EXPRESSION OF RETICULOCYTE IRON EXPORTER GENE *FPN*1B IN THALASSEMIA PATIENTS UNDERGOING BLOOD TRANSFUSION AND IRON CHELATION THERAPY

Pradit Tonpetch[1], Rungtip Putburee[1], Amporn Tripatara[1], Arunee Jetsrisuparb[1,2] and Supan Fucharoen[1]

[1]Center for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences; [2]Department of Pediatrics, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Abstract. Thalassemia is a hereditary disease caused by abnormalities in globin genes, leading to ineffective erythropoiesis and chronic hemolytic anemia. Individuals with thalassemia who have moderate to severe clinical symptoms need to be treated with regular blood transfusion and iron chelation. This study investigated expression levels of ferroportin gene *FPN*1B in reticulocytes (surrogate for erythroblasts) of regularly and irregularly blood transfused thalassemia patients who also received iron chelation treatment compared to healthy controls, and to relate *FPN*1B mRNA levels with those of plasma ferritin and serum malondialdehyde, markers of oxidant damage in tissues and organs. Expression levels of *FPN*1B in both regularly and irregularly transfused thalassemia are significantly higher than that in healthy controls, as were plasma ferritin and serum malondialdehyde levels. Interestingly, levels of *FPN*1B expression correlated with ferritin and malondialdehyde concentrations in α-thalassemia but not β-thalassemia/Hb E patients. These results suggest elevated expression of erythroblast *FPN*1B in these patients may be a contributing factor to iron-associated oxidant stress.

Keywords: ferroportin, *FPN*1B expression, iron overload, oxidant stress, reticulocyte, thalassemia

INTRODUCTION

Thalassemia, an autosomal recessive anemia, is caused by genetic abnormalities to globin genes resulting in imbalances in the amounts of α- and β-globin chains and thereby producing hypochromic red blood cells (Weatherall, 1998). In Thailand alpha-thalassemia (reduced alpha-globin synthesis) (Hb H) and β-thalassemia (reduced beta-globin synthesis)/Hb E (E25K) are common types of thalassemia with clinical manifestations, which vary from mild, moderate to severe anemia, and those with moderate to severe symptoms need to be treated with regular blood transfusion and concomitant iron chelation therapy (Fucharoen and Winichagoon, 2000; Premawardhena *et al*, 2005).

Correspondence: Amporn Tripatara, Center for Research and Development of Medical Diagnostic Laboratiries, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel/Fax: +66 (0) 43 202087
E-mail: amptri@kku.ac.th

Iron is an essential element of the body and is mainly found in the form of complexes with proteins such as hemoglobin. Non-bound iron ("molecular iron") that can react with hydrogen peroxide (Fenton reaction) giving rise to free hydroxyl radicals and leading to oxidant damage of cells and organelles (Arosio and Levi, 2002). Levels of serum malondialdehyde, a product of lipid peroxidation, in β-thalassemia/Hb E patients are higher than those in healthy subjects (Suebpeng *et al*, 2016), suggesting the presence of reactive oxygen species, which could lead to cell and organ damage. In thalassemia patients treated with regular blood transfusion and iron chelation, the amount of iron in the body is still increased (as determined by the levels of ferritin in the plasma or serum and measurements of iron loading in endocrine tissues, the heart and the liver), which is largely due to blood transfusions and, to a lesser extent, the increased absorption of iron from the small intestine (Taher *et al*, 2013; Cappellini *et al*, 2014).

Ferroportin is the only iron exporter so far identified in mammals (Abboud and Haile, 2000; Donovan *et al*, 2000). Ferroportin mRNA and protein levels decrease or increase in response to iron depletion or iron overload (Pietrangelo, 2004) allowing the transporter to play a critical role in maintaining tissue iron homeostasis in mammals (McKie and Barlow, 2004; Cianetti *et al*, 2005; Cianetti *et al*, 2010). Ferroportin FPN1B (also known as FPN1 variant II isoform) in erythroblasts is translated from mRNA lacking a iron-responsive element at the 5′ untranslated region and thus is not under regulation of the iron-responsive element/iron-regulatory protein (IRE/IRP) system (Cianetti *et al*, 2005; Zhang *et al*, 2009; Cianetti *et al*, 2010).

