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Evidence that the expression of transferrin receptor 1 on erythroid marrow cells mediates hepcidin suppression in the liver

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Abstract

Hepcidin is the key regulator of iron absorption and recycling, and its expression is suppressed by red blood cell production. When erythropoiesis is expanded, hepcidin expression decreases. To gain insight into the stage of erythroid differentiation at which the regulation might originate, we measured serum hepcidin levels in archived pure red cell aplasia samples from patients whose block in erythroid differentiation was well defined by hematopoietic colony assays and marrow morphologic review. Hepcidin values are high or high normal in pure red cell aplasia patients in whom erythropoiesis is inhibited prior to the proerythroblast stage, but are suppressed in patients with excess proerythroblasts and few later erythroid cells. These data suggest that the suppressive effect of erythropoietic activity on hepcidin expression can arise from proerythroblasts, the stage at which transferrin receptor 1 expression peaks, prompting the hypothesis that transferrin receptor 1 expression on erythroid precursors is a proximal mediator of the erythroid regulator of hepcidin expression. Our characterization of erythropoiesis, iron status, and hepcidin expression in mice with global or hematopoietic cell-specific haploinsufficiency of transferrin receptor 1 provides initial supporting data for this model. The regulation appears independent of erythroferrone and growth differentiation factor 15, supporting the concept that several mechanisms signal iron need in response to an expanded erythron.

The human body requires ~20–25 mg of iron per day to maintain its daily red cell production. The iron is provided mainly by macrophages that retrieve it from senescent red

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Conflict of interest disclosure

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cells and, in small part, by intestinal iron absorption. Transferrin-bound iron in the blood is then delivered to developing erythroid precursors in the bone marrow, which require transferrin receptor 1 (TFRC) for adequate iron uptake [1,2]. On the basis of ferrokinetic studies, Finch proposed that intestinal iron absorption and the mobilization of iron from stores in macrophages and hepatocytes are controlled by both a stores regulator and an erythroid regulator [3]. The stores regulator is responsible for meeting the body's normal iron requirements and for maintaining iron stores, whereas the erythroid regulator ensures an adequate iron supply to the erythron, regardless of the body's iron balance.

Hepcidin is key to iron metabolism because it is the common mediator of both the stores and erythroid regulators. Hepcidin acts by binding the iron export protein, ferroportin, leading to its degradation [4]; this inhibits dietary iron absorption and macrophage iron recycling. Hepcidin synthesis is increased by an excess of iron and decreased by erythropoietic activity [5–7].

In some human diseases and in murine models (e.g., transferrin-deficient mice [8], β-thalassemia, and congenital dyserythropoietic anemia [9,10]), there is both iron overload and anemia, and thus coexisting signals to both up-regulate and down-regulate hepcidin expression. In these conditions, the erythroid regulator is dominant.

Exactly how an erythroid precursor in the bone marrow communicates its iron need to hepatocytes remains unknown. Data suggest that the regulator is a soluble molecule [11]. The erythrokine growth differentiation factor 15 (GDF-15), which is markedly elevated in β-thalassemic serum, was identified as one possible regulator in pathologic states [12], though some data refute this [13]. Accumulating data suggest that GDF-15 is unlikely to mediate hepcidin suppression in normal erythropoiesis and during acute erythropoietic stress [14–16]. Although the recently characterized erythroid factor erythroferrone (Erfe) is capable of suppressing hepcidin expression under erythropoietic stress [17], its role in homeostasis is unknown.

As the regulator originates from the erythroid marrow [6,7], we studied an instructive group of patients with pure red cell aplasia (PRCA) to determine which stages of erythropoiesis signal hepcidin suppression. PRCA is characterized by severe normochromic, normocytic, or macrocytic anemia associated with reticulocytopenia and the near absence of hemoglobin-containing cells in an otherwise normal marrow aspirate. Therefore, there is maturation arrest at or before the proerythroblast stage [18]. In the study described here, we measured hepcidin levels in stored serum from a cohort of immunologically mediated PRCA patients whose block in erythroid differentiation was previously established [19]. Our data suggest that the erythroid regulator of hepcidin expression can derive from proerythroblasts, but not from less-differentiated erythroid progenitors. Recognizing that TFRC expression peaks on proerythroblasts [20] and after considering the published data regarding hepcidin regulation in murine studies and human disorders, we hypothesized that TFRC is a proximal mediator of the erythroid regulator of hepcidin expression and tested this directly in a murine model of Tfrc haploinsufficiency.

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Methods

PRCA samples, hepcidin and GDF-15 assays, and marrow reviews

Sera, obtained from patients with PRCA who were evaluated at the University of Washington between 1982 and 1992, were stored at −80°C. Patient characteristics were previously published [19]. Hepcidin [21] and GDF-15 assays (Quantikine Human GDF-15 Immunoassay, R&D Systems, Minneapolis, MN, USA) were performed blinded to diagnoses. Hepcidin concentration in a recent PRCA case was similar to those in the archived samples at the same stage of erythroid differentiation block, indicating that hepcidin in archived samples did not significantly degrade. S.C. rereviewed the marrow morphology in available specimens. The University of Washington Human Subjects Committee approved all studies.

Animal studies

Mice with haploinsufficiency of Tfrc were obtained from Nancy Andrews (Duke School of Medicine) on a C57BL/6 background [2] and housed at University of Washington. Polymerase chain reaction (PCR) genotyping was performed on tail biopsy DNA (list of primers is provided in Supplementary Table E1, online only, available at www.exphem.org). Ten million bone marrow cells were transplanted into 6- to 8-week-old Pep3b lethally irradiated recipients. Mice were sacrificed for analyses after stable engraftment and at least 7 weeks posttransplantation. The University of Washington Institutional Animal Care and Use Committee approved all studies. Mice were given 100 U epoetin alfa (Centocor Ortho Biotech, Horsham, PA, USA) intraperitoneally and sacrificed 15 hours later. Linear regression analysis was performed using GraphPad Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA).

Blood analyses

Mice were bled retro-orbitally. Reticulocyte counts were obtained at Phoenix Laboratory (Everett, WA, USA) on an ADVIA120 (Siemens Medical Solutions USA, Malvern, PA). Complete blood counts were obtained on a Hemavet HV950FS (Drew Scientific, Waterbury, CT) analyzer. Erythropoietin was measured using Mouse/Rat EPO Immunoassay Kit (R&D Systems). Serum iron parameters were determined using an iron/TIBC kit (Pointe Scientific, Canton, MI, USA). Nonheme tissue iron content was quantified as described [22]. Soluble transferrin receptor was measured by an enzyme-linked immunosorbent assay (MyBioSource, San Diego, CA, USA).

Quantitative real-time reverse transcription PCR

Mice were sacrificed by cervical dislocation under 2,2,2-tribromoethanol. Total RNA was isolated using Trizol (Life Technologies, Grand Island, NY, USA). Complementary DNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), and murine liver hepcidin, Bmp6, and Id1 messenger RNA (mRNA) levels were determined with 5′-nuclease quantitative PCR assays. Gene expression was quantified by the Pfaffl method [23] using the mean of duplicate samples relative to β-actin expression. Expression of Erfe in FACSAria-sorted hematopoietic cell populations with the FACSAria (BD

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Biosciences, San Jose, CA, USA) was measured by SYBR green quantitative PCR assays and normalized to β-actin expression (primers in Supplementary Table E1, online only, available at www.exphem.org).

