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Control of Systemic Iron Homeostasis by the 3′ Iron-Responsive Element of Divalent Metal Transporter 1 in Mice

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Eight control of intestinal iron absorption is required to avoid both iron insufficiency and excess.1 Dietary nonheme iron is taken up by absorptive enterocytes via the apical iron transporter DMT1 (a.k.a. SLC11A2),2,4 and transcribed into the circulation by ferroportin (FPN, a.k.a. SLC40A1), with the help of a ferroxidase, hephaestin (HEPH).3 FPN activity is controlled by the liver hormone hepcidin,1 but DMT1 seems regulated locally via mechanisms operating within enterocytes.5,6 DMT1 messenger ribonucleic acid (mRNA) exists in four isoforms that differ in their 5′ and 3′ ends.7 Three end diversity results from alternative usage of splicing and polyadenylation sites and yields isoforms that either contain or lack a conserved iron responsive element (IRE) in their 3′ untranslated region (UTR). IRE-containing isoforms are predominant in duodenal enterocytes.

IREs are stem-loop structures that interact with iron regulatory proteins (IRPs, a.k.a. ACO1 and IREB2) in iron-depleted cells.3 IRP binding to multiple IREs in the 3′-UTR of the transferrin receptor 1 (TFRC) mRNA limits its degradation by Regnase-1 (a.k.a. ZC3H12A).8 The presence of an IRE-like motif in DMT1 suggests that DMT1 could be regulated by IRPs, similar to TFRC. However, the single DMT1 IRE contains an additional 3′-bulge in its upper stem, and DMT1 mRNA seems to lack a Regnase-1 binding site.9 Importantly, DMT1 expression only responds to iron fluctuation in a subset of cell lines,10,11 and the DMT1 3′IRE failed to exhibit iron-dependent regulation in reporter assays.11 Furthermore, Dmt1 transcription is controlled by HIF2α (a.k.a. EPAS1),3,6 which itself is regulated by IRPs,1 confirming the study of specific functions of the Dmt1 3′IRE.12 Here, we address the role of this ribonucleic acid (RNA) motif using a mouse model with selective disruption of the Dmt1 3′IRE.

We established a mouse line lacking the 5′ stem, the apical loop and part of the 3′ stem of the Dmt1 3′IRE (Fig. 1A–D) and Supplemental Materials and Methods, http://links.lww.com/HS/A91). Mutagenesis of the Dmt1 3′IRE impairs IRP binding. Importantly, all four Dmt1 transcripts are adequately expressed in homozygous mutant mice, including those that bear the dysfunctional IRE (Supplemental Figure 1, http://links.lww.com/HS/A91). The resulting allele (designated Dmt1IRE−/−) is inherited in Mendelian proportions (Supplemental Table 1, http://links.lww.com/HS/A91). The mean weight is comparable to Dmt1+/+ littermates (Supplemental Table 3, http://links.lww.com/HS/A91) are globally preserved during postnatal growth (2 weeks of age, during a period of high iron demand), early adulthood (3 months of age) and advanced age (9 months).

A flow cytometry analysis did not reveal any abnormality of terminal erythroid differentiation in young adults (Supplemental Figure 2, http://links.lww.com/HS/A91). The mean weight is slightly higher in 2-week-old Dmt1IRE−/− pups but later is comparable to Dmt1+/+, Dmt1−/− (Supplemental Table 3, http://links.lww.com/HS/A91). Spleen, liver, kidney, and heart weights were unchanged (Supplemental Table 3, http://links.lww.com/HS/A91). These data show that while Dmt1 is essential during perinatal life and critical for erythroid iron acquisition,2,3 its 3′IRE is not required under standard laboratory conditions and appears to be dispensable for normal hematopoiesis.

Interestingly, 2-week-old Dmt1IRE−/− male mice display a 40% reduction in serum iron levels, and a decrease in transferrin saturation (Fig. 1E). In spite of the hypoferremia, hepcidin concentration, which is low during postnatal growth, is not affected (Fig. 1F). Similarly, both hepatic and splenic iron stores are small and remain indistinguishable from wild-type animals.
Disruption of the Dmt1 3'IRE decreases serum iron levels during early life but leads to hyperferremia in adulthood.