In this study we investigated *FPN*1B expression in erythroblasts of β-thalassemia/Hb E and α-thalassemia patients receiving regular blood transfusion and iron chelation. Our previous report (Pratummo *et al*, 2014) demonstrated that hepcidin (*HAMP* mRNA) expression in monocytes of β-thalassemia/Hb E patients with regular transfusion and iron chelation was elevated as compared to healthy controls suggesting that local interaction between hepcidin and ferroportin in an autocrine fashion would prevent iron released from monocyte/macrophage store. In order to avoid the necessity of undertaking bone marrow aspirations to obtain erythroblasts, peripheral blood reticulocytes were used as surrogates.

MATERIALS AND METHODS

Subjects

β-Thalassemia/Hb E (30 splenectomized and 30 non-splenectomized) and α-thalassemia (Hb CS with Hb Bart's (CSA2ABart's), Hb CS with Hb Bart's and Hb H (CSA2ABart'sH), Hb CS with Hb E and Hb Bart's (CSEABart's), Hb Bart's with Hb E (EABart's), Hb Bart's with Hb H (A2ABart'sH) ($n = 38$) subjects together with healthy individuals ($n = 30$) were enrolled in the study. The regularly blood transfused β-thalassemia/Hb E subjects were those previously reported (Suebpeng *et al*, 2016). For non-splenectomized and splenectomized β-thalassemia/Hb E patients, the average age is 12.8 and 18.2 years, average amount of transfused blood received 316 and 366 ml/kg body weight/year, and steady-state Hb level 7.2 g/dl and 7.1 g/dl, respectively. Iron chelation therapy (Deferiprone alone or Deferiprone and Desferrioxamine) was conducted according to guidelines of Taher *et al* (2013) and Cappellini *et al*

(2014). The α-thalassemia subjects constituted 30 patients (RegT) who underwent regular transfusion (1-2/month for correction of growth failure, bone change and/or marked hepatosplenomegaly) with plasma ferritin concentration $\geq$ 1,000 ng/ml, and 8 patients (RarT) who rarely required transfusion ($\leq$2/year) with plasma ferritin concentration $\geq$ 800 ng/ml; both groups underwent iron chelation therapy. Inclusion criteria for the healthy subjects were Hb level ≥13 g/dl for males or ≥12 g/dl for females, mean cellular volume >80 fl, Hb typing A_2A, Hb A_2 <4%, and plasma ferritin >15 ng/ml (WHO/CDC, 2004).

The study was approved by Khon Kaen University Ethics Committee for Human Research (HE571262 and HE591562) and prior written informed consent was obtained from each participant.

Sample preparation

Approximately 7 ml of blood were collected in an EDTA-containing tube. Clotted blood was also collected for serum preparation. Reticulocyte fraction was prepared according to Suebpeng *et al* (2016). In brief, the fresh whole blood sample was mixed with 3% dextran, white blood cells were removed and the top layer of red blood cells was collected, washed and mixed with Percoll™ density centrifugation at 1.085 g/ml (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The reticulocyte fraction was washed with 0.85% normal saline solution 3 times and spun down by centrifugation.

Laboratory measurements

Whole blood parameters were determined using an automated hematology analyzer (KX-21; Sysmex, Kobe, Japan). Plasma ferritin and serum malondialdehyde level were assessed by enzyme linked immunosorbent assay (ELISA) using a commercial kit (RayBiotech, Norcross, GA) and thiobarbituric acid-reactive substance assay (Lapenna *et al*, 2001), respectively.

