IRE-dependent Regulation of Intestinal Dmt1 Prevails During Chronic Dietary Iron Deficiency but is Dispensable in Conditions of Acute Erythropoietic Stress

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Iron must be supplied in adequate amounts to sustain erythropoiesis while avoiding toxic iron accumulation.1 Whole body iron levels are largely determined by the rate of dietary iron absorption in the duodenum.2 Divalent metal transporter 1 (DMT1, encoded by Slc11a2) is an essential iron transporter present at the apical membrane of absorptive enterocytes.3 It mediates the influx of nonheme iron from the intestinal lumen into the interior of the cell after reduction of the metal by CYB5R1 (a.k.a. DCYTB); iron is then either stored intracellularly into nanocages made of ferritin-H (FTH1) and -L (FTL1) subunits, or exported into the circulation by the basolaterally expressed transporter ferroportin (FPN, a.k.a. SLC40A1) to be loaded onto transferrin after oxidation of the metal by hematin (HEPH).4

Intestinal iron absorption is systemically modulated by the hormone hepcidin (a.k.a. HAMP), which binds to and inhibits FPN.5 Hepcidin production in hepatocytes augments when iron levels in serum and body iron stores are elevated; conversely, stimulation of erythropoiesis suppresses hepcidin through the erythroid-derived hormone erythroferrone (ERFE, encoded by CYB5R1) and -l (FTL1) subunits, or exported into the circulation by the basolaterally expressed transporter ferroportin (FPN, a.k.a. SLC40A1) to be loaded onto transferrin after oxidation of the metal by hematin (HEPH).4

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Figure 1. The Dmt1 3’IRE is required for full stimulation of duodenal DMT1 expression during chronic dietary iron deficiency. (A) WT (indicated IRE<sup>WT</sup>) and Dmt1<sup>IRE<sup>Δ/Δ</sup></sup> (indicated IRE<sup>Δ/Δ</sup>) mice, respectively, were fed with a low (<10 ppm, gray bars) versus a Ctr iron diet (~200 ppm, white bars) from weaning age on until early adulthood (12 weeks of age); (B) serum iron parameters; (C) hepatic iron store; (D) serum EPO levels and percentage of reticulocytes among TER119<sup>+</sup> blood cells. (E, F) Circulating levels of ERFE and hepcidin; the stress hormone ERFE (E) could not be detected under basal conditions (indicated nd). (G) Top histogram: iron content per mg of dry duodenal tissue. Bottom panels: Perl’s staining (enhanced with diaminobenzidine) in the duodenum revealing iron deposits in intestinal epithelial cells of Dmt1<sup>IRE<sup>Δ/Δ</sup></sup> fed with the Ctr diet; iron remains below the detection limit in mice receiving the low iron regimen, regardless of genotype (counterstain: hämalaun; scale bar: 200 µm). (H) Top histograms: qRT-PCR analysis of iron uptake molecules in the duodenum. Bottom panels: representative western-blot analysis of DMT1 protein expression in the duodenum, showing blunted DMT1 stimulation in Dmt1<sup>IRE<sup>Δ/Δ</sup></sup> versus Dmt1<sup>IRE<sup>WT</sup></sup> mice. DMT1 runs as a smear, the asterisk indicates a cross-reactive band. (I) Duodenal expression of the Fth1 and Ftl1 iron storage molecules was assessed by qRT-PCR (top histograms) and western blotting (lower panels), respectively. The representative western blot shows a marked decrease in ferritin protein levels in iron deficient duodena. (J) Top: qRT-PCR analysis of Fpn (left) and Heph (right) expression in the duodenum. Bottom panels: western blot analysis FPN protein levels. In H to J, qRT-PCR results are presented as fold change over WT mice fed with the Ctr diet. Average mRNA levels of Actb, Tubb5, and Gapdh served as reference except in G, where Dmt1-IRE pre-mRNA levels were calibrated to Actb pre-mRNA expression. In H to J, ACTB served as loading control for western blotting. Statistical significance was determined using 2-way ANOVA with Tuckey’s multiple comparisons test, and a P value inferior to 0.05 was considered significant. In B to C, n = 8 to 15. In D to G, n = 6 to 10. In H to J, n = 14 to 17. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ACTB = beta actin; Ctr = control; Cybrd1 = cytochrome b reductase 1; DMT1 = Divalent metal transporter 1; EPO = erythropoietin; ERFE = erythroferrone; FPN = ferroportin; FTH1 = ferritin heavy chain 1; FTL1 = ferritin light chain 1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HEPH = hephaestin; IRE = iron-responsive element; qRT-PCR = real-time quantitative reverse transcription PCR; sat. = saturation; TUBB5 = tubulin, beta 5 class I; WT = wild-type.
Figure 2. The Dmt1 3IRE is dispensable for duodenal induction of DMT1 in conditions of stress erythropoiesis. (A) Adult WT (IRE+/+) and Dmt1IREΔ/Δ (IREΔΔ) mice were subjected to acute stress erythropoiesis. The animals were either treated with EPO (top) or with PHZ (bottom), as indicated. (B) Spleen index (calculated as square root of 100× spleen weight/body weight). (C) Serum iron parameters. (D) Serum levels of ERFE and hepcidin; ERFE was not detected in saline-injected mice (indicated nd). (E) Serum EPO concentration in mice treated with PHZ. (F) Hepatic iron store. (G, H) Duodenal iron metabolism: the bar graphs on the left display the iron content per mg of dry tissue in EPO (G) versus PHZ (H)-injected animals; the histology pictures show iron staining of duodenal villi (Perl's staining enhanced with diaminobenzidine; counterstain: hæmalaun; scale bar: 200 µm). (I, J) top histograms: qRT-PCR analysis of Dmt1 and Cybrd1 transcripts in the duodenum of animals treated with EPO (I) or with PHZ (J), respectively. Bottom panels: representative western-blot analysis of DMT1 protein expression in the duodenum of EPO (I) versus PHZ (J)-injected mice. The expression of apical iron transport molecules augments in mice exposed to erythropoietic stresses regardless of genotype. In (I) and (J), qRT-PCR results are presented as fold change over WT mice injected with saline. Average mRNA levels of Actb, Tubb5 and Gapdh served as reference except for the analysis of Dmt1-IRE pre-mRNA levels, which were calibrated to Actb pre-mRNA expression. For western blotting, ACTB served as loading control. Statistical significance was determined using 2-way ANOVA with Tuckey’s multiple comparisons test, and a P value inferior to 0.05 was considered significant. In (B) to (G), n = 9 to 10 (EPO) or n = 8 to 12 (PHZ). In (I) and (J), n = 5 (EPO) or n = 9 to 12 (PHZ). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ACTB = beta actin; Cybrd1 = cytochrome b reductase 1; DMT1 = Divalent metal transporter 1; EPO = erythropoietin; ERFE = erythroferrone; IRE = iron-responsive element; PHZ, phenylhydrazine; qRT-PCR = real-time quantitative reverse transcription PCR; sat. = saturation; WT = wild-type.
reported observations. The low iron regimen, however, strongly depletes the duodenal iron content in both WT and mutant mice (Figure 1G, top).

