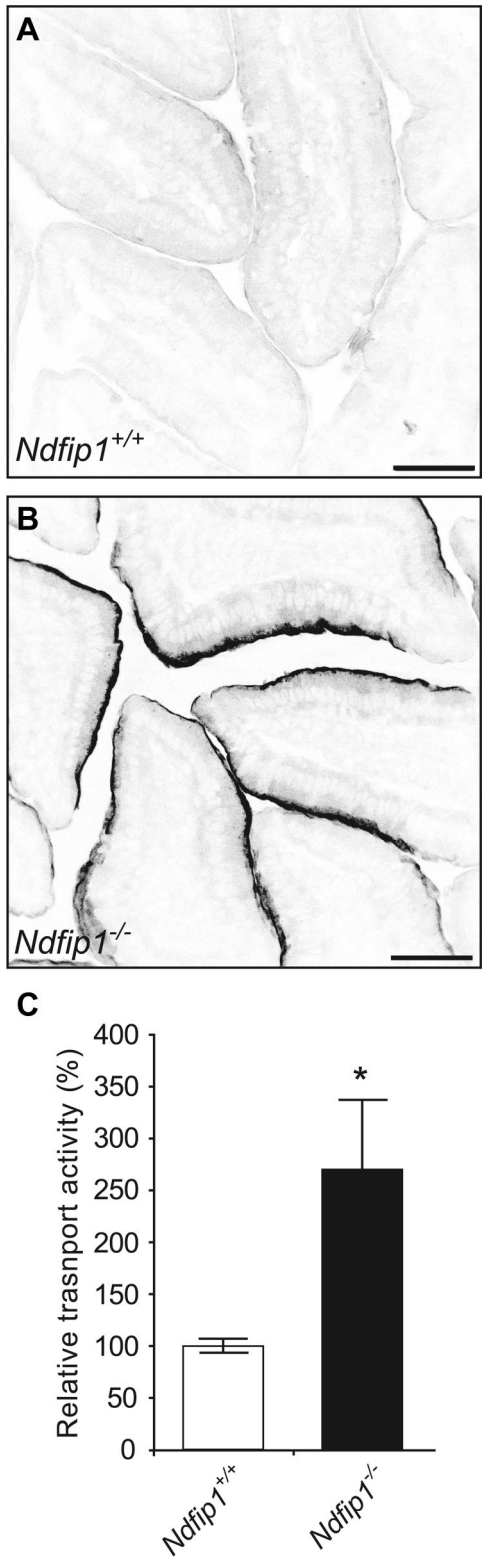

Figure 1. Ndfip1 is important for regulating DMT1 under iron-deficient conditions in vivo. DMT1 expression in the duodenum of (A) *Ndfip1*$^{+/+}$ mice and (B) *Ndfip1*$^{-/-}$ mice fed a low iron diet for 3 weeks. DMT1 levels are increased in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice. Scale bar, 50 μm. (C) DMT1 relative transport activity in enterocytes isolated from *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ mice fed a low iron diet. DMT1 activity is increased in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice. Data represented as mean ± SD,*$P < .05$, n = 3-7.

