RED CELLS, IRON, AND ERYTHROPOIESIS

Transferrin receptor 2 is a potential novel therapeutic target for β-thalassemia: evidence from a murine model

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Introduction

β-thalassemias are genetic disorders characterized by anemia, ineffective erythropoiesis, and iron overload. Current treatment of severe cases is based on blood transfusion and iron chelation or allogeneic bone marrow (BM) transplantation. Novel approaches are explored for nontransfusion-dependent patients (thalassemia intermedia) who develop anemia and iron overload. Here, we investigated the erythropoietin (EPO) receptor partner, transferrin receptor 2 (TFR2), as a novel potential therapeutic target. We generated a murine model of thalassemia intermedia specifically lacking BM Tfr2: because their erythroid cells are more susceptible to EPO stimulation, mice show improved erythropoiesis and red blood cell morphology as well as partial correction of anemia and iron overload. The beneficial effects become attenuated over time, possibly due to insufficient iron availability to sustain the enhanced erythropoiesis. Germ line deletion of Tfr2, including haploinsufficiency, had a similar effect in the thalassemic model. Because targeting TFR2 enhances EPO-mediated effects exclusively in cells expressing both receptors, this approach may have advantages over erythropoiesis-stimulating agents in the treatment of other anemias. (Blood. 2018;132(21):2286-2297)

optimal treatment accessible to only a minority of patients worldwide. A subset of patients not requiring transfusions (thalassemia intermedia) suffers from multiple morbidities and secondary iron overload. The only curative approach for the most severe forms of β-thalassemia is allogeneic bone marrow (BM) transplantation (BMT), an approach limited by the availability of HLA-matched donors and the risk of graft-versus-host disease. Clinical trials of gene therapy are in progress with encouraging results16,17; however, gene therapy will likely become available to a minority of patients. There is a great interest in developing novel treatments that target IE, improving erythroid maturation and controlling iron overload. The most promising are activin ligand traps, molecules that inhibit the transforming growth factor-β pathway18,19 now in a phase 3 clinical trial.20,21 Other approaches aimed at increasing the iron hormone hepcidin, restricting iron for erythropoiesis as the use of hepcidin/minihepcidins,22,23 transferrin,24,25 and inhibitors of the protease TMPRSS626-30 have been tested with beneficial effects mainly in models of thalassemia intermedia. Defining the molecular mechanism of these novel compounds and exploiting potential combinations is a therapeutic challenge for the future.

We recently identified transferrin (TF) receptor 2 (TFR2), a sensor of circulating TF-bound iron, as a regulator of erythropoiesis. TFR2 is a transmembrane protein, mainly expressed in hepatocytes

KEY POINTS

- Deletion of BM Tfr2 ameliorates anemia and iron overload in a murine model of transfusion-independent thalassemia.
- Deletion of Tfr2 enhances transcription of genes involved in cell proliferation and mitochondrial activity.

β-thalassemias are autosomal-recessive disorders caused by β-globin gene mutations resulting in defective hemoglobin synthesis and α-globin excess. The unbalanced synthesis between normal α- and reduced/absent β-globin chains is the pathogenic clue of the disease and causes massive expansion of the immature erythroid precursors. The balance between erythroid progenitor proliferation and erythroid precursor differentiation is subverted: the early erythroid cell pool is abnormally expanded, whereas maturation of erythroblasts is defective because of increased apoptosis.

Erythropoiesis is governed by erythropoietin (EPO), a hormone primarily produced by the kidney. EPO levels, stimulated by hypoxia, are high in β-thalassemia. EPO binding to its receptor (EPOR) on the surface of erythroid precursors activates the JAK2/STAT5-signaling pathway and the transcription of several genes involved in proliferation, differentiation, and survival. Despite high EPO levels, since the globin defect, erythroid differentiation is blocked in β-thalassemia and the resulting hallmarks of the disease are ineffective erythropoiesis (IE) and anemia. Because of the expanded erythropoiesis, the increased erythroid regulator erythropherrone (ERFE) suppresses hepcidin and causes iron overload.