At the molecular level, chronic iron deficiency triggers a substantial and selective increase in the expression of the Dmt1-IRE mRNA isoform in WT animals (Figure 1H, top left histogram), which translates as increased DMT1 protein levels (Figure 1H, bottom panels). This response is accompanied by an upregulation of the immature Dmt1-IRE pre-mRNA (Figure 1H, bottom left histogram). Most likely, iron depletion in duodenal enterocytes activates HIF2, which in turn stimulates Dmt1-IRE transcription.6 This notion is further supported by the concomitant upregulation of another HIF2-target, Cybrd1 (Figure 1H, bottom right histogram).7 Similar to WT, Dmt1IRE/Δ/Δ mice subjected to chronic iron scarcity display higher expression of both the immature Dmt1-IRE pre-mRNA and the Cybrd1 mRNA (Figure 1H, bottom histograms), consistent with increased HIF2 activity. The augmentation of Dmt1-IRE pre-mRNA levels is even twice as high as in WT iron-deficient duodena. However, the expression of the mature Dmt1-IRE mRNA is only mildly augmented and is lower than in WT (Figure 1H, upper left histogram), and DMT1 protein induction is blunted (Figure 1H, bottom panels), indicating that disruption of the Dmt1 3′IRE impairs Dmt1 posttranscriptional stimulation under conditions of chronic dietary iron deficiency. Despite the blunted DMT1 response, systemic iron metabolism parameters remain globally within normal range, potentially due to compensatory effects. In addition to stimulation of apical iron transport molecules, the low iron regimen and subsequent reduction of mucusal iron levels lead to diminished expression of the FTH1 and FTL1 iron storage proteins without concomitant change in the level of the corresponding mRNAs, both in WT and Dmt1IRE/Δ/Δ duodena (Figure 1I), possibly reflecting ferritin degradation6 or suppression of ferritin translation by the IRPs.11 Chronic iron deficiency also augments the expression of FPN at the protein level, and in response in WT mice slightly exceeds that of mutant animals (Figure 1J, lower panels). The low iron diet has globally little or no impact on Fpn and Heph mRNA levels (Figure 1J, histograms). Collectively, these results indicate that stimulation of Dmt1-IRE transcription by HIF2 does not suffice, and that posttranscriptional stimulation of Dmt1 through the 3′IRE is additionally required for full stimulation of intestinal DMT1 expression in response to chronic dietary iron deficiency.