Figure 1.

A) The Dmt1 locus comprises 18 exons. The first exon is transcribed from 2 alternative promoters termed 1a and 1b, respectively. 3' end diversity results from alternative splicing and alternative usage of polyadenylation sites: inclusion of exon 16 gives rise to Dmt1 mRNA isoforms bearing the 3'UTR IRE; non-IRE isoforms arise from the joining of a splice donor located upstream of the IRE within exon 16 with the splice acceptor of exon 17. The IRE stem-loop was mutagenized using a targeting construct encompassing exons 12 to 16; a CD neomycin (Neo) resistance cassette (indicated Neor) flanked with LoxP sites (depicted by grey triangles) was inserted into intron 15 and served as a selection marker. The Dmt1 IRE was replaced by the mutated IRE through homologous recombination in embryonic stem cells. The CD-Neo cassette was subsequently removed via transient expression of CRE recombinase to obtain the Dmt1 IRED allele. (B) Schematic representation of the murine Dmt1 IRE stem-loop structure. The bases highlighted in red are deleted in the Dmt1 IRED allele. (C) Southern-blot analysis of the Dmt1 locus in Dmt1 IRE+/+, Dmt1 IRE+/D, and Dmt1 IRED/D mice with a 3' external probe (P3') after digestion of genomic deoxyribonucleic acid with NcoI, indicated N in (A). (D) Total RNA from wild-type (top) and mutant (bottom) littermates was reverse transcribed and the IRE region of the Dmt1 mRNA was PCR amplified (Supplemental Table 4, http://links.lww.com/HS/A91) and sequenced. The electropherograms confirm proper mutagenesis of the 3'IRE of the Dmt1 mRNA. The nucleotides deleted in the wild-type IRE are underlined. The NcoI site created in the mutated IRE is shown. (E) Serum iron parameters were assessed in male mice at 2 weeks (2 wks), 3 months (3 mos) and 9 months (9 mos) of age (6–13 male mice per group). (F) Serum hepcidin concentration (left) and liver hepcidin mRNA levels (right) in 3-month-old male mice. (G) The liver and splenic iron stores were determined in male mice (8–22 mice per group) at different stages of life, as in (E). The results are expressed as percentage of wild-type littermates for each range of age. Note that the hepatic and splenic iron stores of 2 week-old mice are low compared to adults (liver: ∼100±32 mg nonheme iron/g of dry tissue in 2-week old wild-type males vs 279±51 and 357±62 mg/g at 3 and 9 months of age, respectively; spleen: 227±414 mg/g in 2 week-old wild-type males vs 1927±149 and 3881±744 mg/g at 3 and 9 months of age, respectively). Histograms display averages ± SEM. p: Student t-test (*: p < 0.05; **: p < 0.01; ***: p < 0.001). CD = cytidine deaminase; IRE = iron responsive element; mRNA = messenger ribonucleic acid; RNA = ribonucleic acid.
Figure 2. The Dmt1 3’IRE exerts divergent effects on intestinal DMT1 expression in postnatal vs adult animals. (A) Left: Duodenal nonheme iron levels were analyzed in male mice at different stages of life as in Figure 1E (9–15 mice per group). Right: Perl’s staining in the duodenum of 3-month old male mice showing iron deposition in mucosal cells (counterstain: nuclear fast red). (B) Top: qPCR analysis of Dmt1 mRNA levels in the duodenum of 2 week- (left) vs 3 month- (right) old mice using IRE vs non-IRE-specific primers (Supplemental Table 4, http://links.lww.com/HS/A91). The data display transcript levels as percentage of control (ie, Dmt1IRE+/+) after calibration to Actb (n = 9–16 mice per group); similar results were obtained when using Gapdh and/or Tubb5 as standard (not shown). Middle panels: immunostaining of DMT1 in the duodenum (counterstain: hematoxylin); a higher magnification is presented for adult tissues because the DMT1 signal (arrows) is fainter than in suckling pups. Bottom: representative western-blot analysis of DMT1 in whole duodenal extracts. (C) qPCR analysis of Dmt1 pre-mRNA levels in 2-week- vs 3-month-old male mice, after calibration to the Actb pre-mRNA (similar results were obtained after calibration of Gapdh pre-mRNA, not shown). (D) qPCR (top) and western-blot (bottom) analysis of ferroportin expression in the duodenum of mice at 2 weeks (left) vs 3 months (right) of age. For western blotting (B and D), ACTB was used as loading control. In (A) and (B), pictures were acquired with a DM5000 microscope equipped with a DFC420C camera and a 10× (A), 20× (B, 2 weeks) or 40× (B, 3 months) objectives, and were processed using the Leica Application Suite (Leica Biosystems, Wetzlar, Germany). (E) qPCR analysis of Heph mRNA levels in the duodenum of 2 week- vs 3-month old mice. (F) qPCR analysis of HIF2-target genes in the duodenum of 3-month-old animals. The qPCR data in (D) to (F) display transcript levels as percentage of control (ie, Dmt1IRE+/+) after calibration to Actb (similar results were obtained after calibration on Gapdh and/or Tubb5, not shown). (G) Duodenal scrapings from 3-month old mice were resolved through linear 10% to 50% sucrose density gradients and fractions were collected with constant UV recording (top). Fractions corresponding to untranslated mRNA (mRNP: messenger ribonucleoprotein), monosomes (80S), as well as light and heavy polysomes were collected. The amount of Dmt1 (IRE and non-IRE isoforms) and Actb mRNAs in those fractions was determined by qPCR. The histograms (bottom) represent mRNA levels across the fractions as a percentage (mean ± SEM; n = 7–8) of the sum of all fractions. Histograms display averages ± SEM, p: Student t-test (*, p < 0.05; **, p < 0.01; ***: p < 0.001). IRE = iron responsive element, mRNA = messenger ribonucleic acid, RNA = ribonucleic acid.
(Fig. 1G). In contrast to suckling pups, 3 month-old Dmt1<sup>fl/fl</sup> males exhibit high serum iron and transferrin saturation values (Fig. 1E) and an increase in tissue iron stores (Fig. 1G), accompanied by an augmentation of serum hepcidin (Fig. 1F, left) attributable to stimulation of the hepcidin transcript in liver (Fig. 1F, right). Nine-month-old Dmt1<sup>fl/fl</sup> males display a comparable hyperferremia and a trend towards high serum ferritin (Fig. 1E), associated with hepatic and splenic iron loading (Fig. 1G). Dmt1<sup>fl/fl</sup> female mice show a similar, albeit less pronounced iron phenotype (Supplemental Figure 3, http://links.lww.com/HS/A91) at 2 weeks and 3 months. Enlargement of tissue iron stores in Dmt1<sup>fl/fl</sup> adults is not associated with aberrantly high expression of mRNAs encoding known iron import or sequestration genes, nor with marked suppression of transcripts coding for iron export molecules (Supplemental Figure 4, http://links.lww.com/HS/A91), suggesting that tissue iron loading is secondary to the elevation of serum iron rather than a consequence of aberrant iron management in liver and spleen cells.