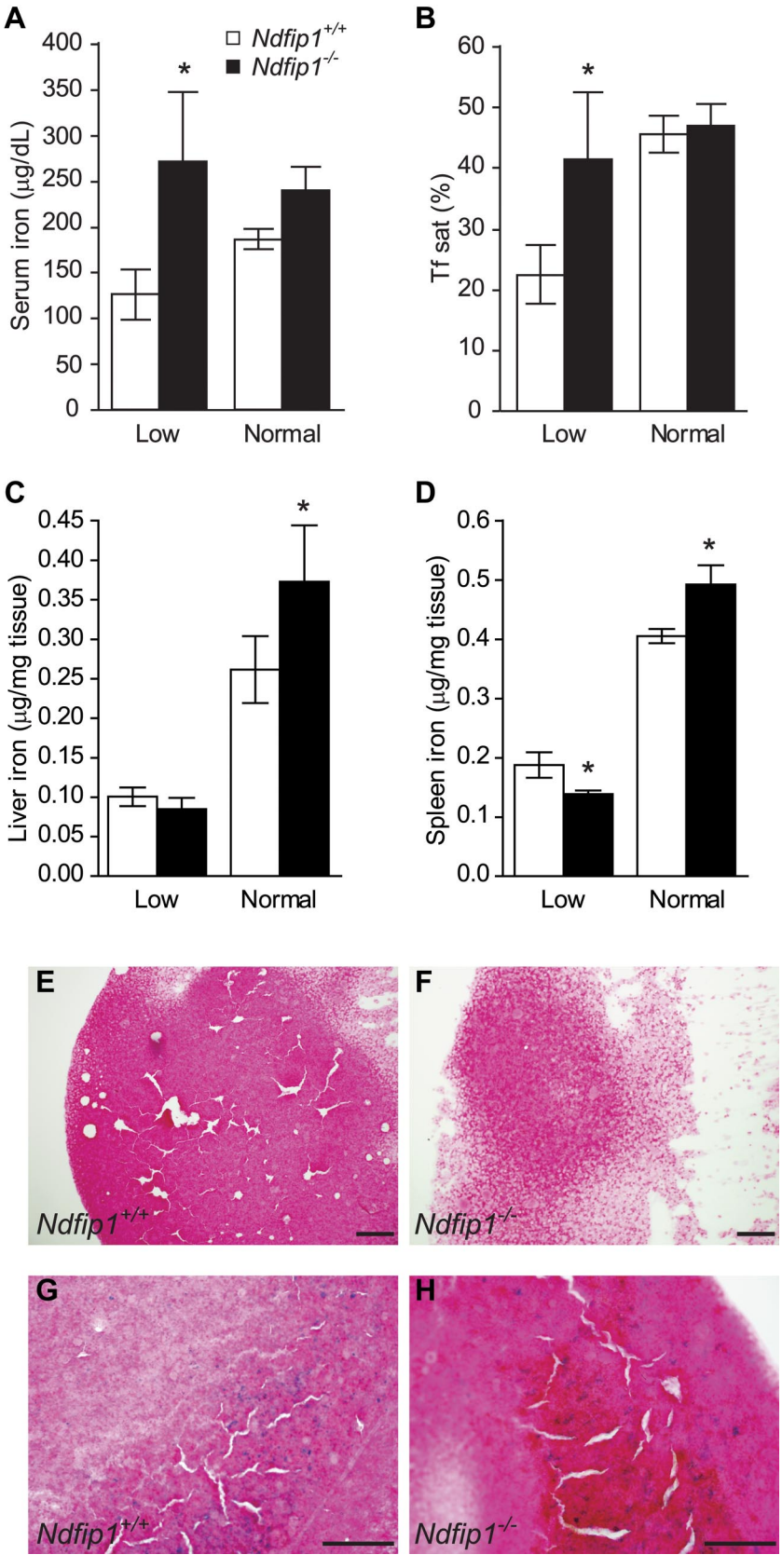

Figure 2. *Ndfip1*$^{-/-}$ mice show increased iron uptake under low iron conditions and increased iron storage under normal iron conditions. (A) Serum iron levels and (B) transferrin saturation (Tf sat) in *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ mice fed a low- or normal iron diet. Both serum iron and transferrin saturation are increased in *Ndfip1*$^{-/-}$ mice on the low-iron diet compared with *Ndfip1*$^{+/+}$ mice. Iron stores in (C) liver and (D) spleen. Data represent mean ± SD, $^*P < .05$, n = 3-7. Bone marrow iron levels in (E) *Ndfip1*$^{+/+}$ mice and (F) *Ndfip1*$^{-/-}$ mice on the low-iron diet and in (G) *Ndfip1*$^{+/+}$ mice and (H) *Ndfip1*$^{-/-}$ mice on the normal diet. Scale bar, 50 μm. Iron stores are significantly increased in *Ndfip1*$^{-/-}$ mice on the normal diet compared with *Ndfip1*$^{+/+}$ mice.