We next assessed the role of the Dmt1 3′IRE in the response to stress erythropoiesis, a condition that acutely increases the intestinal iron absorption machinery. Mice were injected either with recombinant EPO to stimulate erythropoiesis directly,11 or phenylhydrazine (PHZ) to mimic acute hemolytic anemia, a situation that also enhances erythropoiesis (Figure 2A).12 As expected, both treatments trigger extramedullary erythropoiesis, as indicated by a marked increase in spleen size in all genotypes (Figure 2B). Accordingly, EPO treatment augments hemoglobin levels (Suppl. Table S1) as well as reticulocyte frequency in peripheral blood, as measured by flow cytometry (WT: 14.3% ± 1.0% versus 5.8% ± 1.2% reticulocytes among TER119+ cells in EPO-treated versus control mice; Dmt1IRE/Δ/Δ, 14.1 ± 1.1 and 4.6% ± 0.5%, respectively); reticulocytosis could not be assessed reliably in PHZ-treated mice due to autofluorescence from damaged RBCs (not shown). Expansion of the erythroid compartment in EPO-treated mice increases iron consumption and thus lowers serum iron and transferrin saturation values (Figure 2C), while inducing ERFE and suppressing serum hepcidin (Figure 2D). EPO does not affect hepatic iron levels (Figure 2F), but the amount of iron in duodenal villi tends to be reduced (Figure 2G). Differing from the EPO regimen, the release of iron from damaged RBCs in PHZ-treated mice results in an elevation of systemic iron indices in both WT and Dmt1IRE/Δ/Δ animals, with high serum iron and ferritin concentration, high transferrin saturation values, and iron accumulation in the liver (Figure 2C,F). Yet, the marked reduction of RBC counts (Suppl. Table S1) enhances EPO and ERFE production (Figure 2D,E), albeit to a lesser extent in Dmt1IRE/Δ/Δ mice. Despite high ERFE levels, the reduction in hepcidin does not reach statistical significance (Figure 2D). While EPO treatment tends to decrease the duodenal iron content (Figure 2G), this is not the case in PHZ-treated animals (Figure 2H). Consistent with an elevation of iron needs, treatment with either EPO or PHZ significantly increase the levels of the Cybrd1 mRNA and the immature Dmt1-IRE pre-mRNA in Dmt1IRE/Δ/Δ duodena (Figure 2I). However and in sharp contrast to chronic dietary iron deficiency (Figure 1H), the absence of a functional Dmt1 3′IRE in Dmt1IRE/Δ/Δ mice does not weaken the induction of the mature Dmt1-IRE mRNA nor the stimulation of DMT1 protein expression under conditions of stress erythropoiesis (Figure 1J). Mirroring duodenal iron levels, ferritin protein expression is decreased in EPO, but barely affected in PHZ-treated mice, regardless of genotype (Suppl. Figure S1A,B). FPN protein expression is augmented in all genotypes (Suppl. Figure S1C,D), mRNA levels of Fth1, Ftl1, and Heph are barely affected, Fpn mRNA expression is mildly induced by EPO (Suppl. Figure S1A–D). Overall, these data show that the Dmt1 3′IRE is largely dispensable for adequate regulation of key intestinal iron absorption molecules in conditions of acute erythropoietic stress.

We have formerly reported a strong posttranscriptional downregulation of Dmt1-IRE mRNA expression in Dmt1IRE/Δ/Δ duodena during the suckling period,11 a stage of life characterized by high iron needs, low iron stores, and relatively limited iron supply from maternal milk.12,13 This phenomenon, however, was not recapitulated in adult mice under steady-state conditions, uncovering an age-dependent switch in the activity of the Dmt1 3′IRE in the intestine. With the inability to replenish supply from dietary source, WT mice fed with an iron-poor diet over a long period of time respond by enhancing duodenal Dmt1-IRE expression. By contrast and despite a marked stimulation of Dmt1-IRE transcription, adult Dmt1IRE/Δ/Δ mice exhibit low levels of Dmt1-IRE both at the mature mRNA and protein level. Hence, a functional IRE loop in the 3′UTR of the Dmt1 mRNA appears to be needed to properly adjust DMT1 expression during long-term iron scarcity, as observed in sucking mice12 and in animals subjected to dietary iron deficiency (this study). Conceivably, and by analogy to other 3′IRE motifs, IRP binding to the Dmt1-3′IRE stabilizes the transcript, which together with stimulation of Dmt1 transcription by HIF2 is needed to achieve adequate DMT1 protein expression. In contrast to dietary iron restriction, both EPO and PHZ treatments represent acute increases in systemic iron demands in mice already loaded with iron. Our results indicate that the loss of IRE function in Dmt1 does not affect the overall response to acute erythropoietic stress. This implies that the observed upregulation of duodenal DMT1 expression is largely IRE/IRP-independent and is likely mediated by HIF2.13 Among other possibilities, the degree of iron depletion in absorptive enterocytes (more profound in chronic dietary iron deficiency than upon EPO or PHZ treatments) might influence the contribution of IRP/IRE binding and HIF2, to the regulation of DMT1.

In addition to the gene or tissue-dependent effects on DMT1 expression reported previously,6 the Dmt1 3′IRE now appears to also influence DMT1 regulation in a stimulus-dependent manner. Whether the context-dependent activity of the Dmt1 3′IRE involves the luminal or enterocytic iron status, the gut microbiome, and additional molecules whose activity is also situation-dependent warrants further research.
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AUTHOR CONTRIBUTIONS

MQ and BG designed the research. MQ, MB, AE, GS, MP-S, and BG performed experiments. MQ, MB, ZK, and BG analyzed the data. MQ, ZK, and BG wrote and reviewed the article.

DISCLOSURES

The authors have no conflicts of interest to disclose.

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