Flow cytometry

Single-cell suspensions of freshly isolated marrow or spleen were immunostained with antibodies to Ter119, CD44, CD71, B220, and Gr1 (BD Pharmingen, Franklin Lakes, NJ, USA) [24,25]. $B220^{+}Gr1^{+}$ cells were excluded from analyses. Additional cells were fixed with 1% paraformaldehyde, permeabilized with ice-cold methanol, and immunostained with an antibody recognizing phospho-STAT5(Y694). Staining was quantified with a FACSCanto Flow Cytometer (BD Biosciences), and data were analyzed with FlowJo Tree Star (FlowJo, Ashland, OR, USA). The geometric mean fluorescence of CD71 (Tfrc) expression in each population was normalized to the wild-type average of population I/II, which was defined as 100%. Absolute marrow cellularity was calculated according to Colvin et al. [26]. We defined erythroid Tfrc mass as the product of the absolute number of cells in a gated population and the normalized CD71 expression of that population, calculated separately for marrow and spleen populations, and then added together to obtain the absolute Tfrc mass of each population.

Results

Hepcidin expression is not suppressed in samples from PRCA patients with a block in differentiation prior to the proerythroblast stage, but is suppressed in PRCA patients with excess numbers of proerythroblasts

To minimize the confounding effects of transfusion or immunosuppressive therapy on hepcidin expression, we restricted our analysis to those PRCA patients evaluated at disease presentation (serum samples were available from 13 of the 21 patients in the original cohort who met this criterion). Clinical data were previously published (summarized in Supplementary Table E2, online only, available at www.exphem.org). We defined four stages of differentiation blocks: before BFU-E (burst-forming units—erythroid, i.e., no colony growth), BFU-E to CFU-E (colony-forming units—erythroid, i.e., BFU-E are present, but CFU-E are not detected), CFU-E to proerythroblast (i.e., BFU-E and CFU-E are present, but proerythroblasts are rare or absent), and proerythroblast stage (i.e., ample to excess proerythroblasts). In the current study, we repeated a pathologic review of seven available marrow aspirates and one newly diagnosed PRCA patient's aspirate to confirm the diagnosis and enumerate the number of proerythroblasts present relative to total marrow erythroid mononuclear cells. We confirmed that there was a relative increase in proerythroblasts in two marrows (patients 24 and 2). The ratios of proerythroblasts to total marrow erythroid mononuclear cells were 0.82, and 0.77, respectively, whereas the ratio ranged from 0 to 0.50 in the other evaluable patient samples. All 11 patients in whom erythropoiesis was blocked at or prior to CFU-E had high normal (3/11) or elevated (8/11) hepcidin levels (mean hepcidin level was 490.2 ± 110.2 ng/mL compared with 42.0 ± 22.3 ng/mL in control samples, $p = 0.002$) (Supplementary Figure E1A, online only, available at www.exphem.org). In contrast, hepcidin was suppressed in one of the two patients in whom erythroid differentiation was blocked at the proerythroblast stage (patient 2, 12.7 ng/mL).

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Importantly, patient 2 had the highest number of proerythroblasts relative to total marrow mononuclear cells among all PRCA patients analyzed (0.21 vs. 0–0.08) (Supplementary Figure E1B, online only, available at www.exphem.org). Although patient 24 also had a near-complete block in erythroid differentiation at the proerythroblast stage, his hepcidin was not suppressed; of note, he had many fewer proerythroblasts (ratio = 0.08). As GDF-15 is implicated in the inhibition of hepcidin in pathologic conditions [12], we also measured serum GDF-15 levels; these values did not correlate with the site of block or clinical parameters (Supplementary Figure E2, online only, available at www.exphem.org). These data imply that proerythroblasts can signal the liver to suppress hepcidin expression, but do not include or exclude the possibility that later erythroid cells can also signal.

As TFRC expression increases at the CFU-E stage and peaks on proerythroblasts before decreasing [20], we considered whether the abundance of TFRC protein might be a physiologic way that the erythron judges its need for iron. We reasoned that patient 2 had excess proerythroblasts, each with high TFRC expression, and thus a relatively high total TFRC mass that might signal hepcidin suppression. Patients with a block in erythroid differentiation prior to proerythroblasts would have low TFRC protein on all marrow cells and would be unable to signal hepcidin suppression. Recognizing that our hepcidin data have multiple confounding variables (e.g., hepcidin values are known to vary with age, sex, transfusion, time of day, menopausal status, and potential hepcidin degradation caused by prolonged storage of serum samples) and that the hypothesis resulted from an observation in a single patient, we tested the relationship of erythroid cell surface TFRC levels to hepcidin expression directly and quantitatively in a murine model.

Mice with decreased erythroid Tfrc mass are unable to suppress hepcidin expression despite iron-restricted erythropoiesis

Tfrc$^{+/-}$ mice have iron-restricted erythropoiesis characterized by a mild microcytic, hypochromic anemia (Table 1) and a relative and absolute expansion of early marrow erythroid precursors (population III, which represents polychromatophilic erythroblasts [27], $16.8 \pm 2.2\%$ vs. $13.0 \pm 1.2\%$, $p = 0.002$, and $4.9 \pm 1.0 \times 10^7$ vs. $3.5 \pm 0.4 \times 10^7$, $p = 0.004$) (Fig. 1). Splenic analyses did not reveal significant differences in relative or absolute numbers of erythroid precursors as they were not consistently expanded in *Tfrc*$^{+/-}$ mice, confirming that the animals model physiologic and not stress erythropoiesis (Supplementary Figure E3, online only, available at www.exphem.org). Thus, *Tfrc*$^{+/-}$ mice have iron-restricted and mildly expanded erythropoiesis, consistent with previous reports [2].

As expected, Tfrc levels per cell are decreased on *Tfrc*$^{+/-}$ compared with control cells at all stages of terminal erythroid differentiation (Fig. 2). Additionally, the total quantity of cell surface transferrin receptor present on marrow and splenic erythroid precursors calculated as a function of mean fluorescence intensity and cell number (and termed erythroid Tfrc mass) is lower on the *Tfrc*$^{+/-}$ erythron compared with controls despite the early erythroid expansion ($11.7 \pm 2.4 \times 10^7$ vs. $7.9 \pm 1.1 \times 10^7$, $p = 0.003$). As a secondary study, we measured soluble Tfrc concentration in serum [13,28]. Soluble Tfrc concentration was equivalent in *Tfrc*$^{+/-}$ and controls (Table 2); we suspect this is a consequence of the higher absolute numbers of polychromatophilic erythroblasts and reticulocyte counts observed in

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Tfrc$^{+/-}$ compared with control animals, as these populations are known to preferentially contribute to soluble Tfrc levels in vivo [13,29]. Splenic erythropoiesis, which is comparable in *Tfrc*$^{+/-}$ and control mice, constitutes only ~10% of the total steady-state erythroid compartment and, thus, contributes little to the total erythroid Tfrc mass (Supplementary Figure E3, online only, available at www.exphem.org).