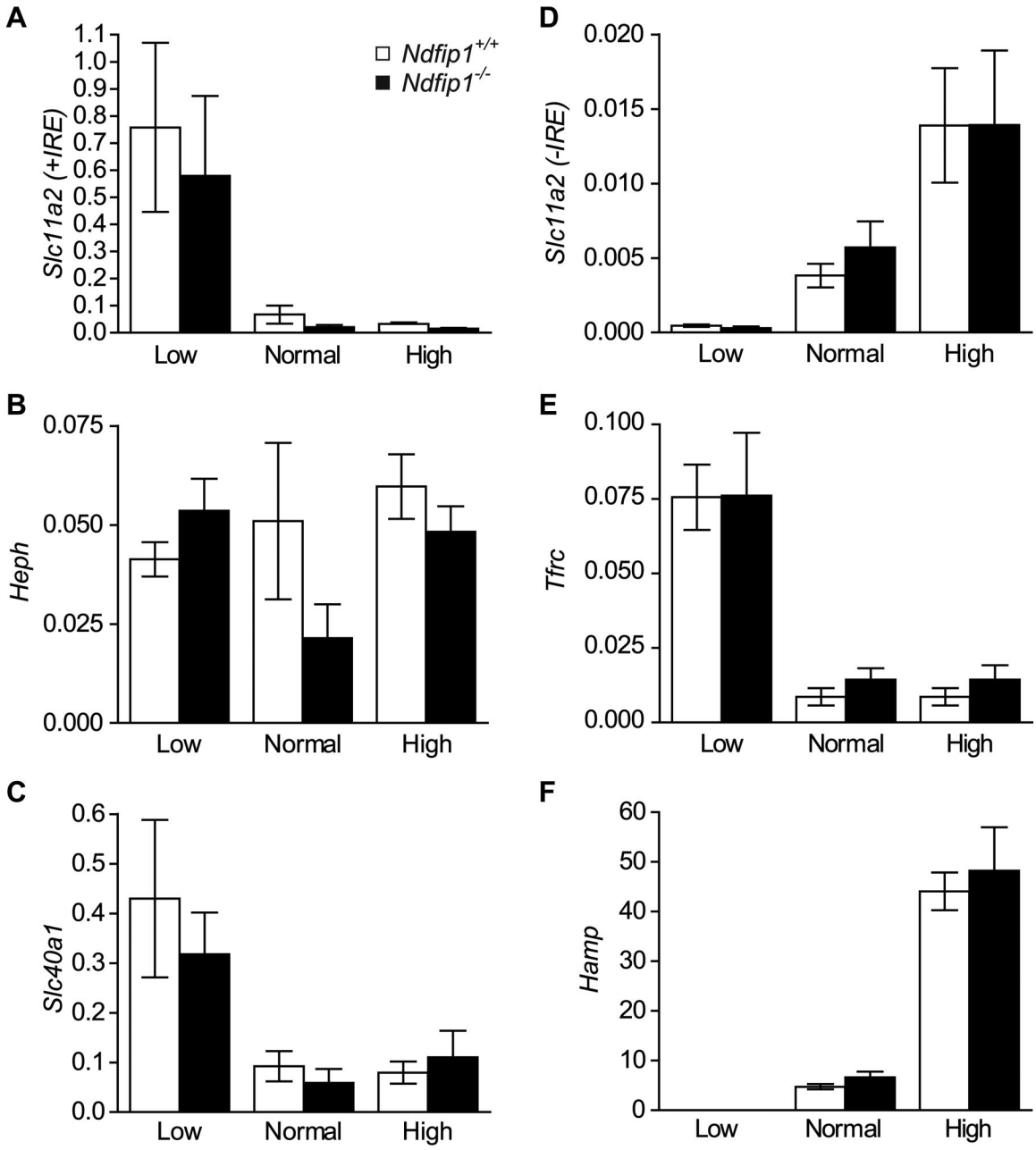

Figure 3. mRNA levels of genes involved in regulating iron homeostasis in *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ mice fed either a low-, normal, or high-iron diet. (A) *Slc11a2* (+IRE isoform), (B) *Heph,* and (C) *Slc40a1* in the duodenum. These genes are involved in iron uptake from the diet. (D) *Slc11a2* (-IRE isoform), (E) *Tfrc1,* and (F) *Hamp* in the liver. These genes are involved in iron storage. *Slc11a2,* DMT1; *Heph*, hephaestin; *Slc40a1*, ferroportin 1; *Tfrc*, transferrin receptor 1; *Hamp*, Hepcidin. All genes respond to the different diets as expected. Data represent mean ± SE, **P* < .05, n = 7-9.

Tissue iron measurement using inductively coupled plasma mass spectroscopy

Small pieces of tissue (liver or spleen) were dried down for 24 hours at 85°C after which they were weighed and then digested in 25% nitric acid in a glass tube at 65°C for 16 hours. The samples were then diluted in 1% nitric acid and analyzed by inductively coupled plasma mass spectroscopy by SA Water. Values were then normalized to dry weight.

Results

Previously, we have reported that Ndfip1 regulates DMT1 by acting as an adaptor between DMT1 and the ubiquitin ligase WWP2, thereby mediating its ubiquitination and subsequent degradation.[12] *Ndfip1*$^{-/-}$ mice fed a standard rodent diet had increased DMT1 levels and activity in the liver, with a concomitant increase in liver iron levels.[12] To further dissect the physiological role of Ndfip1 in iron homeostasis in vivo, we subjected *Ndfip1*$^{-/-}$ mice to a low-, normal, or high-iron diet. Immunostaining of the duodenum showed an increase in DMT1 levels in *Ndfip1*$^{-/-}$ mice fed a low-iron diet compared with *Ndfip1*$^{+/+}$ mice (Figure 1A-B). DMT1 activity in enterocytes isolated from mice fed a low-iron diet was also dramatically increased in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice (Figure 1C). As a result, serum iron levels and transferrin saturation were significantly increased in *Ndfip1*$^{-/-}$

