To the editor:

Increased levels of ERFE-encoding *FAM132B* in patients with congenital dyserythropoietic anemia type II

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Recessive mutations in the *SEC23B* gene cause congenital dyserythropoietic anemia type II (CDAII),[1] a rare hereditary disorder hallmarked by ineffective erythropoiesis, iron overload, and reduced expression of hepatic hormone hepcidin.[2,3] Some erythroid regulators have been proposed as pathological suppressors of hepcidin expression, such as growth differentiation factor 15 (GDF15) in thalassemia, CDAI and II,[4-6] even if alone GDF15 seems unnecessary for physiological hepcidin suppression.[7] The most recently described is the erythroblast-derived hormone erythroferrone (ERFE), a member of the tumor necrosis factor superfamily that specifically inhibits hepcidin production. ERFE-encoding *FAM132B* is an erythropoietin (EPO)-responsive gene in experimental models.[8] However, the function of ERFE in humans remains to be investigated.

To evaluate ERFE expression in CDAII patients, we enrolled 37 *SEC23B*-related nontransfusion-dependent patients (supplemental Table 1, available on the *Blood* Web site). Ten new cases from 9 unrelated pedigrees were described (supplemental Table 2). All patients were young adults (19.1 ± 3.0 years at diagnosis), with high transferrin saturation (71.4% ± 5.5%). For case-control study, 29 age- and sex-matched healthy controls (HCs) were also included (supplemental Table 1) (see supplemental Methods).

We observed a statistically significant overexpression of the *FAM132B* gene either in peripheral blood leukocytes (PBLs) (Figure 1A) or in reticulocytes (supplemental Figure 1a) from CDAII patients compared with HCs. Indeed, a statistically significant correlation between PBLs and reticulocyte *FAM132B* expression from the same patients was observed (Spearman $\rho = 0.78$; $P = .02$) (supplemental Figure 1b). Similarly, a marked upregulation of *Erfe* in both erythroblasts and PBLs from $Hbb^{th3/+}$ mice compared with wild-type (WT) ones was observed (supplemental Figure 1c). Of note, ~0.03% of erythroblasts ($CD71^+/CD45^-$ cells) were found in PBLs from CDAII patients (data not shown).

Consistent with previous studies and with the loss of function of *SEC23B* mutations,[9] we observed a reduced *SEC23B* expression in our patients compared with HCs (supplemental Figure 2a). Indeed, *FAM132B* and *SEC23B* gene expression exhibited an inverse correlation (supplemental Figure 2b). Interestingly, we did not observe in our patients the same trend of expression of the *FAM132A* paralog gene, which shares 69% of transcript identity with *FAM132B* (supplemental Figure 2c). This suggests a possible involvement of ERFE-encoding *FAM132B* in the pathogenesis of hemosiderosis in CDAII.

During the preparation of this study, 2 articles reporting measurement of human ERFE protein were published.[10,11] According to gene expression analysis, we found increased ERFE expression in CDAII patients compared with HCs (Figure 1B).

Of note, we observed increased *ERFE* expression also in β-thalassemia (BT)-intermedia patients, which exhibit iron overload similarly to CDAII patients (supplemental Table 1). Conversely, only a slight increase of *ERFE* expression was observed in patients with mild well-compensated anemia, such as hereditary spherocytosis (HS) (Figure 1C). These data suggested that the markedly increased ERFE expression observed in both CDAII and BT-intermedia patients is mainly due to ineffective erythropoiesis.