Iron dyshomeostasis in Dmt1<sup>IRE<sub>A</sub></sup> mice could result from altered intestinal iron absorption. At 2 weeks of age, duodenal nonheme iron levels are indistinguishable from wild type (Fig. 2A). However, Dmt1<sup>fl/fl</sup> pups exhibit a selective downregulation of the Dmt1-IRE mRNA isoform, associated with a decrease in total DMT1 protein levels and reduced DMT1 immunostaining at the apical surface of enterocytes (Fig. 2B). The downregulation of the Dmt1-IRE mRNA is largely posttranscriptional, since Dmt1-IRE pre-mRNA levels are not significantly altered (Fig. 2C). This is in agreement with the predicted role of 3'UTR IRES, based on analogy to TFR2, where IRP binding decreases mRNA decay. Considering that FPN protein and Hepha mRNA levels are not reduced (Fig. 2D and E), the hypoferremia in Dmt1<sup>fl/fl</sup> pups could be explained by downregulation of intestinal DMT1 expression. During adulthood, Dmt1<sup>IRE<sub>A</sub></sup> mice exhibit an approximately 2-fold increase in enterocyte iron accumulation (Fig. 2A), associated with normal expression of FPN and Hepha (Fig. 2D and E). In contrast to its partial suppression in Dmt1<sup>IRE<sub>A</sub></sup> pups, IRE-containing Dmt1 mRNA is expressed at nearly wild-type levels in adult intestine (Fig. 2B). It is unlikely that this is due to compensatory stimulation of Dmt1 transcription by HIF2. Indeed, mRNA levels of Fpn and Ccd1, both HIF2-target genes, are not increased (Fig. 2D and F). Cytb<sub>6</sub>1, another HIF2-target, appears to be repressed (Fig. 2F). Moreover, Dmt1 pre-mRNA levels are unchanged (Fig. 2C). Hence, the 3'IRE of Dmt1 exerts a positive effect on intestinal DMT1 expression during the postnatal period of growth but not during adult life. This age-dependent effect of the Dmt1 3'IRES appears to be tissue-specific. While it is also observed in heart, it is not detected in spleen or kidney (Supplemental Figures 4 and 5, http://links.lww.com/HS/A91). Surprisingly, while Dmt1<sup>IRE<sub>A</sub></sup> adults express nearly wild-type levels of Dmt1-IRE mRNA, duodenal DMT1 protein expression is increased (Fig. 2B). Although we cannot exclude changes in protein turnover, we speculated that disruption of the Dmt1 3'IRES could alter mRNA translation. Supporting this notion, we observed a significant shift of the Dmt1-IRE mRNA isoform from monosomes to polysomes (Fig. 2G), suggesting that the Dmt1 3'IRES partially represses Dmt1 mRNA translation in the adult duodenum. As we did not detect obvious signs of altered urinary iron excretion (not shown), the relatively mild iron accumulation in Dmt1<sup>IRE<sub>A</sub></sup> animals could reflect a modest increase in dietary iron absorption secondary to intestinal DMT1 protein upregulation, leading to progressive accretion of iron in the body over time.