Humans and mice with iron-restrictive erythropoiesis caused by dietary iron deficiency [21] or a lack of transferrin [8] have early erythroid expansion and suppressed hepcidin [27]. Because *Tfrc*$^{+/-}$ mice have iron-restricted-erythropoiesis and also early erythroid expansion, we expected hepcidin to be lower in *Tfrc*$^{+/-}$ mice than in controls. However, *Tfrc*$^{+/-}$ mice had hepcidin expression comparable to that of control animals (Fig. 3A). Importantly, serum transferrin saturations were comparable to those of control mice, whereas liver iron content was lower in *Tfrc*$^{+/-}$ than in control mice, so any contribution of the stores regulator (which stimulates hepcidin expression) to hepcidin expression in *Tfrc*$^{+/-}$ mice should be equivalent to or less than that of controls (Table 2). Consistent with equivalent serum iron availability, expression of bone morphogenetic protein 6 (Bmp6) and inhibitor of DNA binding 1 (Id1) [30] were not reduced in *Tfrc*$^{+/-}$ mice (Supplementary Figure E4, online only, available at www.exphem.org). These data imply that the erythroid regulator of hepcidin suppression is not active in *Tfrc*$^{+/-}$ mice and are consistent with our working model that Tfrc is needed to suppress hepcidin expression.

Failure to suppress hepcidin expression is hematopoietic-cell intrinsic

The liver is the physiologically important site for modulating hepcidin expression in response to body iron status. Specifically, it is proposed that Hfe–Tfrc complexes on the surface of hepatocytes sense the saturation of iron-bound transferrin in the serum; at low transferrin saturations, Hfe is sequestered by Tfrc, and as transferrin saturation increases, Hfe is dislodged from its overlapping binding site on Tfrc by holotransferrin and is then free to interact with transferrin receptor 2 and signal the upregulation of hepcidin expression [31]. To exclude any confounding liver-specific effect of Tfrc haploinsufficiency on hepcidin expression and, thus, on interpretation of data centered on the erythroid regulator of hepcidin specifically, we transplanted *Tfrc*$^{+/-}$ (or control) marrow into lethally irradiated wild-type recipients and confirmed that, analogous to *Tfrc*$^{+/-}$ mice, mice with hematopoietic cell-specific *Tfrc* haploinsufficiency also have iron-restricted erythropoiesis (Table 3), reduced Tfrc levels per cell at all stages of terminal erythroid differentiation (Fig. 4), and reduced erythroid Tfrc mass ($3.3 \pm 0.6 \times 10^7$ vs. $7.1 \pm 2.4 \times 10^7$, n = 3 mice per group, p = 0.058), and percentage transferrin saturation is not significantly reduced compared with that of control mice (Table 4). Hepcidin levels are higher in these mice than in controls, and this difference is significant among male mice (3.9 ± 1.4 vs. 1.0 ± 0.6, $p = 0.03$) (Fig. 3B). Thus, the lack of hepcidin suppression in the face of iron-restricted erythropoiesis observed in *Tfrc*$^{+/-}$ mice holds in the transplant model.

Hepcidin expression correlates with erythroid Tfrc mass

Given that hepcidin expression is not suppressed in *Tfrc*$^{+/-}$ mice under basal conditions, we inquired whether this relationship holds under an acute erythropoietic stress. We treated mice with erythropoietin, sacrificed the animals 15 hours later, characterized their

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erythropoiesis, and determined liver hepcidin expression. As expected [32], erythropoietin treatment significantly increases Tfrc expression in all early marrow erythroid precursor populations in *Tfrc*$^{+/-}$ mice and control mice (I–III) (Fig. 2), resulting in an increase in total erythroid Tfrc mass ($16.2 \pm 1.8 \times 10^7$ in control mice vs. $10.9 \pm 1.7 \times 10^7$ in *Tfrc*$^{+/-}$ mice, $p = 0.02$). The increase in the Tfrc mass of erythroid populations I–III correlates with hepcidin expression (Fig. 5A), consistent with the hypothesis that Tfrc signals the erythroid regulator of hepcidin expression; this regulator, in turn, mediates a signal to the liver to suppress hepcidin expression. We restricted our analysis to erythroid populations I–III, as those cells demonstrate erythropoietin responsiveness as evident by increased transferrin receptor expression in response to erythropoietin (see Fig. 2). Of note, 15 hours after erythropoietin treatment, the total number of erythroid cells had not yet increased in control mice ($7.8 \pm 1.7 \times 10^7$ vs. $9.1 \pm 0.9 \times 10^7$, $p = 0.28$) or in *Tfrc*$^{+/-}$ mice ($11.1 \pm 3.4 \times 10^7$ vs. $9.4 \pm 0.9 \times 10^7$, $p = 0.44$) and did not correlate with hepcidin expression ($R^2 = 0.24$). This allowed us to separate a correlation between Tfrc mass and hepcidin expression from one between total erythroid cell number and hepcidin expression. Together, this work implies that the erythroid Tfrc mass is a proximal mediator of the erythroid regulator of hepcidin expression, both at baseline and in response to an acute erythropoietic stress.

Secreted molecule erythroferrone does not function downstream of Tfrc *to suppress hepcidin expression*

Kautz et al. [17,33] identified Erfe as a soluble molecule that transmits the erythron's iron needs to the liver [17]. Erythroferrone mRNA expression increases before hepcidin mRNA expression decreases in mice subjected to an acute erythropoietic stress (i.e., phlebotomy or erythropoietin therapy), and mice lacking Erfe fail to suppress hepcidin in response to phlebotomy or erythropoietin treatment. Considering these data within the context of our finding that erythroid Tfrc mass correlates with hepcidin expression, we asked whether Tfrc might signal a suppression in hepcidin expression by increasing *Erfe* transcription. We confirmed the findings of Kautz et al. that *Erfe* expression is high in early erythroid precursors (Supplementary Figure E5, online only, available at www.exphem.org) and increases in response to the acute erythropoietic stress of erythropoietin therapy, where its expression correlates with hepcidin expression (Fig. 5B). We then measured Erfe expression in our animals at baseline (basal conditions) and after erythropoietin therapy (pharmacologic stress erythropoiesis). Given hepcidin expression is not suppressed in *Tfrc*$^{+/-}$ mice, if Erfe functions as the erythroid regulator of hepcidin expression and functions downstream of Tfrc, one would expect Erfe to be lower in *Tfrc*$^{+/-}$ mice than in controls. However, Erfe expression is not reduced in *Tfrc*$^{+/-}$ mice during basal erythropoiesis or after erythropoietin therapy (Fig. 6). These data suggest that Erfe and Tfrc function to suppress hepcidin expression after erythropoietin therapy independently. Consistent with this finding, Erfe and Tfrc [32] are both transcriptionally regulated by Stat5, and Stat5 activation in response to erythropoietin therapy was comparable in *Tfrc*$^{+/-}$ and control mice (Supplementary Figure E6, online only, available at www.exphem.org).

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Discussion

Erythroid cells are dependent on TFRC for their cellular iron uptake [2]. Expression is highest on proerythroblasts through basophilic erythroblasts, decreases with further erythroid differentiation [20], and is upregulated both by iron deficiency (posttranscriptional regulation via IRP/IRE interactions [5,34]) and by increased erythropoiesis (transcriptional regulation via erythropoietin and Stat5 signaling [32]). When there is a need for increased red cell production (and, thus, increased iron delivery to marrow erythroid cells), the erythron expands, Tfrc mass increases, and hepcidin is suppressed. If iron delivery is compromised, early erythroid precursors expand and the Tfrc protein expression per cell increases; thus, Tfrc mass also increases in this setting. A normal Tfrc mass maintains iron delivery for basal erythropoiesis, and alternatively, when the Tfrc mass is reduced (post-cytoreductive chemotherapy), erythropoiesis ceases, and there is no further need for iron. Thus, the erythroid Tfrc mass reflects the erythron's iron needs. These observations and our findings in PRCA patients led us to hypothesize that transferrin receptor 1 on erythroid cells is a proximal mediator of the erythroid regulator of hepcidin suppression.