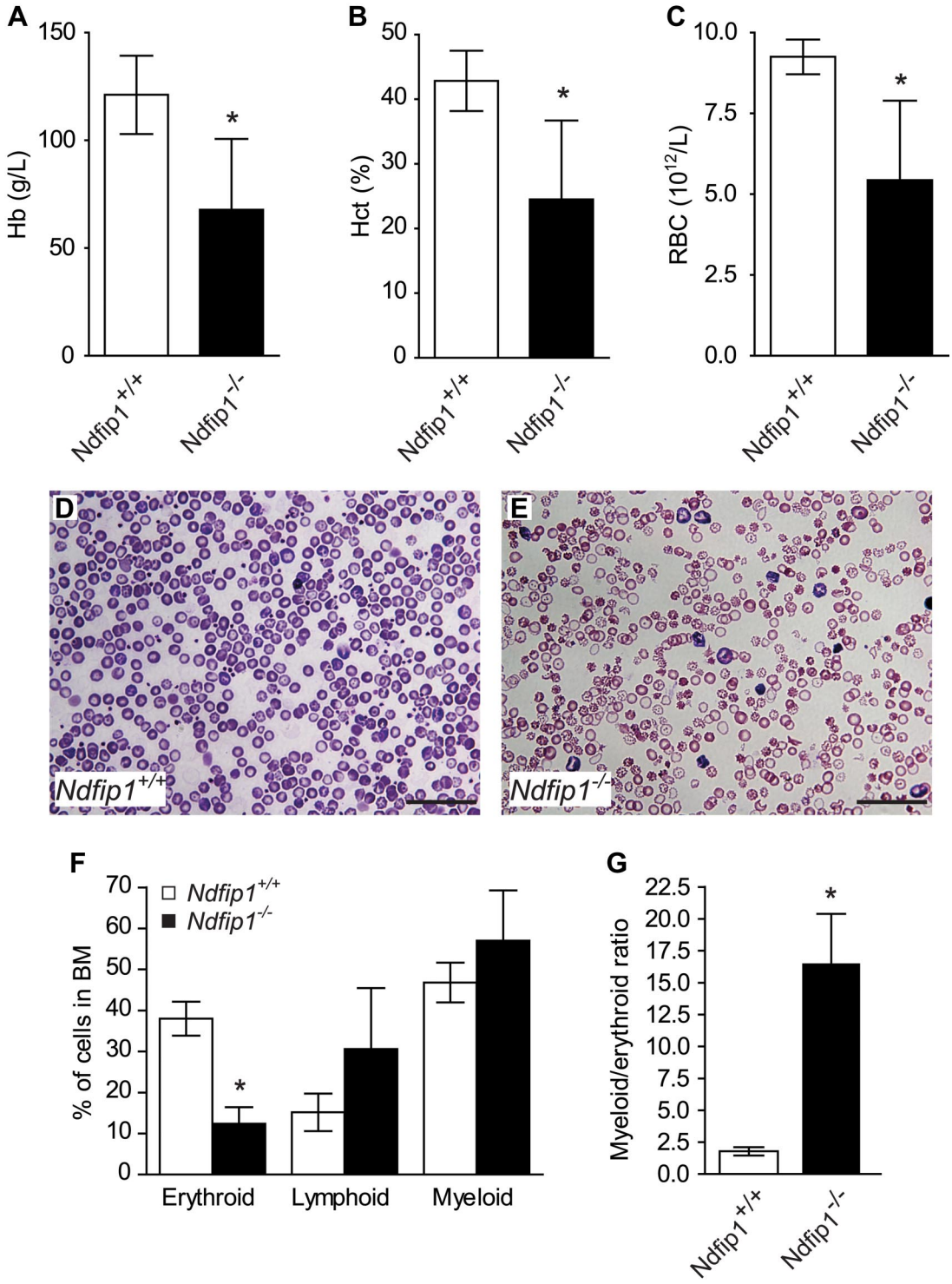

Figure 4. *Ndfip1*$^{-/-}$ mice fed a low-iron diet develop hypochromic, microcytic anemia. (A) Hemoglobin (Hb), (B) hematocrit (Hct), and (C) RBC count are decreased in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice. Blood smears from (D) *Ndfip1*$^{+/+}$ mice and (E) *Ndfip1*$^{-/-}$ mice show RBCs from *Ndfip1*$^{-/-}$ mice are microcytic and hypochromic compared with their wild-type counterparts. Scale bar, 50 μm. (F) Erythroid, lymphoid, and myeloid series as a percentage of total cells in the bone marrow smears. (G) The myeloid to erythroid ratio in bone marrow. The percentage of erythroid series cells is decreased in *Ndfip1*$^{-/-}$ mice, and the myeloid to erythroid ratio is increased, indicating impaired erythropoiesis. Data represent mean ± SD, $*P < .05$, n = 4-7.

compared with *Ndfip1*$^{+/+}$ mice (Figure 2A-B). Total iron binding capacity, serum transferrin, and serum ferritin were not significantly different (supplemental Table 2).

Because serum iron levels can be highly variable in mice, we measured tissue iron stores as a more accurate indication of iron balance. In mice fed a normal diet, hepatic and splenic iron stores were significantly higher in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice (Figure 2C-D). Stainable iron could also be found in the bone marrow (Figure 2G-H). The increase in liver and splenic iron in *Ndfip1*$^{-/-}$ mice on the normal iron diet with no significant increase in serum iron and transferrin saturation (Figure 2) may be attributable to a maximum transferrin saturation

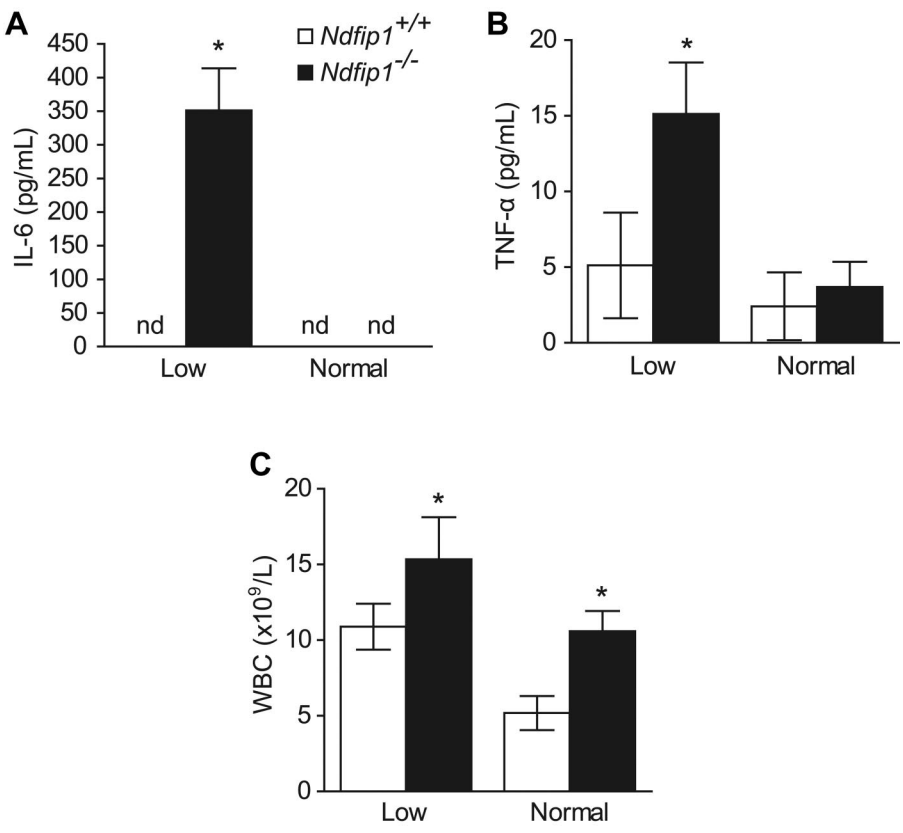

Figure 5. The anemia of *Ndfip1*$^{-/-}$ mice under iron-deficient conditions is partly contributable to inflammation. (A) IL-6 and (B) TNF-α are increased in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice fed a low-iron diet but are not significantly different on the normal diet. (C) WBC count is significantly increased in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice fed both the low-iron and normal diet. These factors are indicative of anemia of inflammation. nd, not detected. Data represent mean ± SD, $^*P < .05$, n = 3-7.