Current treatment of β-thalassemia is based on lifelong blood transfusions and iron chelation, a costly, demanding and far from
and erythroid cells. Hepatic TFR2 induces hepcidin transcription in response to increased circulating iron\textsuperscript{31-33} and, although the molecular mechanisms remain to be clarified, TFR2 mutations cause hemochromatosis type 3.\textsuperscript{31}

In erythroid precursors, TFR2 associates with EPOR in the endoplasmic reticulum.\textsuperscript{34} Both in hepatic and erythroid cells, TFR2 is stabilized on the cell membrane by diferric transferrin\textsuperscript{35,36}; as a sensor of circulating iron, TFR2 adjusts both hepcidin and red cell production to the available iron.\textsuperscript{37,38} Understanding the erythroid TFR2 function started from the intriguing finding that, when challenged with iron deficiency, Tfr2\textsuperscript{2/-} mice developed erythrocytosis, whereas animals with hepatic-specific Tfr2 deletion did not.\textsuperscript{39} We confirmed the hypothesis that erythrocytosis in Tfr2\textsuperscript{2/-} animals was determined by the loss of erythroid Tfr2 by generating mice with BM-specific inactivation of Tfr2 (Tfr2\textsuperscript{BMKO} mice). These mice showed erythrocytosis, increased EPO sensitivity of erythroid cells, and a reduced apoptosis rate of late erythroblasts mimicking mild iron deficiency.\textsuperscript{40}

Altogether, these results led us to conclude that TFR2 might be a novel therapeutic target for the treatment of \(\beta\)-thalassemia. We speculate that loss of Tfr2 in thalassemia intermedia may both enhance erythroid precursor survival and mimic iron deficiency, a condition reported to partially correct the \(\beta\)-thalassemia phenotype.\textsuperscript{22,26-30,41} To verify this hypothesis, we used the Hbb\textsuperscript{th3/+} murine model of thalassemia intermedia, generated mice lacking Tfr2 in the whole body or in BM and demonstrated a long-lasting amelioration of the phenotype in both cases.

### Methods

**Mouse models and BMT**

Hbb\textsuperscript{th3/+} mice (with heterozygous deletion of \(\beta1\) and \(\beta2\) genes)\textsuperscript{42} on a pure C57BL/6N background (The Jackson Laboratory, Bar Harbor, ME) and Tfr2\textsuperscript{2/-} mice on a pure 129S2 background\textsuperscript{33} were crossed obtaining Tfr2\textsuperscript{2/-} and Tfr2\textsuperscript{2/-}/Hbb\textsuperscript{th3/+} progenies on a mixed C57/129S2 background; these animals were backcrossed generating Tfr2\textsuperscript{2/-}/Hbb\textsuperscript{th3/+}, and Hbb\textsuperscript{th3/+} mice. Mice were fed a standard diet; blood was collected from tail-vein puncture for hematological analyses at 4, 10, 15, 20, 25, 29, 33, and 37 weeks of age from both sex animals. A cohort of animals was euthanized at 10 weeks, another at 37 weeks of age. At euthanization, blood was collected for
Figure 2. Analysis of erythropoiesis and serum EPO and ERFE levels of Tfr2BMKO/Hbbth3/ mice. Mice were analyzed 9 and 22 weeks after transplantation with thalassemic (Tfr2wt/Hbbth3/) or Tfr2−/−/Hbbα/β (Tfr2BMKO/Hbbα/β) BM. Mice were fed a standard diet. In the figure are graphed: (A) representative gating strategy for analysis of Ter119 subpopulations in Hbbth3/ and Tfr2BMKO/Hbbth3/ mice. Viable cells (impermeable to propidium iodide [PI]) from BM were analyzed for Ter119/CD44 expression. Ter119 were gated and further analyzed with respect to forward scatter (FSC) and CD44 surface expression for subpopulation composition (gated clusters: proerythroblasts [I], basophilic erythroblasts [II], polychromatic erythroblasts [III], orthochromatic erythroblasts and immature reticulocytes [IV], and mature red cells [V]); (B) blood smears stained with May-Grunwald-Giemsa showing the morphology of RBCs of representative Hbbth3/ and Tfr2BMKO/Hbbth3/ mice (original magnification ×40); (C) percentage of Ter119+ cells on alive
transferrin saturation (TS) determination; liver and spleen were weighed and dried for iron quantification.