Our murine studies provide initial supporting data for this hypothesis. *Tfrc*$^{+/-}$ mice have iron-restricted erythropoiesis, yet hepcidin is not suppressed in these animals. In the *Tfrc*$^{+/-}$ mice, each erythroid precursor is iron-restricted and the animals demonstrate a compensatory increase in the number of early erythroid precursors. In this setting (i.e., early erythroid expansion), hepcidin suppression would be expected, but as the Tfrc mass (more cells × less Tfrc per cell) is reduced compared with that of control mice, there is no hepcidin suppression. Important support for this hypothesis is that the same observations held in mice with hematopoietic cell-specific Tfrc deficiency in which the percentages of transferrin saturation and liver iron content were equivalent in experimental and control animals, thus excluding any possible confounding stimulatory signal to hepcidin expression from a hepatocyte-derived stores regulator.

Additionally, this hypothesis is consistent with the findings of prior human and murine studies and accounts for previously difficult-to-reconcile data (Table 5). For example, the hypothesis explains why hepcidin is suppressed in patients with recovering erythropoiesis after marrow transplantation [6] and in healthy volunteers who receive erythropoietin [15] (i.e., the abrupt increase in erythropoietin results in a predominance of proerythroblasts and basophilic erythroblasts as well as more TFRC per erythroid cell [29]), yet is normal (baseline) in persons with chronic (compensated) hemolysis in whom erythropoietin levels are only marginally elevated and erythroid hyperplasia is mild and exists across all stages of erythroid differentiation.

That hematopoietic cell-specific *Stat5* null mice have iron-restricted erythropoiesis yet have high hepcidin expression is also consistent with this hypothesis, because erythropoietin transcriptionally regulates Tfrc expression via Stat5, and thus, these mutant mice have low Tfrc expression on early progenitors [32]. The hypothesis further implies that iron deficiency leads to hepcidin suppression via both stores regulation and erythroid regulation, because Tfrc expression by an iron-restricted erythron is high. This would also explain the dramatic suppression of hepcidin when there is iron-restricted erythropoiesis (mild

microcytosis) but no anemia (thus normal erythropoietin), such as in patient A4 (Supplementary Figure E1A, online only, available at www.exphem.org). It also explains why hepcidin expression is low in hypotransferrinemic mice (high Tfrc/cell because of lack of erythron iron) and why it recovers but does not normalize (as Tfrc expression persists) when these mice receive red cell transfusions to correct the hemoglobin, suppress reticulocyte production, and presumably normalize erythropoietin levels [8].

Several erythroid regulators of hepcidin expression may exist. Murine studies have revealed that Erfe, a tumor necrosis factor α superfamily member, regulates hepcidin production after phlebotomy and after erythropoietin administration. Additionally, Erfe levels are higher in a mouse model of β-thalassemia characterized by ineffective erythropoiesis [17,33]. As Erfe expression was equivalent in *Tfrc*$^{+/-}$ and control mice, we suspect that Erfe acts via a distinct signaling cascade (Fig. 7).

What is the mechanism by which Tfrc might increase marrow production of a soluble regulator? The observations in hypotransferrinemic mice in which iron delivery to the erythron is restricted argue that this is not a consequence of the marrow's ability to take up iron or synthesize heme. We favor the possibility that Tfrc functions as a signaling molecule because the alternative possibility, that soluble Tfrc (released from erythroid cells) directly suppresses liver hepcidin expression, is inconsistent with published work [15,35,36] and our data in *Tfrc*$^{+/-}$ mice. In support of this mechanism, some data suggest a role for Tfrc in signaling erythroblast survival and proliferation separate from its iron transport function [37]. Future studies are needed to provide definitive proof for our provocative hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Trenor CC III, Campagna DR, Sellers VM, Andrews NC, Fleming MD. The molecular defect in hypotransferrinemic mice. Blood. 2000; 96:1113–1118. [PubMed: 10910930]
2. Levy JE, Jin O, Fujiwara Y, Kuo F, Andrews NC. Transferrin receptor is necessary for development of erythrocytes and the nervous system. Nat Genet. 1999; 21:396–399. [PubMed: 10192390]
3. Finch C. Regulators of iron balance in humans. Blood. 1994; 84:1697–1702. [PubMed: 8080980]
4. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004; 306:2090–2093. [PubMed: 15514116]
5. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: Molecular control of mammalian iron metabolism. Cell. 2004; 117:285–297. [PubMed: 15109490]
6. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. Blood. 2006; 108:3730–3735. [PubMed: 16882706]
7. Vokurka M, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. Physiol Res. 2006; 55:667–674. [PubMed: 16497104]
8. Bartnikas TB, Andrews NC, Fleming MD. Transferrin is a major determinant of hepcidin expression in hypotransferrinemic mice. Blood. 2011; 117:630–637. [PubMed: 20956801]

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9. Kearney SL, Nemeth E, Neufeld EJ, et al. Urinary hepcidin in congenital chronic anemias. Pediatr Blood Cancer. 2007; 48:57–63. [PubMed: 16220548]
10. Papanikolaou G, Tzilianos M, Christakis JI, et al. Hepcidin in iron overload disorders. Blood. 2005; 105:4103–4105. [PubMed: 15671438]
11. Weizer-Stern O, Adamsky K, Amariglio N, et al. Downregulation of hepcidin and haemojuvelin expression in the hepatocyte cell-line HepG2 induced by thalassaemic sera. Br J Haematol. 2006; 135:129–138. [PubMed: 16939499]
12. Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med. 2007; 13:1096–1101. [PubMed: 17721544]
13. Fertrin KY, Lanaro C, Franco-Penteado CF, et al. Erythropoiesis-driven regulation of hepcidin in human red cell disorders is better reflected through concentrations of soluble transferrin receptor rather than growth differentiation factor 15. Am J Hematol. 2014; 89:385–390. [PubMed: 24860871]
14. Kanda J, Mizumoto C, Kawabata H, et al. Serum hepcidin level and erythropoietic activity after hematopoietic stem cell transplantation. Haematologica. 2008; 93:1550–1554. [PubMed: 18641032]
15. Ashby DR, Gale DP, Busbridge M, et al. Erythropoietin administration in humans causes a marked and prolonged reduction in circulating hepcidin. Haematologica. 2010; 95:505–508. [PubMed: 19833632]
16. Tanno T, Rabel A, Lee YT, Yau YY, Leitman SF, Miller JL. Expression of growth differentiation factor 15 is not elevated in individuals with iron deficiency secondary to volunteer blood donation. Transfusion. 2010; 50:1532–1535. [PubMed: 20210929]
17. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet. 2014; 46:678–684. [PubMed: 24880340]
18. Keel, S.; Abkowitz, J. Pure red cell aplasia.. In: Young, NS.; Gerson, SL.; High, KA., editors. Clinical Hematology. Elsevier (Mosby); Maryland Heights. Mo: 2006.
19. Charles RJ, Sabo KM, Kidd PG, Abkowitz JL. The pathophysiology of pureredcellaplasia:Implicationsfor therapy. Blood. 1996; 87:4831–4838. [PubMed: 8639856]
20. Iacopetta BJ, Morgan EH, Yeoh GC. Transferrin receptors and iron up-take during erythroid cell development. Biochim Biophys Acta. 1982; 687:204–210. [PubMed: 6284220]
21. Ganz T, Olbina G, Girelli D, Nemeth E, Westerman M. Immunoassay for human serum hepcidin. Blood. 2008; 112:4292–4297. [PubMed: 18689548]
22. Torrance, JD.; Bothwell, TH. Tissue iron stores.. In: Cook, JD., editor. Methods in Hematology: Iron. Churchill Livingstone; New York: 1980. p. 90-115.
23. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45. [PubMed: 11328886]
24. Chen K, Liu J, Heck S, Chasis JA, An X, Mohandas N. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci U S A. 2009; 106:17413–17418. [PubMed: 19805084]
25. Socolovsky M, Nam HS, Fleming MD, Haase VH, Brugnara C, Lodish HF. Ineffective erythropoiesis in Stat5a–/–5b–/–mice due to decreased survival of early erythroblasts. Blood. 2001; 98:3261–3273. [PubMed: 11719363]
26. Colvin GA, Lambert JF, Abedi M, et al. Murine marrow cellularity and the concept of stem cell competition: Geographic and quantitative determinants in stem cell biology. Leukemia. 2004; 18:575–583. [PubMed: 14749701]
27. Liu S, Bhattacharya S, Han A, et al. Haem-regulated eIF2alpha kinase is necessary for adaptive gene expression in erythroid precursors under the stress of iron deficiency. Br J Haematol. 2008; 143:129–137. [PubMed: 18665838]
28. Huebers HA, Beguin Y, Pootrakul P, Einspahr D, Finch CA. Intact transferrin receptors in human plasma and their relation to erythropoiesis. Blood. 1990; 75:102–107. [PubMed: 2294984]
29. R'Zik S, Loo M, Beguin Y. Reticulocyte transferrin receptor (TfR) expression and contribution to soluble TfR levels. Haematologica. 2001; 86:244–251. [PubMed: 11255270]