Determination of reticulocyte expression level

Total RNA was extracted from reticulocytes using Trisure® reagent [Bioline (Aust), Alexandria, NSW, Australia]. Measurement of *FPN*1B expression was based on quantitative (q)RT-PCR using a commercial kit (SensiFAST™ SYBR No-ROX One-Step kit; Bioline, Taunton, MA). Primers (Invitrogen, Carlsbad, CA) used (for *FPN*1B) were 5′CGAGATGGATGGGTCTCCTA3′ (sense) and 5′ACCACATTTTCGACGTAGCC 3′ (antisense) (Kijima *et al*, 2008), and (for *GAPDH*) 5′GAAGGTGAAGGTCGGAGTC3′ (sense) and 5′GAAGATGGTGATGGGATTTC3′ (antisense) (Pratummo *et al*, 2014). In brief, the reaction mixture (consisting of approximately 0.1 μg of total RNA and 400 nM final concentration of each primer, SensiFAST™ SYBR No-ROX One-Step mix, reverse transcriptase, RNase inhibitor, DEPC treated water in 20 μl total volume) was incubated at 42°C for 10 minutes, followed by 95°C for 10 minutes and then thermocycling (in Illumina® Eco™ Real-Time PCR System; Illumina, San Diego, CA) for 40 cycles of 94°C for 15 seconds, 60°C for 15 seconds and 72°C for 15 seconds. The level of *FPN*1B transcript relative to that of *GAPDH* (internal control) was quantified using the equation $2^{-\Delta\Delta Ct}$ (where Ct is threshold cycle) (Livak and Schmittgen, 2001).

Statistical analysis

Data are reported as mean ± standard deviation (SD). Statistical analysis was performed using SPSS statistics package, version 17 (IBM, Armonk, NY). Calculations for significant differences among the various groups of subjects were carried

out using nonparametric Kruskal-Wallis and Mann-Whitney *U* tests. Correlation between the parameters studied in the patients was calculated using Spearman rho test. A p-value <0.05 is considered statistically significant.

RESULTS

Median age of α-thalassemia RegT and RarT patients was 13.9 years (range 8-25 years) and 13.4 years (range 8-19 years), median steady state Hb level 8.1 g/dl (range 7.0-8.5 g/dl) and 8.8 g/dl (range 8.3-9.3 g/dl), and average volume of blood received during the previous year 201.5 and 31.0 ml/kg body weight, respectively. The median age of the healthy controls was 24 years (range 22-39 years).

Mean reticulocyte *FPN*1B expression level (normalized to *GAPDH*) relative to that of control group demonstrate significant elevation expression for regularly transfused splenectomized and non-splenectomized β-thalassemia/Hb E patients, as well as those of α-thalassemia RegT and RarT patients (Table 1). There are no significant differences in relative *FPN*1B mRNA level among non-splenectomized and splenectomized β-thalassemia/Hb E and α-thalassemia RegT patients but that of α-thalassemia RarT is significantly lower.

Levels of plasma ferritin and serum malondialdehyde of all patients' groups are significantly higher than those of the control group (Table 1). For those with β-thalassemia/Hb E, ferritin and malondialdehyde levels in splenectomized patients are significantly higher than those in non-splenectomized ones, and ferritin and malondialdehyde levels in both groups of β-thalassemia/Hb E patients are also significantly higher than α-thalassemia RegT patients with those of α-thalassemia RarT patients being the lowest.

Reticulocyte *FPN*1B mRNA levels in α-thalassemia patients are significantly correlated with plasma ferritin (Fig 1A) and serum malondialdehyde levels (Fig 1B). There were no such correlations in β-thalassemia/Hb E patients (data not shown).

DISCUSSION

The current work investigated the expression levels of reticulocyte (surrogate for erythroblast) iron exporter gene *FPN*1B in β-thalassemia/Hb E (receiving regular blood transfusion and iron chelation) and α-thalassemia patients (receiving regular and irregular blood transfusion, but both groups undergoing iron chelation to assess the amount of iron stored in the body and oxidative stress state under recommended chelation therapy and the expression of erythroid-specific iron exporter gene. Plasma ferritin and serum malondialdehyde levels were employed as markers of oxidant stress arising from tissue iron overload.

Higher reticulocyte *FPN*1B expression levels of both β-thalassemia/Hb E and α-thalassemia patients compared to normal subjects suggest an elevation of iron exported from bone marrow of thalassemia patients. This not unexpected as translation of FPN1B mRNA is not under IRE/IRP control (Cianetti *et al*, 2005; Zhang *et al*, 2009). The majority of iron in the body is mobilized to the bone marrow for heme biosynthesis (Vaisman *et al*, 1997). Thus, in thalassemia erythroblasts, where there is a net decrease in hemoglobin content, increased synthesis of ferroportin provides a means to remove excess unwanted iron not required for hemoglobin biosynthesis. This notion is consistent with the observation that reticulocyte *FPN*1B mRNA amounts are

Table 1
Levels of reticulocyte ferroportin *FPN*1B mRNA, blood plasma ferritin and serum malondialdehyde of thalassemia patients and healthy controls.