As recently reported, the ablation of Erfe in thalassemic mice induces a slight amelioration of ineffective erythropoiesis but does not improve the anemia.[12] In order to explore the possible interplay between the levels of erythroferrone and the degree of anemia in CDAII patients, we divided our cohort into 2 subgroups: low and high FAM132B. Unlike thalassemic Erfe-haploinsufficient mice, we observed a statistically significant reduction in hemoglobin level in the high-FAM132B subset compared with the low-FAM132B one. Of note, we focused on nontransfusion-dependent patients, thus the expression level of *FAM132B* was not biased by transfusion regimen; however, ERFE expression did not correlate even with the occasional need for transfusions (Table 1). The higher expression of ERFE seems to reflect the increase of iron demand for Hb production as well as the expanding abnormal erythropoiesis, characterizing CDAII, as attested by the increased levels of soluble transferrin receptor and EPO in high-FAM132B patients. This in turn leads to reduced hepcidin and hepcidin-to-ferritin ratio in the high-FAM132B group compared with the low-FAM132B one, resulting in augmented iron delivery to the erythron. Indeed, an inverse correlation between *FAM132B* expression and hepcidin levels was observed (Spearman r, −0.49; $P = .004$). However, the iron balance data do not differ significantly between the 2 CDAII subgroups, even if a trend to increased transferrin saturation was observed in high-FAM132B patients (Table 1). Thus, ERFE upregulation may contribute to the inappropriate suppression of hepcidin, but does not solely account for the subsequent hemosiderosis observed in CDAII patients.

High expression of *FAM132B* in ex vivo EPO-differentiating human erythroblasts has been documented.[8] Accordingly, we confirmed a progressive increase of *FAM132B* expression in healthy $CD34^+$ progenitor cells EPO induced to erythroid differentiation. Particularly, we observed a significant increase of *FAM132B* in the transition from basophilic (9 days, 3.93 ± 1.32) to polychromatic (11 days, 14.30 ± 2.46; $P = .02$) erythroblasts (Figure 1D). Of note, the onset of multinuclearity in an in vitro model of CDAII involves polychromatic and orthochromatic precursors.[13] Thus, the accumulation of highly expressing *FAM132B* polychromatic erythroblasts in CDAII marrow could account for the pathological overexpression observed in CDAII patients. Our observation is consistent with the demonstration that polychromatic erythroblasts are the main source of Erfe in BT mice. Indeed, similarly to CDAII erythropoiesis, BT is characterized by an accumulation of polychromatic erythroblasts that do not mature to the orthochromatic stage. Thus, the increased Erfe expression in