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30. Kautz L, Meynard D, Monnier A, et al. Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. Blood. 2008; 112:1503–1509. [PubMed: 18539898]
31. Schmidt PJ, Toran PT, Giannetti AM, Bjorkman PJ, Andrews NC. The transferrin receptor modulates Hfe-dependent regulation of hepcidin expression. Cell Metab. 2008; 7:205–214. [PubMed: 18316026]
32. Zhu BM, McLaughlin SK, Na R, et al. Hematopoietic-specific Stat5-null mice display microcytic hypochromic anemia associated with reduced transferrin receptor gene expression. Blood. 2008; 112:2071–2080. [PubMed: 18552213]
33. Kautz L, Jung G, Nemeth E, Ganz T. The erythroid factor erythroferrone and its role in iron homeostasis. Blood. 2013; 122:4. [PubMed: 23828884]
34. Schranzhofer M, Schifrer M, Cabrera JA, et al. Remodeling the regulation of iron metabolism during erythroid differentiation to ensure efficient heme biosynthesis. Blood. 2006; 107:4159–4167. [PubMed: 16424395]
35. Flanagan JM, Peng H, Wang L, et al. Soluble transferrin receptor-1 levels in mice do not affect iron absorption. Acta Haematol. 2006; 116:249–254. [PubMed: 17119325]
36. Shintani N, Kohgo Y, Kato J, et al. Expression and extracellular release of transferrin receptors during peripheral erythroid progenitor cell differentiation in liquid culture. Blood. 1994; 83:1209–1215. [PubMed: 8118025]
37. Coulon S, Dussiot M, Grapton D, et al. Polymeric IgA1 controls erythroblast proliferation and accelerates erythropoiesis recovery in anemia. Nat Med. 2011; 17:1456–1465. [PubMed: 22019886]
38. Origa R, Galanello R, Ganz T, et al. Liver iron concentrations and uri-nary hepcidin in beta-thalassemia. Haematologica. 2007; 92:583–588. [PubMed: 17488680]
39. Libani IV, Guy EC, Melchiori L, et al. Decreased differentiation of erythroid cells exacerbates ineffective erythropoiesis in beta-thalassemia. Blood. 2008; 112:875–885. [PubMed: 18480424]
40. Ramos P, Guy E, Chen N, et al. Enhanced erythropoiesis in Hfe-KO mice indicates a role for Hfe in the modulation of erythroid iron homeostasis. Blood. 2011; 117:1379–1389. [PubMed: 21059897]

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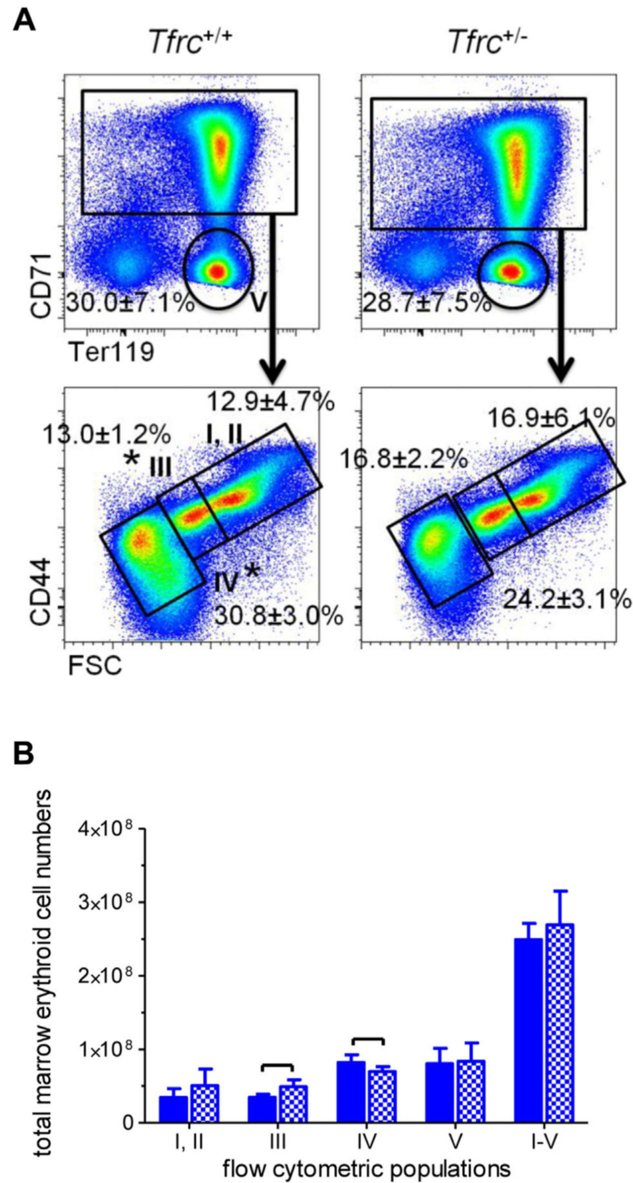

Figure 1.