	β-thalassemia/Hb E patients with regular blood transfusion		α-thalassemia patients		Healthy controls
	Non-splenectomized ($n = 30$)	Splenectomized ($n = 30$)	RegT ($n = 30$)	RarT ($n = 8$)	($n = 30$)
Relative *FPN*1B mRNA (mean ± SD)	2.56 ± 1.41*	2.65 ± 1.48**	2.98 ± 0.63^{***a}	1.67 ± 0.25****	1.00 ± 0.29
Ferritin (ng/ml) (mean ± SD)	1,190.84 ± 198.12^{*bcd}	1,548.76 ± 905.90^{**ef}	896.00 ± 47.76^{***a}	305.59 ± 174.05****	34.18 ± 22.58
Malondialdehyde (μM) (mean ± SD)	3.11 ± 0.46^{*bcd}	4.15 ± 0.94^{**ef}	2.40 ± 0.35^{***a}	1.71 ±0.34****	1.18 ±0.08

RarT, irregularly blood transfused; RegT, regularly blood transfused; SD, standard deviation.
* $p< 0.05$, non-splenectomized β-thalassemia/Hb E patients compared to healthy controls.
** $p< 0.05$, splenectomized β-thalassemia/Hb E patients compared to healthy controls.
*** $p< 0.05$, α-thalassemia RegT patients compared to healthy controls.
**** $p< 0.05$, α-thalassemia RarT patients compared to healthy controls.
^{a} $p< 0.05$, RegT compared to RarT (α-thalassemia patients).
^{b} $p< 0.05$, non-splenectomy compared to splenectomy (β-thalassemia/Hb E patients).
^{c} $p< 0.05$, non-splenectomized β-thalassemia/Hb E patients compared to α-thalassemia RegT patients.
^{d} $p< 0.05$, non-splenectomized β-thalassemia/Hb E patients compared to α-thalassemia RarT patients.
^{e} $p< 0.05$, splenectomized β-thalassemia/Hb E patients compared to α-thalassemia RegT patients.
^{f} $p< 0.05$, splenectomized β-thalassemia/Hb E patients compared to α-thalassemia RarT patients.

higher in β-thalassemia/Hb E (splenectomized and non-splenectomized) and α-thalassemia RegT than in α-thalassemia RarT patients.

All four groups of thalassemia patients have some degree of iron-associated oxidant tissue damage as evidenced by the elevation in plasma ferritin and serum malondialdehyde levels compared to control despite blood transfusion and appropriate iron chelation regimen (Taher *et al*, 2013; Cappellini *et al*, 2014). As expected α-thalassemia RarT patients were affected the least. This residual oxidant tissue damage probably reflects an inability of the chelating agents to neutralize all molecular iron present in tissues and organs of the thalassemia subjects, especially those receiving frequent (every 3-4 weeks) blood transfusions.

The correlations of reticulocyte *FPN*1B mRNA levels with plasma ferritin study and serum malondialdehyde levels present in α-thalassemia but β-thalassemia/Hb E patients might indicate that iron exported from bone mar-