maintained by hepcidin and other homeostatic regulators. As expected, the hepatic, splenic, and bone marrow iron stores were lower in *Ndfip1*$^{-/-}$ mice and *Ndfip1*$^{+/+}$ mice fed low-iron diet compared with mice fed with normal iron diet. There was no significant difference in hepatic and bone marrow iron stores in *Ndfip1*$^{-/-}$ mice and *Ndfip1*$^{+/+}$ mice fed low iron diet (Figure 2C-F). However, spleen iron stores were reduced in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice. This may be due to increased iron demand in *Ndfip1*$^{-/-}$ mice due to severe anemia and also the difference in splenic architecture between the 2 genotypes.[14]

The body maintains normal iron balance by fine tuning the expression of a number of genes in response to changes in dietary iron levels. We therefore examined the expression of genes involved in iron uptake in the duodenum and iron storage in the liver of mice fed a low-, normal, or high-iron diet using qPCR. At the transcript level, both *Ndfip1*$^{-/-}$ and *Ndfip1*$^{+/+}$ mice respond as expected[16,17] to varying levels of iron in the diet (Figure 3). Given that there is no difference between the genotypes, these data support a role for Ndfip1 in the posttranscriptional regulation of DMT1.

To investigate further the effects of loss of *Ndfip1*, a complete blood count was performed. Surprisingly, *Ndfip1*$^{-/-}$ mice fed a low iron diet developed severe microcytic, hypochromic anemia as indicated by significantly reduced Hb and Hct (Figure 4A-B). This was also clearly evident in the blood smears (Figure 4D-E). The MCV, MCH, and MCH concentration were significantly lower in mice fed the low-iron diet compared with mice fed a normal iron diet. Although there was no significant difference in MCV and MCH between the genotypes, the RBC count and Hb were significantly lower in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice fed the low-iron diet (Figure 4A,C). This may suggest suppressed effective erythropoiesis in *Ndfip1*$^{-/-}$ mice fed the low-iron diet (Figure 4A-C and supplemental Table 3). This is further supported by examination of the bone marrow, which demonstrated reduced erythropoiesis in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice fed low-iron, as indicated by a reduction in the percentage of erythroid series cells and an increase in the myeloid to erythroid ratio (Figure 4F-G). Although lymphoid and myeloid series cells were increased in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice fed low iron, the difference was not statistically significant (Figure 4F).

Ndfip1$^{-/-}$ mice develop inflammatory disease due to hyperactivated T cells.[14] *Ndfip1*$^{-/-}$ mice on the low-iron diet showed significant increases in IL-6 and TNF-α compared with *Ndfip1*$^{+/+}$ mice (with no significant differences seen in mice on the normal diet; Figure 5A-B). WBC count was increased in *Ndfip1*$^{-/-}$ mice compared with *Ndfip1*$^{+/+}$ mice on both the low-iron and normal diets; Figure 5C), indicative of anemia of chronic disease.[18] This did not seem to be attributable to any single cell type, although neutrophils (myeloid series) and lymphocytes (lymphoid series) made up the majority of the cells (supplemental Table 3 and Figure 4). To further investigate the contribution of each disease process (iron deficiency and inflammation) toward the severe anemia of *Ndfip1*$^{-/-}$ mice fed the low-iron diet, we measured ferritin and ferroportin levels in the liver and ferroportin levels in duodenum (supplemental Figure 1). As expected, the hepatic ferritin expression was higher in all mice fed the normal iron diet compared with mice fed the low-iron diet; however, there was no difference between *Ndfip1*$^{-/-}$ mice and *Ndfip1*$^{+/+}$ mice fed with the same diet. The hepatic ferroportin expression was increased in mice fed the low-iron diet (supplemental Figure 1A) compared with mice fed the normal iron diet (supplemental Figure 1B), but there was no difference between genotypes on the same diet. The