Mice with *Tfrc* haploinsufficiency have a mild expansion of bone marrow polychromatophilic erythroblasts. (**A**) Flow cytometric analyses of bone marrow from a representative control and $Tfrc^{+/-}$ mouse immunostained with antibodies against CD71, CD44, Ter119, Gr1, and B220. $CD71^+$ cells are gated in the lower panel and plotted as CD44 versus forward scatter (FSC) to define the terminal stages of erythroid differentiation: proerythroblasts and basophilic erythroblasts (I/II), polychromatophilic erythroblasts (III), orthochromatophilic erythroblasts and reticulocytes (IV), and mature red blood cells (V). Percentages of cells among cells shown in the CD71 by Ter119 plot are shown (mean ± standard deviation). Control n = 7, $Tfrc^{+/-}$ n = 7. *p* values between the same individual control and $Tfrc^{+/-}$ populations: I/II = 0.20, III = 0.002, IV = 0.002, V = 0.73. (**B**) Absolute numbers of marrow erythroid precursors in each population and populations I–V combined. *Solid bars* represent wild-type mice, and *checkered bars* represent $Tfrc^{+/-}$ mice. Mean ±

standard deviation. Control n = 7, *Tfrc*$^{+/-}$ n = 7. *p* values: I, II = 0.11, III = 0.004, IV = 0.02, V = 0.8, I–V = 0.32. Erythroid precursors were enriched by excluding B220^{+} and Gr1^{+} cells from the analyses in (**A**) and (**B**).

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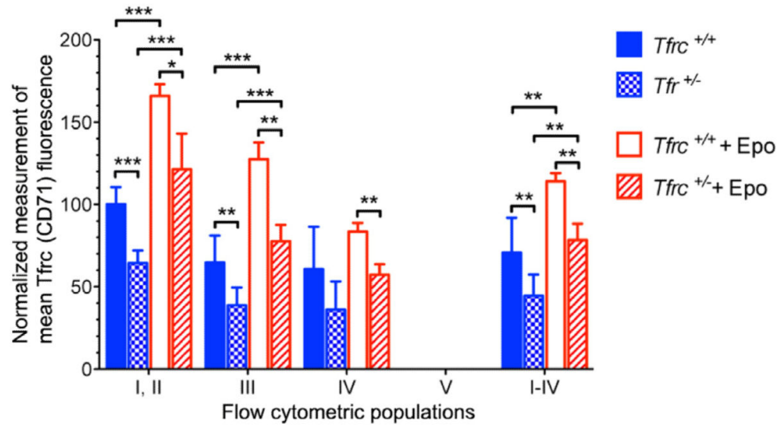

Figure 2.

Transferrin receptor expression on developing erythroblasts is markedly reduced in *Tfrc* haploinsufficient mice. Tfrc expression by flow cytometric analysis on bone marrow erythroblasts from control (*solid bars*) and *Tfrc*$^{+/-}$ (*checkered bars*) mice at baseline or from control (*open bars*) and *Tfrc*$^{+/-}$ (*hashed bars*) mice 15 hours after treatment with 100 U erythropoietin. Mean Tfrc fluorescence intensities of each population and of populations I–IV combined are expressed relative to the mean Tfrc fluorescence intensity of populations I, II from untreated control mice. Mean ± standard deviation of three to seven mice per group. *p* values: *<0.05, ** 0.01, *** 0.001; all remaining *p* values >0.05.

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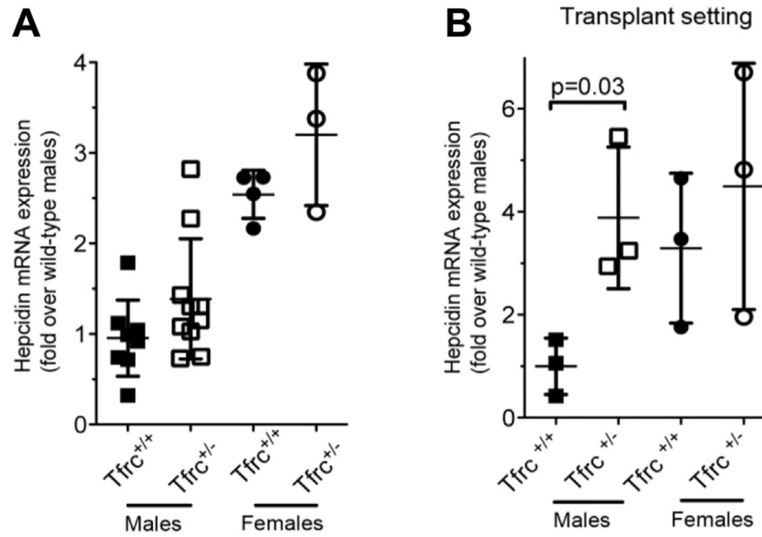

Figure 3.

Hepcidin mRNA expression of *Tfrc*$^{+/-}$ mice is comparable to that of control mice. (**A**) Liver hepcidin mRNA levels in control and *Tfrc*$^{+/-}$ mice. Data presented as mean values ± standard deviations. Control males, n = 8; *Tfrc*$^{+/-}$ males, n = 10; control females, n = 4; *Tfrc*$^{+/-}$ females, n = 3. All p values >0.05. (**B**) Liver hepcidin mRNA levels in mice transplanted with wild-type or *Tfrc*$^{+/-}$ marrow. Mean values ± standard deviations, n = 3 in each group. Messenger RNA levels were measured by reverse transcriptase quantitative real-time polymerase chain reaction. Sample polymerase chain reactions were performed in duplicate, normalized to β-actin mRNA levels, and expressed as fold over control male mRNA levels.

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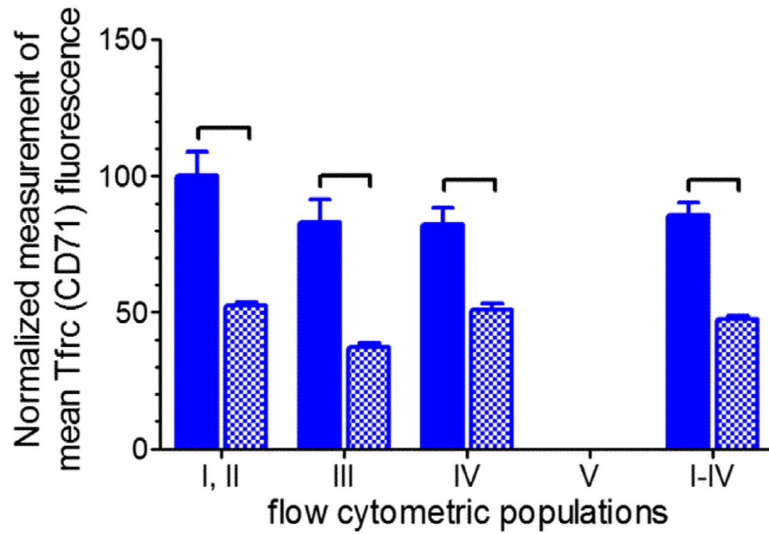

Figure 4.

Transferrin receptor expression on developing erythroblasts is reduced in hematopoietic cell-specific *Tfrc* haploinsufficient mice. Data are for Pep3b mice transplanted with wild-type or *Tfrc*$^{+/-}$ marrow and analyzed 7 weeks posttransplant. Tfrc expression by flow cytometric analysis on bone marrow erythroblasts from control (*solid bars*) and *Tfrc*$^{+/-}$ (*checkered bars*) mice. Mean Tfrc fluorescence intensities of each population and of populations I–IV combined are expressed relative to the mean Tfrc fluorescence intensity of population I, II from control mice. Means ± standard deviations. Control n = 3, *Tfrc*$^{+/-}$ n = 3, all p values   0.001. Populations I–IV are defined in Figure 1A. The percentages of cells and absolute numbers of cells in each population are comparable 7 weeks posttransplant in control and *Tfrc*$^{+/-}$ mice.