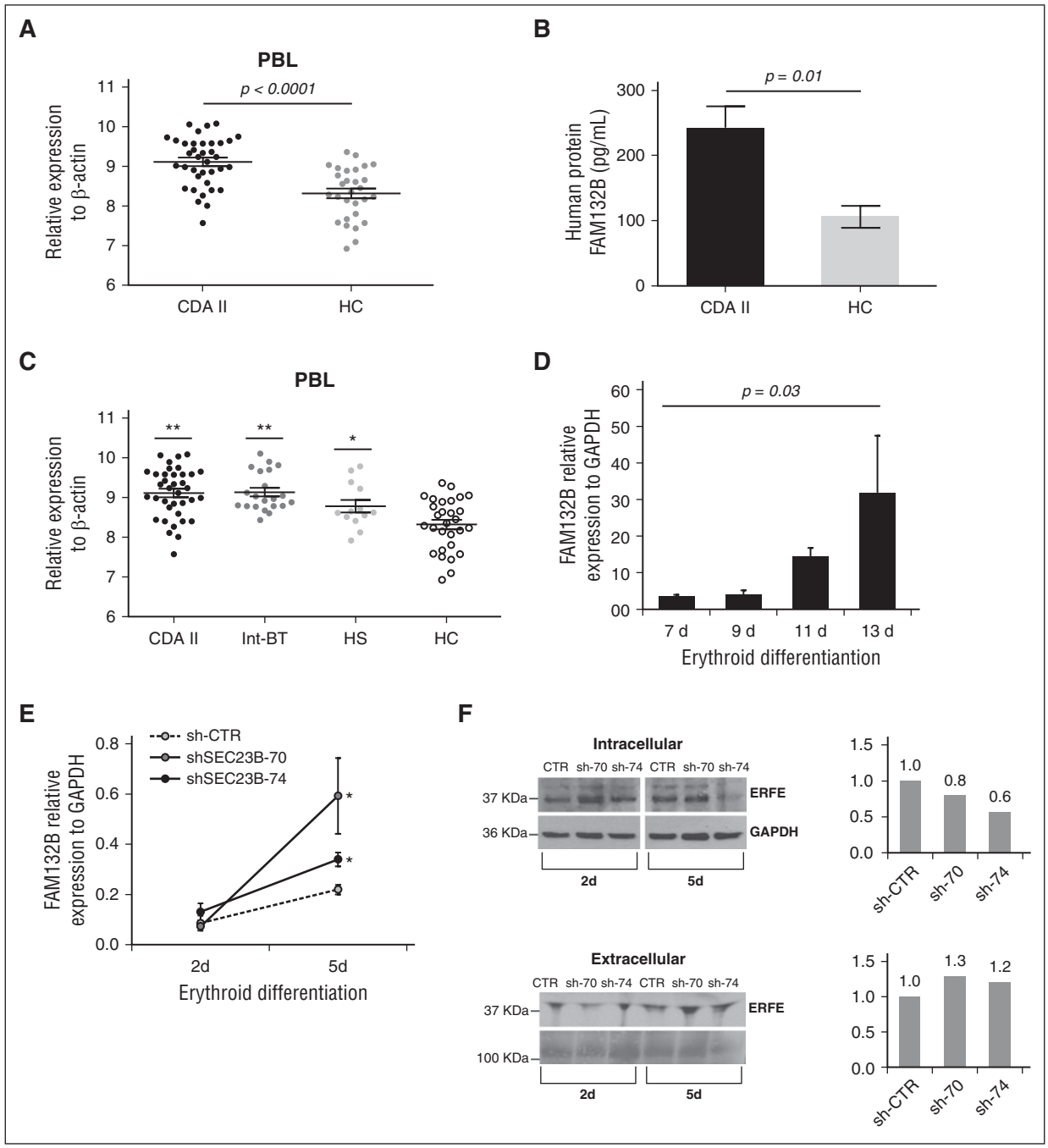

Figure 1. ERFE expression in CDAII patients and during erythroid differentiation in normal and SEC23B-deficient cells. (A) *FAM132B* messenger RNA (mRNA) levels (normalized to *β-actin*) in PBLs from 37 CDAII patients and 29 HCs. Overexpression of the *FAM132B* gene in CDAII patients (9.11 ± 0.10) compared with HCs (8.32 ± 0.12; $P < .0001$) was observed. Data are presented as mean ± standard error of the mean (SEM). *P* value by the Student *t* test. (B) Human protein ERFE evaluation in plasma samples from 29 CDAII patients and 12 HCs. Data are presented as mean ± SEM. *P* value by the Student *t* test. (C) *FAM132B* mRNA levels (normalized to *β-actin*) in PBLs from 37 CDAII, 21 BT-intermedia, 13 HS patients, and 29 HCs. Data are presented as mean ± SEM. $^{**}P < .0001$; $^{*}P < .05$ vs HC group (Student *t* test). (D) *FAM132B* mRNA levels (normalized to glyceraldehyde-3-phosphate dehydrogenase [*GADPH*]) in normal $CD34^{+}$ cells induced to erythroid differentiation by EPO at 7, 9, 11, and 13 days. Data are presented as mean ± SEM of 3 experiments. *P* value by analysis of variance (ANOVA) test. (E) *FAM132B* mRNA levels (normalized to *GADPH*) in stable clones of K562 silenced for SEC23B at 2 and 5 days of erythroid differentiation by hemin. Data are presented as mean ± SEM of 3 experiments. $^{*}P < .05$ (Student *t* test). (F) Immunoblot of ERFE protein in total cell lysate and medium samples from SEC23B-silenced K562 cells induced to erythroid differentiation at 2 and 5 days; GAPDH was loading control for total cell lysate, whereas medium samples were normalized by Ponceau red staining of the blots. Densitometric analysis of the blot showed on the left (5 days): ERFE protein levels of both SEC23B-silenced K562 clones were normalized on sh-CTR K562 clone.