BM cells isolated from a subset of 10-week-old Tfr2<sup>−/−</sup>/Hbb<sup>th3+/−</sup> and Hbb<sup>th3+/−</sup> male mice were used for BMT, as described (Nai et al 40; supplemental Methods, available on the Blood Web site). Transplanted mice were fed a standard diet until 9 weeks after BMT when a cohort of animals was euthanized. A second cohort was fed an iron-deficient (ID) (<3 mg/kg carbonyl iron; SAFE, Augy, France) or iron-balanced (IB) (200 mg/kg carbonyl iron; SAFE) diet starting 9 weeks until 22 weeks after BMT. Blood was collected for hematological analyses every 4 weeks. At euthanization, animals were analyzed as previously described. In addition, serum EPO and ERFE (courtesy of Tom Ganz and Elizabeta Nemeth, University of California Los Angeles, Los Angeles, CA) were measured. Liver, spleen, kidneys, and heart were weighed, dissected, and snap-frozen immediately for RNA analysis, dried for tissue iron quantification, or processed for fluorescence-activated cell sorter (FACS) analysis. BM cells were harvested and processed for FACS or RNA analysis.

All mice were maintained in the animal facility of San Raffaele Institute in accordance with the European Union guidelines. The study was approved by the institutional animal care and use committee of the San Raffaele Institute.

Hematological analysis, flow cytometry, and tissue iron quantification

Complete blood count, blood smears, TS, serum EPO, and tissue iron content measurement were performed by standard methods (supplemental Methods). FACS analyses were performed as in Nai et al 40 and serum ERFE measurement as in Kautz et al 12

Quantitative RT-PCR

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed by standard methods (supplemental Methods).

RNAseq analysis

RNA was prepared by standard methods (supplemental Methods). Strand-specific RNA sequencing (RNAseq) library preparation was performed with the Illumina Stranded Truseq mRNA kit (Illumina, San Diego, CA). Libraries were multiplexed and run in paired-end mode on an Illumina Nextseq 500 platform (read length, 80 bases). Fastq files were demultiplexed, checked for quality control using the FastQC program, and mapped against the mm10 mouse genome with the STAR package to generate bam files. Reads were annotated against the GRCm38.p5 reference gene set (Ensembl) and counted using the summarizeOverlaps function of the GenomicAlignments package (Bioconductor). Differentially expressed genes were obtained with the Deseq2 package 43 using the independent hypothesis weighting function of the IHW package for false discovery rate (FDR) calculation. 44 Genes with FDR <10% and with log2 fold change less than −0.5 (downregulated) or more than +0.5 (upregulated) were used for GOr term analysis with the topGO package (Bioconductor). Canonical pathways were investigated based on the log2 fold change of the entire gene set using the gene set enrichment analysis (GSEA) package (Broad Institute).

The RNAseq fastq file can be obtained from the Arrayexpress platform under the accession number E-MTAB-6606 (https://www.ebi.ac.uk/arrayexpress/experiments/e-mtab-6606/).

Statistics

Data are presented as mean plus or minus standard error (SE). The unpaired 2-tailed Student t test was performed using GraphPad Prism 5.0 (GraphPad). P <.05 was considered statistically significant.

Results

Deletion of erythroid Tfr2 induces a sustained amelioration of anemia in Hbb<sup>th3+/−</sup> mice

To investigate whether Tfr2 loss ameliorates the thalassemic phenotype, to overcome any possible interference of iron-loading typical of Tfr2<sup>−/−</sup> mice, we developed a model of specific Tfr2 inactivation in BM cells of Hbb<sup>th3+/−</sup> animals, which fully recapitulates the characteristics of β-thalassemia intermedia in humans 45.

Lethally irradiated wild-type (wt) mice were transplanted with BM cells from Hbb<sup>th3+/−</sup> or Tfr2<sup>−/−</sup>/Hbb<sup>th3+/−</sup> donor mice coisogentic for the allelic form of the CD45 antigen. Engraftment of donor cells was almost complete both in the BM and in the spleen (supplemental Figure 1). Hemoglobin (Hb) levels and red blood cell (RBC) count are stable over time in Hbb<sup>th3+/−</sup> mice. A consistent and persistent increase of RBC count (Figure 1A) and Hb levels (Figure 1B) is observed in thalassemic mice lacking BM Tfr2 (Tfr2<sup>BMKO</sup>/Hbb<sup>th3+/−</sup>). This occurs in the presence of a further decrease of both erythrocyte indexes, mean corpuscular volume (MCV; Figure 1C), and mean corpuscular hemoglobin (MCH; Figure 1D), indicating iron-restricted erythropoiesis. Only at −22 weeks after BMT does the improvement fade in double mutants: Hb and MCV levels return comparable to values of Hbb<sup>th3+/−</sup> mice. However, in Tfr2<sup>BMKO</sup>/Hbb<sup>th3+/−</sup> mice, RBC count persists higher and MCH levels lower than in thalassemic controls, even if MCH also drops at this time point in Hbb<sup>th3+/−</sup> animals.