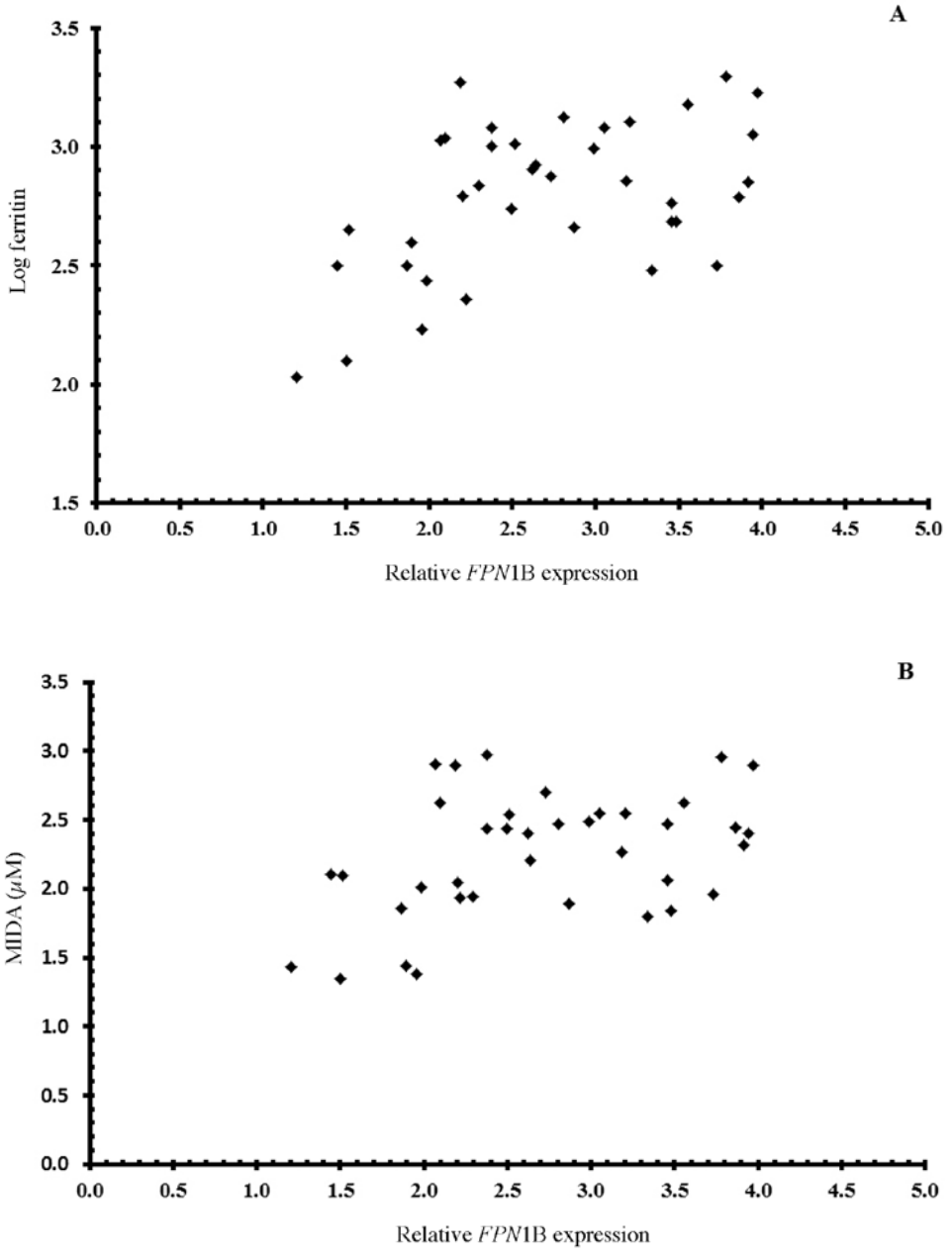

Fig 1-Scatterplot illustrating relationship between levels of reticulocyte *FPN*1B mRNA and log plasma ferritin (A) and serum malondialdehyde (MDA) (B) of α-thalassemia patients. *FPN*1B mRNA content was measured using quantitative RT-PCR normalized to *GAPDH* mRNA and reported as relative *FPN*1B expression. Plasma ferritin was determined by an enzyme-linked immunosorbent assay and serum malondialdehyde by a thiobarbituric acid-reactive substances assay. Spearman rho test was used to calculate the correlation. A. Correlation coefficient $r = 0.451$, $p = 0.005$. B. Correlation coefficient $r = 0.348$, $p = 0.032$.

row in α-thalassemia contributed to iron overload stress whereas this was not the situation in β-thalassemia/Hb E. However, further studies will be required to understand this intriguing phenomenon.