erythroblasts from $Hbb^{Th3/+}$ mice compared with WT mice is mainly due to the relative abundance of polychromatic erythroblasts.[12]

CDAII is still lacking a reliable animal model because SEC23B deficiency results in different phenotypes in humans and mice.[14,15] So far, the only reliable in vitro model for CDAII is the SEC23B-silenced K562 cells.[1] We established K562 stable clones silenced for the SEC23B gene by lentiviral shRNAmir targeting human SEC23B, which ensures high-efficiency silencing. When we induced to erythroid differentiation K562 stable clones silenced for SEC23B, we observed a higher expression of *FAM132B* at 5 days of erythroid differentiation

Table 1. *FAM132B* expression and clinical correlations in CDAII patients

	Low *FAM132B*, n = 20	High *FAM132B*, n = 17	*P**
Age, y	25.3 ± 4.9 (16.0; 19)	12.1 ± 2.5 (10.0; 17)	.03
Onset symptoms, y	7.5 ± 2.5 (5.0; 16)	3.2 ± 1.3 (1.3; 16)	.14
Sex, female/male	9 (45.0)/11 (55.0)	9 (52.9)/8 (47.1)	.63
Complete blood count			
RBC, $10^6/\mu L$	3.6 ± 0.2 (3.5; 20)	3.2 ± 0.1 (3.3; 17)	.05
Hb, g/dL	10.7 ± 0.5 (10.4; 20)	9.2 ± 0.4 (9.5; 17)	.02
Ht, %	31.7 ± 1.4 (30.6; 20)	27.5 ± 1.2 (28.0; 17)	.03
MCV, fL	89.7 ± 1.8 (90.2; 20)	86.0 ± 2.2 (84.7; 17)	.20
MCH, pg	30.6 ± 0.7 (31.0; 18)	28.9 ± 0.9 (27.9; 17)	.12
MCHC, g/dL	33.8 ± 0.4 (33.5; 19)	33.3 ± 0.3 (33.1; 16)	.32
RDW, %	19.9 ± 2.5 (18.9; 12)	21.8 ± 1.2 (22.0; 15)	.48
PLT, $10^3/\mu L$	373.0 ± 41.1 (290.0; 17)	459.2 ± 69.2 (390.0; 17)	.30
Retics abs count, $10^3/\mu L$	67.4 ± 9.2 (59.2; 20)	87.3 ± 17.5 (79.7; 16)	.30
Retics, %	2.0 ± 0.3 (1.5; 20)	2.7 ± 0.6 (2.2; 16)	.25
Reticulocyte index	1.3 ± 0.2 (1.2; 20)	1.7 ± 0.3 (1.5; 16)	.38
Iron balance			
Hepcidin/ferritin	0.04 ± 0.01 (0.02; 16)	0.01 ± 0.003 (0.006; 16)	.01
Hepcidin, nM	5.8 ± 1.9 (2.7; 17)	1.0 ± 0.3 (0.6; 16)	.02
Ferritin, ng/mL	372.1 ± 107.7 (200.0; 19)	168.5 ± 36.0 (99.8; 17)	.10
Ferritin level/dosage age†	32.9 ± 17.2 (14.9; 18)	26.1 ± 8.6 (12.7; 17)	.73
Transferrin saturation, %	67.7 ± 6.8 (62.5; 19)	81.8 ± 7.8 (86.0; 8)	.23
Serum iron, μg/dL	157.8 ± 13.6 (159.5; 18)	162.7 ± 20.4 (172.0; 13)	.84
sTfR, mg/L	3.7 ± 0.4 (3.7; 12)	5.1 ± 0.5 (5.7; 8)	.04
Laboratory data and transfusion regimen			
EPO, mIU/mL	82.5 ± 19.1 (61.9; 14)	154.3 ± 14.5 (170.1; 13)	.01
GDF15, pg/mL	814.9 ± 251.1 (503.5; 13)	781.9 ± 140.6 (804.0; 9)	.92
Total bilirubin, mg/dL	3.7 ± 0.8 (2.5; 19)	2.3 ± 0.3 (2.1; 16)	.15
Unconjugated bilirubin, mg/dL	3.1 ± 0.8 (2.2; 17)	1.9 ± 0.3 (1.5; 12)	.22
Transfusion need, yes/no	7 (46.7)/8 (53.3)	10 (58.8)/7 (41.2)	.49