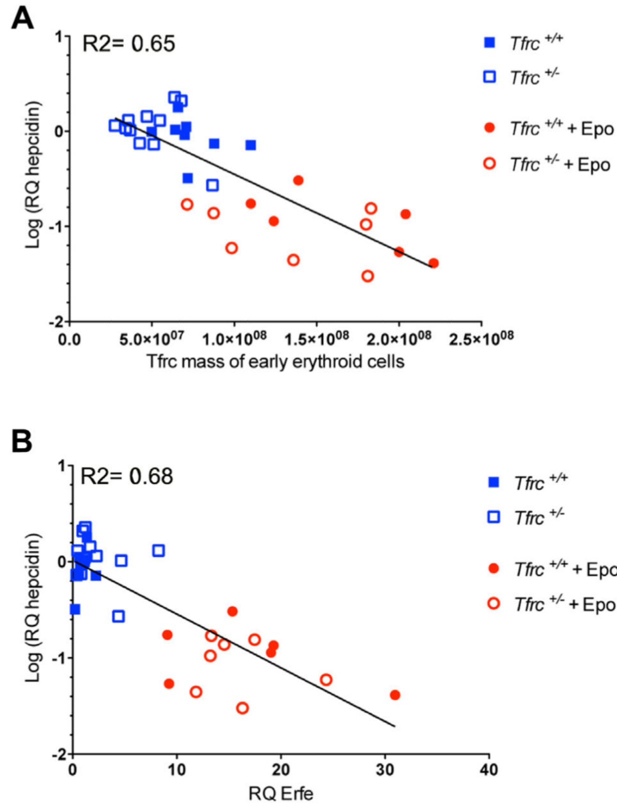

Figure 5.

Hepcidin expression correlates with erythroid transferrin receptor mass and marrow erythroferrone (*Erfe*) expression. Linear correlation analyses of hepcidin mRNA levels and either total early erythroblast (populations I–III, bone marrow, and spleen combined) transferrin receptor mass (**A**) or total marrow *Erfe* mRNA levels (**B**). Data represent a total of 32 control and *Tfrc*$^{+/-}$ male mice that were either not treated or treated with erythropoietin as indicated. Each group includes data from at least six mice. RQ hepcidin and RQ Erfe = quantification of mRNA expression relative to the mean of untreated male mice.

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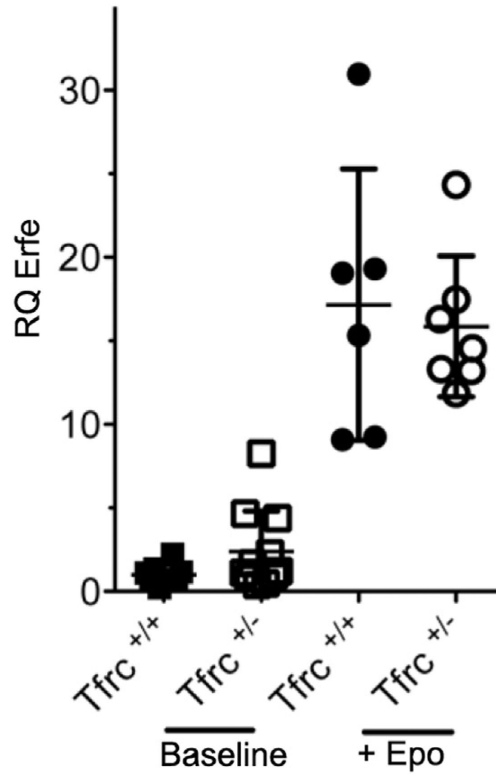

Figure 6.

Erythroferrone expression of *Tfrc*$^{+/-}$ mice is comparable to that of control mice. Bone marrow erythroferrone mRNA levels in control and *Tfrc*$^{+/-}$ male mice. Sample polymerase chain reactions were performed in duplicate, normalized to β-actin mRNA levels, and expressed relative to the mean of untreated control mice (*solid squares*). Baseline *Tfrc*$^{+/-}$ mice (*open squares*), control (*solid circles*), and *Tfrc*$^{+/-}$ (*open circles*) mice 15 hours after erythropoietin treatment. Mean values ± standard deviations from at least six mice per group.

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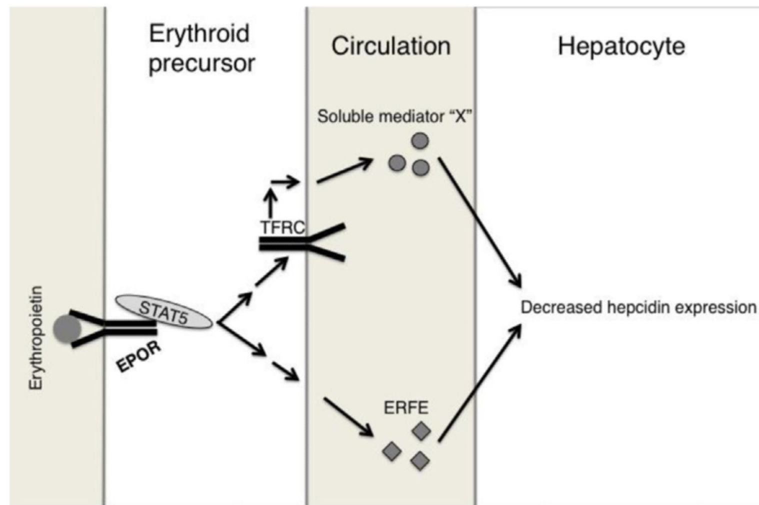

Figure 7.

Model of erythroid regulation of liver hepcidin expression. Erythropoietin binds to its receptor and activates STAT5. This, in turn, stimulates erythroid *TFRC* and *ERFE* expression and results in increased TFRC and ERFE protein expression. ERFE inhibits liver hepcidin expression. In parallel, TFRC on erythroid precursors signals the release of a soluble factor "X" into the circulation that suppresses liver hepcidin expression. Our data are also consistent with the possibility that TFRC functions by increasing ERFE expression posttranscriptionally or by increasing ERFE release.

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Table 1

Hematological parameters in 12-week old $Tfrc^{+/-}$ and control mice

	$Tfrc^{+/+}$ (n = 7)	$Tfrc^{+/-}$ (n = 7)	p Value
Hemoglobin (g/dL)	14.6 ± 0.5	13.8 ± 0.3	0.003
Hematocrit (%)	53.4 ± 7.6	46.8 ± 5.4	NS
Red blood cells (M/μL)	10.4 ± 0.6	11.5 ± 0.3	0.002
Mean corpuscular volume (fL)	51.2 ± 4.8	40.8 ± 3.9	0.0008
Mean corpuscular hemoglobin (pg)	14.1 ± 0.5	12.1 ± 0.4	<0.0001
Reticulocytes (K/μL)	355.1 ± 64.0	451.3 ± 41.8	0.006
Erythropoietin (pg/mL)	491.7 ± 392.8	326.3 ± 164.5	NS
Platelets (K/μL)	713.6 ± 265.7	764.9 ± 117.6	NS
White blood cells (K/μL)	11.0 ± 4.1	10.2 ± 4.9	NS

NS = not significant.