Overall, these results demonstrate that deletion of erythroid Tfr2 induces a sustained amelioration of anemia in thalassemic mice, although loss of the beneficial effect on Hb levels occurs long-term.

Tfr2<sup>BMKO</sup>/Hbb<sup>th3+/−</sup> mice show improved erythropoiesis and reduced iron accumulation

To assess whether the loss of Tfr2 in Hbb<sup>th3+/−</sup> mice improves IE, we evaluated the erythroid differentiation (Figure 2A) in Tfr2<sup>BMKO</sup>/Hbb<sup>th3+/−</sup> and Hbb<sup>th3+/−</sup> animals at both 9 and 22 weeks

[Figure 2 (continued) cells and subpopulation composition (gated cluster I-V) based on Ter119/CD44 expression and FSC (reflecting cell size) both in the BM and in the spleen; D) percentage of reticulocytes in peripheral blood; E) serum EPO levels; F) spleen weight normalized to body weight and (G) serum ERFE levels. Bars indicate SE. Asterisks refer to statistically significant differences between age-matched Hbb<sup>th3+/−</sup> and Tfr2<sup>BMKO</sup>/Hbb<sup>th3+/−</sup> mice: *P < .05; **P < .01; ***P < .005. FSC-A, forward scatter area; SSC-A, side scatter area.]

TARGETING TFR2 IN β-THALASSEMIA
after BMT. At 9 weeks, the erythropoiesis of Hbb\textsuperscript{th3/} mice is highly compromised (Figure 2B-D), whereas it is moderately improved by the absence of BM Tfr2, as shown by the reduced percentage of circulating reticulocytes (Figure 2D). Twenty-two weeks after BMT, double mutants show a further erythropoiesis improvement, with reduced percentage of circulating reticulocytes (Figure 2D) and of stage IV (orthochromatic erythroblasts-reticulocytes) both in BM and spleen, increased stage V cells (mature RBCs) in the BM (Figure 2C), and amelioration of RBC morphology (Figure 2B). This erythropoietic improvement likely maintains the RBC count high despite the initial reduction of Hb levels. The amelioration of anemia and erythropoiesis in double mutants is accompanied by the expected reduction of serum EPO levels at both time points (Figure 2E). Surprisingly, despite this improvement, the percentage of Ter119\textsuperscript{+} cells and the spleen sizes are unchanged in double mutant mice at both time points (Figure 2F).

In agreement with low EPO concentration, serum levels of the erythroid regulator ERFE,\textsuperscript{11} an EPO target gene, are reduced in Tfr2\textsuperscript{BMKO}/Hbb\textsuperscript{th3/} animals relative to Hbb\textsuperscript{th3/} 9 weeks after BMT, whereas, despite lower EPO levels in Tfr2\textsuperscript{BMKO}/Hbb\textsuperscript{th3/} animals, they are comparable between the 2 genotypes at 22 weeks (Figure 2G).

TS is similar between the 2 groups at both time points (Table 1), but is significantly lower in older mice (Hbb\textsuperscript{th3/}}
22 vs 9 weeks: P < .001; Tfr2BMKO/Hbbth3/+ vs Hbbth3/+ mice. Iron concentration in liver (LIC), spleen (SIC), kidney, and heart is as well comparable in thalassemic mice with or without erythroid Tfr2 9 weeks after BMT. However, deletion of erythroid Tfr2 prevents further iron accumulation with aging in liver and kidney. Consistent with LIC, hepatic hepcidin (Hamp) expression is unaffected by the lack of erythroid Tfr2 at 9 weeks and reduced at 22 weeks (Table 1). Notably, SIC is decreased to normal levels40 in Tfr2BMKO/Hbbth3/+ mice (Table 1).