Data are not available for all patients. For quantitative variables, data are presented as mean ± SEM (median; n). For qualitative variables, data are presented as n (%)/n (%).

Hb, hemoglobin; Ht, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelet; RBC, red blood cell; RDW, red blood cell distribution width; sTfR, soluble transferrin receptor.

*The Student *t* test for quantitative unpaired data; χ^2 test for categorical data.

†Normalization of ferritin by means of "ferritin level/dosage age ratio."[16]

in K562 SEC23B-silenced cells compared with not-silenced ones (Figure 1E). Conversely, *SEC23B* expression was lower in both K562 SEC23B stably silenced cell lines compared with K562 sh-CTR cells at 2 and 5 days of differentiation (supplemental Figure 2d). Thus, we confirmed the ex vivo data about inverse correlation between *FAM132B* and *SEC23B* expression observed in our patients.

Literature data lack information about human ERFE protein. In order to validate our data at the protein level, we first evaluated the good performance of the commercial anti-FAM132B antibody we used (supplemental Figure 3a-d). We also provided the first demonstration that ERFE protein is N-glycosylated; indeed, the higher band obtained by western blotting (WB) determination is the glycosylated isoform of the same protein, as demonstrated by the digestion pattern after peptide-N4-(acetyl-β-glucosaminyl)-asparagine amidase treatment (supplemental Figure 3e). According to gene expression data, we observed an increased level of secreted ERFE in the extracellular medium of K562 SEC23B-silenced cells compared with not-silenced ones at 5 days of erythroid differentiation, whereas the ERFE intracellular level was lower (Figure 1F).

Although the mechanisms of hemin-induced differentiation are quite different from EPO-induced ones, we can hypothesize that ERFE overexpression is related to the maturative arrest and the subsequent increased number of erythroid precursors.

This study provides the first analysis on ERFE expression in a human model of dyserythropoietic anemia with ineffective erythropoiesis such as CDAII. Our ex vivo and in vitro data indicate that ERFE overexpression in CDAII patients might be most likely related to both physiological and pathological mechanisms leading to hepcidin suppression in a condition of dyserythropoiesis. Indeed, we clearly demonstrated that in 2 different genetic conditions sharing common clinical findings and similar pathogenesis, such as CDAII and BT intermedia, *FAM132B* overexpression is related to abnormal erythropoiesis. Nevertheless, the absence of a clear correlation between ERFE levels and CDAII iron balance suggests that ERFE cannot be the only erythroid regulator of hepcidin suppression, at least in CDAII patients.

The online version of this article contains a data supplement.

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Contribution: R.R. and A.I. designed and conducted the study, and prepared the manuscript; I.A. established K562 SEC23B-silenced clones, performed FAM132B dosages, and prepared the manuscript; L. De Falco prepared $CD34^+$ cultures; F.M. performed quantitative real-time polymerase chain reaction and sequencing analysis; M.B. performed GDF15 and EPO dosages; A.G. cared for CDAII patients; G.D.R. performed WB analysis; A.M. provided $Hbb^{th3/+}$ and WT mice; P.R. cared for BT-intermedia patients; D.G. performed hepcidin dosage; and L. De Franceschi designed the mouse model experiment and contributed to critical review of the manuscript.

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