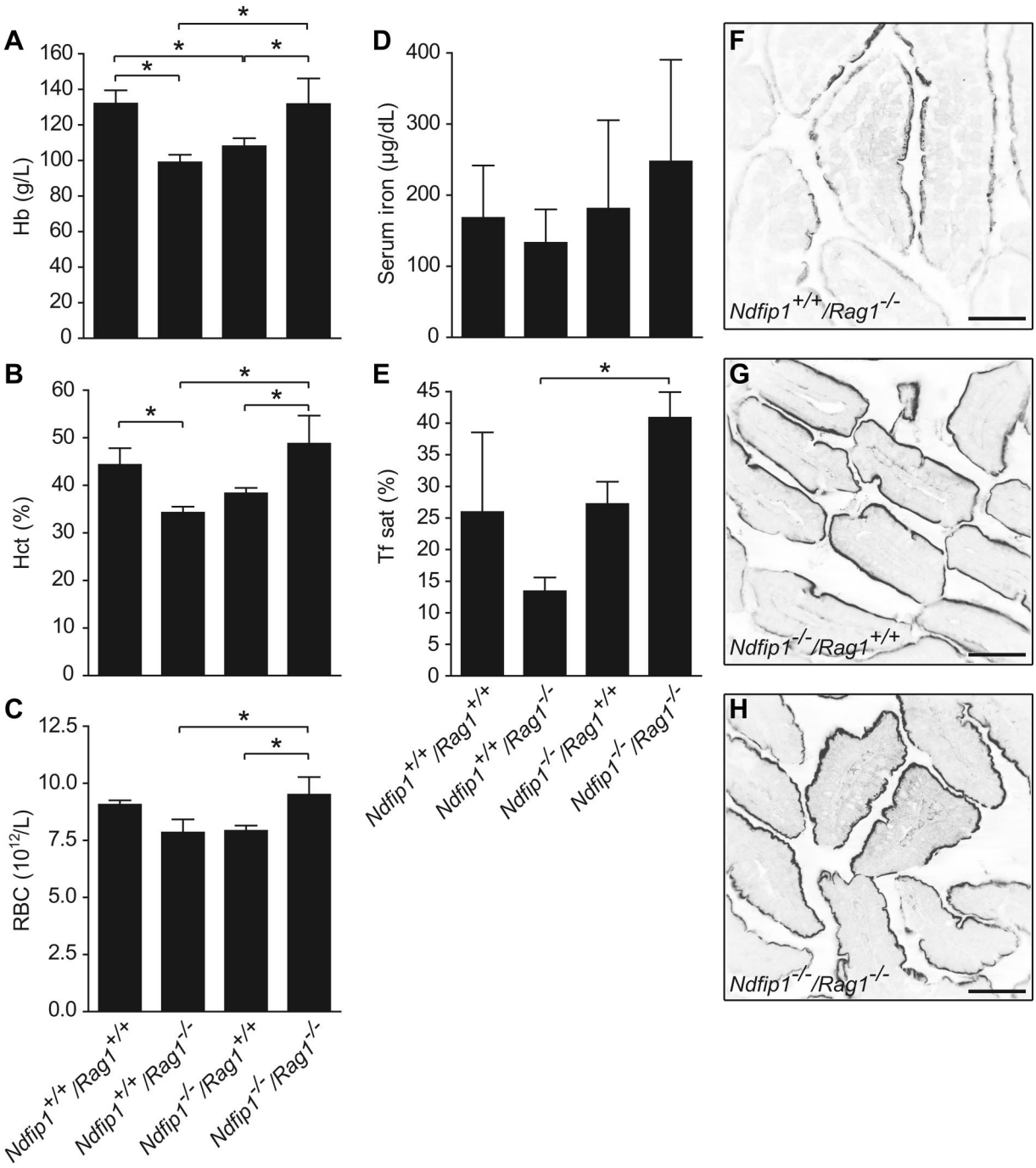

Figure 6. Removing inflammation reveals further evidence for the importance of Ndfip1 in the regulation of DMT1 in vivo. (A) Hb, (B) Hct, and (C) RBC count are significantly increased in *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice compared with single knockout *Ndfip1*$^{+/+}$/*Rag1*$^{-/-}$ mice and *Ndfip1*$^{-/-}$/*Rag1*$^{+/+}$ and are restored to *Ndfip1*$^{+/+}$/*Rag1*$^{+/+}$ levels. (D) Serum iron and (E) transferrin saturation in (*Ndfip1*$^{+/+}$/*Rag1*$^{+/+}$) as for other genotypes *Ndfip1*$^{-/-}$/*Rag1*$^{+/+}$, *Ndfip1*$^{+/+}$/*Rag1*$^{-/-}$, and *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice. Both serum iron and transferrin saturation are elevated in the double knockout mice. DMT1 expression in the duodenum of (F) *Ndfip1*$^{+/+}$/*Rag1*$^{-/-}$ mice, (G) *Ndfip1*$^{-/-}$/*Rag1*$^{+/+}$ mice, and (H) *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice. DMT1 levels are increased in mice lacking *Ndfip1* (*Ndfip1*$^{-/-}$/*Rag1*$^{+/+}$ and *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice) compared with *Ndfip1*$^{+/+}$/*Rag1*$^{-/-}$ controls. All mice were fed a low-iron diet for 3 weeks, data represent mean ± SD, *$P < .05$, n = 3-4.

duodenal ferroportin expression was not different between *Ndfip1*$^{-/-}$ mice and *Ndfip1*$^{+/+}$ mice fed with low-iron diet (supplemental Figure 1C-D). Although *Ndfip1*$^{-/-}$ mice fed the low-iron diet have higher transferrin saturation and serum iron compared with *Ndfip1*$^{+/+}$ mice fed the low-iron diet, both groups of animals were still iron deficient. Iron deficiency and anemia down-regulate hepcidin (*Hamp*) expression (Figure 3F), which in turn increases ferroportin stability (*Hamp* being a negative regulator of ferroportin).[19] Thus, the lack of difference in *Hamp* expression in *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ mice fed the low-iron diet would explain why ferroportin expression (both RNA and protein) was not significantly different between the 2 genotypes.

The inflammatory markers (IL-6, TNF-α, and WBC) were significantly higher in *Ndfip1*$^{-/-}$ mice fed the low-iron diet compared with *Ndfip1*$^{-/-}$ mice fed the normal iron diet (Figure 5). Because *Ndfip1*$^{-/-}$ mice eventually develop inflammatory disease regardless of diet, these data suggest that iron deficiency may accentuate the inflammatory phenotype. Normally, iron deficiency

down-regulates while inflammation up-regulates *Hamp* expression.[19] We observed no significant difference in *Hamp* levels in the livers of *Ndfip1*$^{-/-}$ and *Ndfip1*$^{+/+}$ mice fed the low-iron diet (Figure 3F), and, consistent with previous work,[20] this suggests that iron deficiency may be a more powerful regulator of *Hamp* compared with inflammation.