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Table 2

Iron parameters in 11- to 15-week-old male *Tfrc*$^{+/-}$ and control mice

	Tfrc$^{+/+}$ **(n = 8)**	***Tfrc***$^{+/-}$ **(n = 10)**	***p* Value**
Serum iron (μg/dL)	95.5 ± 15.9	84.2 ± 10.2	0.08
Transferrin saturation (%)	28.8 ± 4.2	27.1 ± 4.8	0.45
Liver iron (μg/g)	56.1 ± 9.6	42.5 ± 8.6	0.01
Kidney iron (μg/g)	44.2 ± 3.6	38.8 ± 4.3	0.01
Spleen iron			
μg/g	400.6 ± 96.7	284.7 ± 99.1	0.02
μg	25.0 ± 5.9	18.0 ± 5.9	0.02
Soluble transferrin receptor (ng/mL)	5.1 ± 2.6	4.4 ± 2.5	0.57
Erythropoietin (pg/mL)	504 ± 372	514 ± 310	0.95

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Table 3

Hematologic parameters in mice transplanted with control or *Tfrc*$^{+/-}$ marrow 7–8 weeks after transplant

	Tfrc$^{+/+}$ **(n = 10)**	***Tfrc***$^{+/-}$ **(n = 10)**	***p* Value**
Hemoglobin (g/dL)	14.6 ± 0.4	13.7 ± 1.0	0.02
Hematocrit (%)	47.0 ± 1.2	43.3 ± 2.2	<0.001
Red blood cells (M/μL)	10.1 ± 0.3	11.5 ± 0.6	<0.001
Mean corpuscular volume (fL)	46.7 ± 0.4	37.8 ± 0.9	<0.001
Mean corpuscular hemoglobin (pg)	14.5 ± 0.2	12.0 ± 0.4	<0.001
Reticulocytes (K/μL)	337 ± 25	400 ± 47	0.11 (n = 3)
Erythropoietin (pg/mL)	1033 ± 318	1676 ± 441	0.11 (n = 3)
Platelets (K/μL)	881 ± 100	885 ± 145	0.95
White blood cells (K/μL)	16.4 ± 3.6	16.4 ± 3.5	1.0

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Table 4

Iron parameters in mice transplanted with *Tfrc*$^{+/-}$ or control marrow

	Male		Female	
	Tfrc$^{+/+}$ **(n = 3)**	***Tfrc***$^{+/-}$ **(n = 3)**	***Tfrc***$^{+/+}$ **(n = 3)**	***Tfrc***$^{+/-}$ **(n = 3)**
Serum				
Iron (μg/dL)	96.5 ± 19.1	92.0 ± 34.8	98.4 ± 41.4	109.4 ± 28.7
Total iron bonding capacity (μg/dL)	293.2 ± 20.1	308.8 ± 66.0	317.2 ± 58.1	305.2 ± 15.3
Transferrin saturation (%)	32.8 ± 4.5	29.4 ± 7.4	33.0 ± 17.2	35.8 ± 9.0
Liver iron (μg/g)	47.9 ± 14.6	65.7 ± 13.2	90.4 ± 53.0	89.9 ± 47.4

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Table 5

Summary of studies on hepcidin levels in mouse models and human disorders supporting the hypothesis that transferrin receptor 1 (TFRC) expression on erythroid precursors is a proximal mediator of the erythroid regulator of hepcidin expression

Condition	Stores regulator (stimulates hepcidin expression)	Erythroid regulator (suppresses hepcidin expression)	Hepcidin	Comments	Total erythroid TFRC/Tfrc protein expression (marrow and spleen)
β-Thalassemia	(**On**) Increased serum/tissue iron and percentage transferrin saturation	(**On**) Ineffective erythropoiesis	Suppressed [38] Erythroid regulator trumps stores regulator	Increased numbers of bone marrow and spleen $CD71^{+}Ter119^{+}$ erythroid cells [39]	Increased owing to large numbers of early erythroid precursors
Hereditary hemochromatosis	(**Off**) Molecular defect prevents hepcidin expression		Suppressed	HFE knockout mice have decreased Tfrc protein expression on erythroid cells (by flow) [40]	Normal Increased erythropoiesis, but less TFRC/cell
Pure red cell aplasia with excess proerythroblasts	Could be on or off	(**On**)	Suppressed	Excess proerythroblasts	Increased (see text)
Recovering erythropoiesis (after bone marrow transplant)	(Should be **On**) Generally these patients are iron overloaded from transfusions	(Must be **On** to explain suppressed hepcidin observed)	Suppressed[14]	As marrow recovers, initially there is expansion of early erythroid precursors	Increased owing to large numbers of early erythroid precursors
Ablation of erythropoiesis (chemotherapy, irradiation, or anti-erythropoietin antibody)	(**On**) Increased serum iron and percentage transferrin saturation	(**Off**)	Increased [6,7]		Decreased Reduced numbers of erythroid precursors at all stages of differentiation
Healthy volunteer given erythropoietin	(Should be **Off**) No change in transferrin saturation or ferritin [15]	(Must be **On** to explain the suppressed hepcidin observed)	Suppressed [15]	Erythropoietin promotes early erythropoiesis	Increased owing to increase in early erythroid precursors
Human hereditary spherocytosis, or several days post-phenylhydrazine treatment in a mouse	(**Off**)	(Must be normal [**baseline**] to explain baseline hepcidin observed)	Not suppressed (baseline) [9]		Only mildly increased; in compensated hemolysis there is no preferential expansion of early erythroid precursors
Hematopoietic cell-specific *Stat-5* null mice	(**On**) Increased serum/tissue iron and percentage transferrin saturation	(Must be **Off** to explain increased hepcidin observed.)	Increased	Stat5 stimulates Tfrc mRNA expression, and Tfrc protein expression is lower on $Ter119^{+}$ cells in *Stat-5* null mice [32]	Decreased owing to reduced expression of Tfrc protein on erythroid precursors at all stages of differentiation
Iron deficiency	(**Off**) Decreased serum/tissue iron and percentage	(Should be **On**) Erythropoiesis persists in humans and is expanded in mouse (similar to $Tf^{hpx/hpx}$ mouse [8])	Suppressed	Increased bone marrow and spleen $CD71^{+}Ter119^{+}$ erythroid cells [5] With iron deficiency, TFRC mRNA expression per cell	Increased owing to increased number of early erythroid precursors, as well as

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Condition	Stores regulator (stimulates hepcidin expression)	Erythroid regulator (suppresses hepcidin expression)	Hepcidin	Comments	Total erythroid TFRC/Tfrc protein expression (marrow and spleen)
	transferrin saturation transferrin saturation			increases because of IRP/IRE regulation	increased TFRC expression per cell
Hypotransferrinemic mouse (*Tf*$^{hpx/hpx}$)	(**Off**)	(Must be **On** to explain suppressed hepcidin observed) Morphologically, there is expanded erythropoiesis (termed *hypotransferrinemic erythropoiesis*) [8]	Suppressed [8]	Tfrc mRNA expression is increased in *Tf*$^{hpx/hpx}$ spleens [8]	Increased Large numbers of erythroid precursors and increased Tfrc mRNA are reported

IRP/IRE = iron-responsive element-binding proteins/iron responsive elements; TFRC = transferrin receptor 1.

The status of the stores and erythroid regulators ("On" or "Off") and observed hepcidin levels are listed in columns 1–4, respectively. Column 5 contains additional data regarding the quantities of erythroid cells and TFRC/Tfrc expression in the erythron. Column 6 describes the total quantity of TFRC/Tfrc protein that would be expected on the basis of these data, noting that TFRC/Tfrc expression initially increases at the CFU-E stage and is highest on intermediate normoblasts (~CD71^{+}Ter119^{+} cells). In each condition, there is an inverse relationship between total TFRC/Tfrc protein expression in the erythroid compartment (column 6) and hepcidin (column 4).