Overall, these results suggest that increased Hb levels in double mutant mice are accompanied by a more effective erythropoiesis and are not due to, but are the cause of, the reduced iron accumulation observed.
The EPO-EPOR-signaling pathway is overactive in erythroid precursors of Tfr2\(^{BMKO/Hbb^h3/+}\) mice

To elucidate the molecular mechanisms by which the genetic loss of Tfr2 ameliorates the thalassemic phenotype, we investigated whether the EPO-EPOR-signaling pathway is overactive as we reported in Tfr2-null erythroid cells.\(^{40}\) The expression levels of the EPO-JAK2-STAT5 target genes Ertf\(^{11}\) (Figure 3A,D) and Bcl-x\(^{13,14}\) (Figure 3B,E) are comparable in the 2 groups of mice 9 weeks after BMT both in BM and spleen, whereas they are slightly lower in the BM of Tfr2\(^{BMKO/Hbb^h3/+}\) relative to Hbb\(^{h3/+}\) animals at 22 weeks. In parallel, Fast, a proapoptotic molecule inhibited by the EPO-phosphatidylinositol 3-kinase-AKT pathway,\(^{6}\) is significantly reduced in Tfr2\(^{BMKO/Hbb^h3/+}\) mice at both time points (Figure 3C,F). Taking into account the remarkably lower serum EPO levels measured in Tfr2\(^{BMKO/Hbb^h3/+}\) mice as compared with Hbb\(^{h3/+}\) controls (Figure 2C), these results are consistent with the EPO-EPOR pathways being activated by the loss of Tfr2 in thalassemia, as observed in wt cells.\(^{40}\)

Transcription of genes of cell proliferation, mitochondrial activity, and oxidative stress response is enhanced in Tfr2\(^{BMKO/Hbb^h3/+}\) mice

To obtain a global view of cellular processes that explain the remarkable amelioration of the phenotype in Tfr2\(^{BMKO/Hbb^h3/+}\) mice, we evaluated how the transcriptome is modulated by the absence of Tfr2. We performed RNAseq analysis on spleen samples from double mutant mice and Hbb\(^{h3/+}\) controls at 22 weeks after BMT, a time point of maximal amelioration of the hematological phenotype of double mutant mice, a cohort of Tfr2\(^{BMKO/Hbb^h3/+}\) and Hbb\(^{h3/+}\) animals was fed an ID diet for 13 weeks, starting 9 weeks after BMT. RBC count and Hb levels of Hbb\(^{h3/+}\) animals are moderately increased after 4 weeks of diet, likely because of ID-induced TFR2 removal from erythroid surface\(^{36}\) and then persist constant. Increased RBC count and Hb levels are noticed in Tfr2\(^{BMKO/Hbb^h3/+}\) mice only from 17 weeks, when iron deficiency was likely achieved, reaching maximal levels at 21 weeks and starting to decline at 22 weeks (Figure 5A-B). Notwithstanding a mild reduction in the percentage of BM Ter119\(^{+}\) cells, the maturation pattern of erythroid cells of Tfr2\(^{BMKO/Hbb^h3/+}\) mice analyzed at sacrifice appears significantly improved as compared with both Hbb\(^{h3/+}\) controls (Figure 5C) and to animals fed a standard diet (Figure 2A). This confirms that the corrective effect of restricting iron to thalassemic erythropoiesis\(^{41}\) also occurs in the absence of Tfr2.

Principal component analysis of the transcription profile of single samples shows a distinct clustering into 2 groups, which correspond to the 2 genotypes (supplemental Figure 2A). In total, we identified 2796 genes differentially regulated between the 2 genotypes (supplemental Figure 2B; supplemental Data File 1). Among them, 1997 are protein coding. The expression levels of the EPOR-JAK2-STAT5 target genes\(^{45}\) are comparable between 2 genotypes (supplemental Figure 2B; supplemental Data File 1). Gene ontology analysis of iron-related genes reveals an overrepresentation of genes involved in lipid and cholesterol handling, leukocyte/lymphocyte differentiation, and coagulation. In addition, most of the antioxidant and protective targets activated by the canonical NF-kB-signaling pathway,\(^{36}\) a recognized target of EPO-EPOR signaling,\(^{47}\) are overexpressed in spleen samples of double mutants as compared with thalassemic controls (Table 2).

In summary, transcriptomic data suggest a more pronounced erythroid commitment of cells lacking Tfr2, accompanied by increased activity of mitochondria and proteasome and sustained activation of the antioxidant response.