To overcome the confounding effects of inflammation, we crossed *Ndfip1* knockout mice into a *Rag1*$^{-/-}$ immunodeficient background. Interestingly, compared with *Ndfip1*$^{-/-}$/*Rag1*$^{+/+}$ and *Ndfip1*$^{+/+}$/*Rag1*$^{-/-}$ mice, *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice fed a low-iron diet showed significantly higher Hb, Hct, and RBC (Figure 6A-C). These parameters in *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice were comparable with *Ndfip1*$^{+/+}$/*Rag1*$^{+/+}$ animals (Figure 6A-C). These results suggest that the anemia in *Ndfip1*$^{-/-}$ mice on the low-iron diet is, at least in part, due to inflammation. As predicted, *Ndfip1*$^{-/-}$/*Rag1*$^{-/-}$ mice exhibited increased DMT1 levels in the duodenum compared with single knockout mice (Figure 6F-H), as well as elevated serum iron levels (although not significant, Figure 6D) and a significant increase in transferrin saturation (Figure 6E).

Discussion

In previous studies, we had demonstrated that Ndfip1 and Ndfip2 proteins act as adaptors to regulate the ubiquitination of DMT1 by Nedd4 family members, such as WWP2 and Nedd4-2.[12,13] Those observations provided a novel mechanism of the regulation of iron transport via ubiquitination and possible degradation of DMT1. In the current study, we have extended previous observations to show that Ndfip1 is a key regulator of DMT1 expression in vivo. As such *Ndfip1*$^{-/-}$ mice fed a normal diet have increased iron stores in the liver, spleen, and bone marrow and higher serum iron concentration. These observations are consistent with iron overload due to increased DMT1 levels and activity, as would be predicted in *Ndfip1*$^{-/-}$ mice.

The control of iron transport and homeostasis are highly dependent on dietary iron.[2,3] As reported in this study, Ndfip1 plays a critical role in iron homeostasis, especially under low-iron dietary conditions. We found that *Ndfip1*$^{-/-}$ mice fed a low-iron diet had significantly more DMT1 expression and transport activity in duodenal enterocytes than in the *Ndfip1*$^{+/+}$ mice under the same conditions. More importantly and contrary to our expectations, *Ndfip1*$^{-/-}$ mice fed a low-iron diet developed more severe microcytic, hypochromic anemia than their wild-type *Ndfip1*$^{+/+}$ counterparts. Although *Ndfip1*$^{-/-}$ mice had higher serum iron and transferrin saturation than *Ndfip1*$^{+/+}$ mice, when fed a low-iron diet, the iron stores were significantly lower than in the mice of either genotype fed the normal iron diet. This suggests that *Ndfip1*$^{-/-}$ mice fed a low-iron diet are still iron deficient, and increased DMT1 activity in these mice is unable to compensate completely for reduced iron supplement. An important contributing factor for the severe anemia is inflammatory diseases in *Ndfip1*$^{-/-}$ mice.[14] Although the iron feeding experiments were carried out before the onset of severe inflammatory disease, *Ndfip1*$^{-/-}$ mice fed low-iron diet showed more inflammation compared with *Ndfip1*$^{-/-}$ mice fed normal iron diet. These data suggest that iron deficiency may accentuate the inflammatory phenotype in *Ndfip1*$^{-/-}$ mice. The removal of inflammation by crossing *Ndfip1*$^{-/-}$ mice into a *Rag1*$^{-/-}$ background resulted in preventing the onset of anemia, confirming that inflammation is important for the anemic phenotype.

Although the effect of inflammation on iron homeostasis is well established, there is limited literature assessing the effect of iron deficiency on the inflammatory process. In vitro studies demonstrate that iron chelation triggers inflammatory response in human intestinal epithelial cells, alveolar macrophages, and human acute monocytic leukemia cell line, THP1.[21,22] Another study showed that iron deficiency enhances inflammation through p38 MAPK–NF-κB–extracellular matrix metalloproteinase inducer and matrix metalloproteinase-9 pathways.[23] The data presented here suggest that a combination of iron deficiency and inflammation results in a more severe anemia in *Ndfip1*$^{-/-}$ mice. This is a unique murine model linking iron deficiency, inflammation, and anemia. The inflammation leads to suppression of erythropoiesis and ineffective use of iron. On the other hand, iron deficiency seems to exacerbate the inflammatory phenotype of *Ndfip1*$^{-/-}$ mice. The combination of iron deficiency and inflammation is a common clinical problem. Most of the patients suffering with systemic illness such as systemic lupus erythematosus, inflammatory bowel disease, and malignancies frequently develop iron deficiency either due to low iron intake and/or blood loss.

In conclusion, our study provides strong evidence that Ndfip1 is critical for DMT1 regulation and iron homeostasis in vivo. We predict that deregulated levels of Ndfip1 expression or modulation of its function by mutations or polymorphisms may have pathological consequences in humans.

Acknowledgments

We thank the staff of SA Pathology animal house for help with animal care and maintenance.

This work was supported by a project grant (508085) from the National Health and Medical Research Council of Australia.