The functionality of the DMT1 3'IRE has been questioned. Although it exhibits weaker affinity for IRPs than other IRES, the DMT1 3'IRE does bind IRP1 in the native cellular environment. Our work confirms that the 3'IRES of Dmt1 plays a role in controlling DMT1 expression and systemic iron homeostasis and reveals an age-dependent switch in its activity. During postnatal growth, the Dmt1 3'IRE promotes intestinal DMT1 expression and secures iron sufficiency; in adulthood, it suppresses DMT1 and prevents systemic iron loading.

Surprisingly, the IRE of Dmt1 seems to influence different aspects of RNA fate in the intestine at different times – abundance and stability during early life and mRNA translation in adulthood. The molecular details of this age-related switch in the function of the Dmt1 3'IRE remain unknown. 3'UTR-based gene regulation is often mediated through the binding of proteins and/or microRNAs to specific RNA sequences and/or structures. We speculate that factors with tissue- and/or age-specific activity or expression might influence the way the Dmt1 3'IRES modulates DMT1 levels. Of note, single 3'IRESs have been identified in other transcripts, including the Calc14a and Fpn2 mRNAs. Like DMT1, the regulation of those mRNAs appears to be cell-type specific and different from the regulation of TFR2. Whether those other single 3'IRESs regulate gene expression through a mechanism similar to the DMT1 3'IRES remains to be determined.

It is well established that Dmt1 regulation in the duodenal mucosa is strongly dependent on HIF2. In that context, the precise role of the age-dependent switch in the activity of the Dmt1 3'IRES remains to be defined. The Dmt1 3'IRES may contribute to maintaining baseline homeostasis of immature intestinal absorption early in life, whereas, in adulthood, the 3'IRES may help to fine-tune DMT1 expression to avoid excessive iron assimilation.

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