Iron deficiency improves erythropoiesis of Tfr2\(^{BMKO/Hbb^h3/+}\) mice

To investigate whether iron deficiency has a further effect on the hematological phenotype of double mutant mice, a cohort of Tfr2\(^{BMKO/Hbb^h3/+}\) and Hbb\(^{h3/+}\) animals was fed an ID diet for 13 weeks, starting 9 weeks after BMT. RBC count and Hb levels of Hbb\(^{h3/+}\) animals are moderately increased after 4 weeks of diet, likely because of ID-induced TFR2 removal from erythroid surface\(^{36}\) and then persist constant. Increased RBC count and Hb levels are noticed in Tfr2\(^{BMKO/Hbb^h3/+}\) mice only from 17 weeks, when iron deficiency was likely achieved, reaching maximal levels at 21 weeks and starting to decline at 22 weeks (Figure 5A-B). Notwithstanding a mild reduction in the percentage of BM Ter119\(^{+}\) cells, the maturation pattern of erythroid cells of Tfr2\(^{BMKO/Hbb^h3/+}\) mice analyzed at sacrifice appears significantly improved as compared with both Hbb\(^{h3/+}\) controls (Figure 5C) and to animals fed a standard diet (Figure 2A). This confirms that the corrective effect of restricting iron to thalassemic erythropoiesis\(^{41}\) also occurs in the absence of Tfr2.

The iron-poor diet significantly decreases TS in both groups of mice as compared with wt IB animals (Figure 5D) and completely prevents iron loading. Indeed, LIC (Figure 5E) of both Hbb\(^{h3/+}\) and Tfr2\(^{BMKO/Hbb^h3/+}\) mice and SIC (Figure 5F) of Hbb\(^{h3/+}\) overlap the mean values of wt mice. Interestingly, SIC of Tfr2\(^{BMKO/Hbb^h3/+}\) is even lower than in wt (Figure 5F), indicating exhaustion of spleen iron stores, likely due to an increased iron consumption by the enhanced erythropoiesis.

Germ line deletion of one or both Tfr2 alleles ameliorates anemia of Hbb\(^{h3/+}\) mice

To confirm the role of TFR2 in β-thalassemia, and propose it as a therapeutic target, we verified whether germ line homozygous or heterozygous Tfr2 deletion also improves anemia in Hbb\(^{h3/+}\) animals. Both RBC number (Figure 6A; supplemental Figure 4A) and Hb levels (Figure 6B; supplemental Figure 4B) are increased in mice lacking Tfr2, an effect that, for Hb levels, is maintained for at least 29 weeks. Interestingly, a gene dosage effect is observed on Hb and RBC improvement. In contrast with results obtained in transplanted mice, MCV (Figure 6C; supplemental Figure 4C) and MCH (Figure 6D; supplemental Figure 4D) are comparable between Hbb\(^{h3/+}\) mice with both or a single Tfr2 allele, whereas MCV is increased in animals lacking both copies of Tfr2, likely because of their high iron burden. The improvement of the hematological phenotype occurs despite the expected severe iron overload of Tfr2\(^{-/-}\)/Hbb\(^{h3/+}\) mice, which show higher LIC (Figure 6E) and TS (Figure 6F) than Hbb\(^{h3/+}\) mice. SIC is comparable between thalassemic mice with or without Tfr2.

Proteasome activity and an underrepresentation of genes involved in lipid and cholesterol handling, leukocyte/lymphocyte differentiation, and coagulation. In addition, most of the antioxidant and protective targets activated by the canonical NF-κB-signaling pathway,\(^{36}\) a recognized target of EPO-EPOR signaling,\(^{47}\) are overexpressed in spleen samples of double mutants as compared with thalassemic controls (Table 2).
Tfr2 deletions have not been reported in selective inactivation of macrophages, although phenotype alterations have not been reported in selective inactivation of macrophages, although phenotype alterations persist in all mice lacking 1 Tfr2 allele. These mice are characterized by a modest increase of TS (Figure 6F) relative to thalassemic controls when 10 weeks old. In contrast, 37 week old Tfr2 BMKO/Hbbth3/2 mice have normal TS as Hbbth3/2 animals and slightly increased LIC.

Overall, these results demonstrate that, irrespective of the iron loading, germ line Tfr2 deletion induces a long-lasting amelioration of anemia in thalassemic mice, indicating that the beneficial effect of Tfr2 loss is iron independent.

Discussion

Current treatments of β-thalassemia are unsatisfactory or unavailable worldwide; thus, the search for novel targeted therapies is a clinical need. Here, we show that the genetic loss of Tfr2 results in a long-lasting improvement of anemia due to more efficient erythropoiesis in the Hbbth3/2 mouse model. This benefit persists at least up to 5 months in animals lacking Tfr2 both ubiquitously and selectively in the BM, making Tfr2 a novel therapeutic target for β-thalassemia. We describe the positive effect to erythroid Tfr2 deletion; however, we cannot exclude a contribution of Tfr2 deletion in other BM-derived cells as macrophages, although phenotype alterations have not been reported in selective inactivation of macrophage Tfr2.