In summary, we observed an increase in the expression level of *FPN*1B in the reticulocytes of thalassemia patients who received blood transfusion (regular and irregular) together iron chelation therapy. This elevation of ferroportin in red cell precursors might lead to release of iron from bone marrow into tissues and organs and, as in the situation of α-thalassemia, could contribute to iron-associated oxidant damage. The role of bone marrow-derived iron in iron overload status of thalassemia patients clearly needs further investigation.

ACKNOWLEDGEMENTS

This study was supported by the Specific Health Problem in Greater Mekong Sub-region (SHeP-GMS) under the National Research University Project and the Center for Research and Development of Medical Diagnostic Laboratories (CMDL), Khon Kaen University.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Abboud S, Haile DJ. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J Biol Chem* 2000; 275: 19906-12.

Arosio P, Levi S. Ferritin, iron homeostasis, and oxidative damage. *Free Radic Biol Med* 2002; 33: 457-63.

Cappellini MD, Cohen A, Porter J, Taher A, Viprakasit V, eds. Guidelines for the management of transfusion dependent thalassaemia (TDT). 3rd ed. Nicosia: Thalassaemia International Federation, 2014: 28-97.

Cianetti L, Segnalini P, Calzolari A, *et al*. Expression of alternative transcripts of ferroportin-1 during human erythroid differentiation. *Haematologica* 2005; 90: 1595-606.

Cianetti L, Gabbianelli M, Sposi NM. Ferroportin and erythroid cells: an update. *Adv Hematol* 2010;2010. pii: 404173. doi: 10.1155/2010/404173.

Donovan A, Brownlie A, Zhou Y, *et al*. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* 2000; 403: 776-81.

Fucharoen S, Winichagoon P. Clinical and hematologic aspects of hemoglobin E beta-thalassemia. *Curr Opin Hematol* 2000; 7: 106-12.

Kijima H, Sawada T, Tomosugi N, Kubota K. Expression of hepcidin mRNA is uniformly suppressed in hepatocellular carcinoma. *BMC Cancer* 2008; 8: 167.

Lapenna D, Ciofani G, Pierdomenico SD, Giamberardino MA, Cuccurullo F. Reaction conditions affecting the relationship between thiobarbituric acid reactivity and lipid peroxides in human plasma. *Free Radic Biol Med* 2001; 31: 331-5.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001; 25: 402-8.

McKie AT, Barlow DJ. The SLC40 basolateral iron transporter family (IREG1/ferroportin/MTP1). *Pflugers Arch* 2004: 801-6.

Pietrangelo A. The ferroportin disease. *Blood Cells Mol Dis* 2004; 32: 131-8.

Pratummo K, Jetsrisuparb A, Fucharoen S, Tripatara A. Hepcidin expression from monocyte of splenectomized and non-splenectomized patients with HbE-β-thalassemia. *Hematology* 2014; 19: 175-80.

Premawardhena A, De Silver S, Arambepola M, *et al*. Hemoglobin E-beta-thalassemia: progress report from the International

Study Group. *Ann N Y Acad Sci* 2005; 1054: 33-9.

Suebpeng Y, Jetsrisuparb A, Fucharoen S, Tripatara A. Frataxin expression in reticulocytes of non-splenectomized and splenectomized patients with HbE-β-thalassaemia. *Clin Biochem* 2016;49:463-6.

Taher A, Vichinsky E, Musallam K, Cappellini MD, Viprakasit V. In: Weatherall D, ed. Guidelines for the management of non transfusion dependent thalassaemia (NTDT). Nicosia: Thalassaemia International Federation, 2013: 45-60.

Vaisman B, Fibach E, Konijn AM Utilization of intracellular ferritin iron for hemoglobin synthesis in developing human erythroid precursors. *Blood* 1997; 90: 831-8.

Weatherall DJ. Pathophysiology of thalassaemia. *Baillieres Clin Haematol* 1998; 11: 127-46.

WHO/CDC. Assessing the iron status of populations. Joint World Health Organization/ Centers for Disease Control and Prevention Technical Consultation on the Assessment of Iron Status at the Population Level. Geneva: WHO/CDC, 2004: 1-112.

Zhang DL, Hughes RM, Ollivier-Wilson H, Ghosh MC, Rouault TA. A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression. *Cell Metab* 2009; 9: 461-73.