Authorship

Contribution: N.J.F., L.E.D., H.E.D., and S.K. designed experiments; N.J.F., Y.A.L., L.E.D., H.E.D., and K.H. performed the experimental work; N.J.F., Y.A.L., L.E.D., H.E.D., D.H., and S.K. analyzed data and wrote the paper; D.H. analyzed all hematological data; L.Z. and M.D.G. generated antibodies and provided technical advice; and B.Y. generated genetically modified mice.

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

Correspondence: Professor Sharad Kumar, Department of Hematology, Centre for Cancer Biology, SA Pathology, PO Box 14, Rundle Mall, Adelaide, Australia; e-mail: sharad.kumar@health.sa.gov.au.

References

1. Isom HC, McDevitt EI, Moon MS. Elevated hepatic iron: a confounding factor in chronic hepatitis C. *Biochim Biophys Acta.* 2009;1790(7):650-662.
2. Anderson GJ, Vulpe CD. Mammalian iron transport. *Cell Mol Life Sci.* 2009;66(20):3241-3261.
3. Garrick MD, Garrick LM. Cellular iron transport. *Biochim Biophys Acta.* 2009;1790(5):309-325.
4. Mims MP, Prchal JT. Divalent metal transporter 1. *Hematology.* 2005;10(4):339-345.
5. Canonne-Hergaux F, Gruenheid S, Ponka P, Gros P. Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. *Blood.* 1999;93(12):4406-4417.
6. Gruenheid S, Canonne-Hergaux F, Gauthier S, Hackam DJ, Grinstein S, Gros P. The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. *J Exp Med.* 1999;189(5):831-841.
7. Rotin D, Kumar S. Physiological functions of the

HECT family of ubiquitin ligases. *Nat Rev Mol Cell Biol.* 2009;10(6):398-409.

8. Shearwin-Whyatt L, Dalton HE, Foot N, Kumar S. Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. *Bioessays.* 2006;28(6):617-628.
9. Jolliffe CN, Harvey KF, Haines BP, Parasivam G, Kumar S. Identification of multiple proteins expressed in murine embryos as binding partners for the WW domains of the ubiquitin-protein ligase Nedd4. *Biochem J.* 2000;351(3):557-565.
10. Harvey KF, Shearwin-Whyatt LM, Fotia A, Parton RG, Kumar S. N4WBP5, a potential target for ubiquitination by the Nedd4 family of proteins, is a novel Golgi-associated protein. *J Biol Chem.* 2002;277(11):9307-9317.
11. Shearwin-Whyatt LM, Brown DL, Wylie FG, Stow JL, Kumar S. N4WBP5A (Ndfip2), a Nedd4-interacting protein, localizes to multivesicular bodies and the Golgi, and has a potential role in protein trafficking. *J Cell Sci.* 2004;117(16):3679-3689.
12. Foot NJ, Dalton HE, Shearwin-Whyatt LM, et al. Regulation of the divalent metal ion transporter DMT1 and iron homeostasis by a ubiquitin-dependent mechanism involving Ndfips and WWP2. *Blood.* 2008;112(10):4268-4275.
13. Howitt J, Putz U, Lackovic J, et al. Divalent metal transporter 1 (DMT1) regulation by Ndfip1 prevents metal toxicity in human neurons. *Proc Natl Acad Sci U S A.* 2009;106(36):15489-15494.
14. Oliver PM, Cao X, Worthen GS, et al. Ndfip1 protein promotes the function of itch ubiquitin ligase to prevent T cell activation and T helper 2 cell-mediated inflammation. *Immunity.* 2006;25(6):929-940.
15. Kuo HC, Smith JJ, Lis A, et al. Computer-identified nuclear localization signal in exon 1A of the transporter DMT1 is essentially ineffective in nuclear targeting. *J Neurosci Res.* 2004;76(4):497-511.
16. Ludwiczek S, Theurl I, Bahram S, Schumann K, Weiss G. Regulatory networks for the control of body iron homeostasis and their dysregulation in HFE mediated hemochromatosis. *J Cell Physiol.* 2005;204(2):489-499.
17. Dupic F, Fruchon S, Bensaid M, et al. Duodenal mRNA expression of iron related genes in response to iron loading and iron deficiency in four strains of mice. *Gut.* 2002;51(5):648-653.
18. Weiss G. Iron metabolism in the anemia of chronic disease. *Biochim Biophys Acta.* 2009;1790(7):682-693.
19. Nemeth E, Ganz T. The role of hepcidin in iron metabolism. *Acta Haematol.* 2009;122(2-3):78-86.
20. Darshan D, Frazer DM, Wilkins SJ, Anderson GJ. Severe iron deficiency blunts the response of the iron regulatory gene Hamp and proinflammatory cytokines to lipopolysaccharide. *Haematologica.* 2010;95(10):1660-1667.
21. Choi EY, Kim EC, Oh HM, et al. Iron chelator triggers inflammatory signals in human intestinal epithelial cells: involvement of p38 and extracellular signal-regulated kinase signaling pathways. *J Immunol.* 2004;172(11):7069-7077.
22. O'Brien-Ladner AR, Blumer BM, Wesselius LJ. Differential regulation of human alveolar macrophage-derived interleukin-1beta and tumor necrosis factor-alpha by iron. *J Lab Clin Med.* 1998;132(6):497-506.
23. Fan Y, Wang J, Wei L, He B, Wang C, Wang B. Iron deficiency activates pro-inflammatory signaling in macrophages and foam cells via the p38 MAPK-NF-kappaB pathway [published ahead of print July 30, 2010]. *Int J Cardiol.* doi:10.1016/j.ijcard.2010.07.005.