The improved erythropoiesis increases iron utilization at degrees that may become counterproductive because depletion of spleen stores causes excessive iron restriction, neutralizing the benefit. This side effect, also suggested by TS reduction over time, strengthens the need to monitor body iron according to the degree of Tfr2 inactivation.

We observed a gene dosage effect of germ line Tfr2 deletion: Tfr2-haploinsufficient thalassemic animals maintain higher Hb levels until 37 weeks old, whereas their iron phenotype is comparable to thalassemic controls. In contrast, Hb levels of (geminal) Tfr2-thalassemic mice deteriorate over time, in parallel with iron overload. These observations suggest that even a partial inhibition of the receptor might ameliorate the thalassemic phenotype without substantially altering systemic iron homeostasis.

Surprisingly, despite the hematological improvement, spleen size remains unchanged in double mutant mice. This is consistent with the positive effect on splenomegaly, with negligible improvement of anemia observed with JAK2 inhibitor treatment of both thalassemic mice and patients.

To unequivocally elucidate the pathway/s altered by the loss of Tfr2, we performed RNAseq analysis on spleen samples at a time of maximally improved erythropoiesis in double mutants.

We found that Tfr2 deletion activates signaling pathways involved in cell proliferation (ribosomal proteins, RNAPolimerase, cell cycle), mitochondrial activity, proteasome function, and antioxidant response, while reducing coagulation, leukocyte/lymphocyte proliferation and differentiation, and lipid metabolism pathways. This analysis is compatible with increased sensitivity of Tfr2-deficient erythropoiesis to EPO stimulation, as observed in wt mice. The RNAseq patterns indicate an increased erythroid commitment and a mitochondrial metabolic switch. Interestingly, a metabolic shift occurs during hematoipoiesis with the induction of mitochondrial activity, which sustains proliferation and differentiation of erythroid cells. EPO directly stimulates the metabolic activity and mitochondrial gene expression in adipocytes, and we speculate that an analogous effect is also induced in erythroid cells. Another contributor to the improved differentiation is increased proteasome activity because proteasomal degradation is required for enucleation of erythroblasts to form mature erythrocytes and improved enucleation ameliorates the thalassemic phenotype following iron restriction. Finally, the activation of the NF-κB antioxidant response may control ineffective erythropoiesis because reactive oxygen species increased in ineffective erythropoiesis of thalassemia and may contribute to transcriptional down-regulation of Fast. Of note, NF-κB activation is reported

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**Table 2. Expression level of NF-κB target genes involved in ROS protection from RNAseq analysis of the spleen of Hbbth3/2 and Tfr2BMKO/Hbbth3/2 mice**

<table>
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<th>Gene name</th>
<th>Hbbth3/2</th>
<th>Tfr2BMKO/Hbbth3/2</th>
<th>P (DESeq2)*</th>
<th>padj (IHW)†</th>
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<td>Sod2 (MnSOD)</td>
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<td>.056003</td>
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<td>Sod1 (CuZnSOD)</td>
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<td>.045185</td>
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<td>.030397</td>
<td>.134317</td>
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<td>Tlx1</td>
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<td>735.73 ± 80.39</td>
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<td>.195756</td>
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<td>171.08 ± 17.92</td>
<td>.00604</td>
<td>.059385</td>
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</tbody>
</table>

Data are indicated as median of the TPM plus minus standard error of the mean.

The adjusted P value has been calculated using the DESeq2 algorithm.

The adjusted P value has been obtained using the IHW correction.

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*The value has been calculated using the DESeq2 algorithm.
†The adjusted P value has been obtained using the IHW correction.
secondary to EPO effect. Thus, based both on RT-PCR of EPO target genes and RNAseq findings, and considering the low EPO levels of Tfr2BMKO/Hbbth3/1 mice, we conclude that the EPO pathway is more active in these animals than in thalassemic controls.

The analysis of iron-related genes showed decreased expression of Fpn, Hmox1, and Alas2, genes that respond to iron, oxidative stress, and heme. Their downregulation could be secondary to decreased hemolysis and/or free heme accumulation in thalassemic cells or to the reduced splenic iron. Indeed, because of the spleen iron reduction of aged Tfr2BMKO/Hbbth3/1, we cannot formally exclude a contribution of iron deficiency to the transcriptional modifications observed.

Recent in vitro findings demonstrate that the iron-mediated lysosomal trafficking of TFR2 controls EPOR surface expression and activity through quantitative variations of Scribble, a scaffold
In the absence of Tfr2, Scribble would be stabilized increasing protein involved in receptors trafficking and cell polarization. In our model, both pathways seems to be enhanced in the absence of Tfr2. However, Scribble correct localization is important to regulate AKT signaling because when mislocalized, Scribble activates the AKT–mammalian target of rapamycin pathway. Additional studies are required to clarify this point.

As in wt mice, in the thalassemic model, deletion of erythroid Tfr2 does not directly modulate iron homeostasis: 9 weeks after BMT, iron accumulation is not prevented, but further iron overload is precluded in double mutant mice over time. These results suggest that the thalassemia amelioration driven by the lack of erythroid Tfr2 is not due to a reduction of available iron. We propose that the more effective erythropoiesis mobilizes iron, especially from the spleen, a major site of erythropoiesis in mice. This process would require hepcidin suppression that apparently does not occur. Nine weeks after BMT, hepcidin levels are similar in the 2 groups of mice, whereas at 22 weeks, they are slightly decreased in Tfr2<sup>2<sup>−/−</sup></sup>/Hbb<sup>θ<sup>3/2</sup></sup> mice. However, considering the lower degree of anemia (and hypoxia) as compared with thalassemic controls, hepcidin levels are inappropriately low and markedly and persistently high in the double mutants. As observed in wt animals, genetic loss of Tfr2 resets iron homeostasis in favor of iron recycling and absorption to meet the requirement of the increased erythropoiesis.

The strong reduction of spleen iron may explain the mild Hb decrease observed in Tfr2<sup>2<sup>−/−</sup></sup>/Hbb<sup>θ<sup>3/2</sup></sup> mice at 22 weeks, despite improved erythropoiesis. Overall, our findings strengthen the need for adequate iron availability for erythropoietic activity.

Despite the impressive systemic iron overload of Tfr2<sup>2<sup>−/−</sup></sup>/Hbb<sup>θ<sup>3/2</sup></sup> mice, their hematological phenotype is significantly and persistently improved, suggesting that iron overload is not a major determinant of anemia in thalassemic mice. In line with this assumption, exacerbation of iron overload through iron dextran in thalassemia was even associated with increased Hb in 1 study. In contrast, iron depletion induced by a prolonged ID
diet induces only a transient modest improvement of Hb in both groups of mice.

In conclusion, erythroid TFR2 appears to be an attractive therapeutic target to potentiate erythropoiesis in beta-thalassemia; our findings on TfR2 haploinsufficient thalassemic mice suggest that TFR2-specific targeting by antisense oligonucleotides or small interfering RNAs could be proposed to ameliorate anemia in nontransfusion-dependent patients. Mechanistic studies aimed at characterizing the TFR2-EPOR interaction may lead to the design of interfering molecules, mimicking an erythroid-specific TFR2 depletion. Finally, targeting TFR2 enhances EPO-mediated effects exclusively in erythroid cells, an advantage over erythropoiesis-stimulating agents. For this reason, it might become an option for other disorders, characterized by anemia and/or ineffective erythropoiesis.

Acknowledgments
The authors thank Tomas Ganz and Elizabeta Nemeth (University of California, Los Angeles, Los Angeles, CA) for murine serum erythroferrone measurement.

This work was supported in part by the Cooley’s Anemia Foundation (Research Fellowship), the Cariplo Foundation (“Young Investigator” grant no. 2017-0916), and the European Hematology Association (José-Carreras Junior Research fellowship) (A.N.); by the European Hematology Association (Advanced Research fellowship) (S.A.); and by the Telethon Foundation (SR-TIGET Core Grant) (G.F.).

Authorship
Contribution: I.A. and M.R.L. performed experiments, analyzed data, and contributed to the writing of the manuscript; S.A., G.M., and M.P. performed research and analyzed data; M.U.M. contributed to the writing of the paper; L.S. and G.F. contributed to the experimental design, data analysis, and manuscript writing; C.C. conceived the experiments and critically reviewed the paper; A.N. designed and performed research, analyzed data, and wrote the manuscript; and all authors approved the final version of the manuscript.

Conflict-of-interest disclosure: C.C. is an advisor of Vifor Iron Core and received honoraria from Vifor Pharma. A.N., L.S., and C.C. have filed a patent application (US provisional no. 62/483 172) on the results presented in the paper. The remaining authors declare no competing financial interests.

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