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Intestinal DMT1 Is Essential for Optimal Assimilation of Dietary Copper in Male and Female Mice with Iron-Deficiency Anemia

Xiaoyu Wang,[1] Shireen RL Flores,[1] Jung-Heun Ha,[1] Caglar Doguer,[1] Regina R Woloshun,[1] Ping Xiang,[1,3] Astrid Grosche,[2] Sadasivan Vidyasagar,[2] and James F Collins[1]

Departments of [1]Food Science and Human Nutrition and [2]Radiation Oncology, University of Florida, Gainesville, FL; and [3]State Key Lab of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, China

Abstract

Background: Divalent metal-ion transporter 1 (DMT1) may transport copper, but studies to date on this topic have been equivocal. Previously, an ex vivo experiment showed that intestinal copper transport was impaired in Dmt1-mutant Belgrade rats.

Objective: In this study, we tested the hypothesis that intestinal DMT1 transports copper in vivo.

Methods: Intestine-specific Dmt1 knockout ($Dmt1^{int/int}$) mice and normal (control) littermates ($Dmt1^{fl/fl}$) were used. In study 1, intestinal copper absorption was assessed in 7-wk-old mice of both sexes and genotypes by oral-intragastric gavage of ^{64}Cu under normal and iron-deficiency anemia (IDA) conditions. In study 2, both sexes and genotypes of 8-wk-old mice were fed diets with adequate iron concentrations [72 parts per million (ppm)] plus adequate (9 ppm) or excessive (183 ppm) copper concentrations for 4 wk. Iron- and copper-related physiologic variables were subsequently assessed.

Results: Study 1 showed that intestinal copper transport was enhanced in normal (~11% increase in males, 35% in females) and anemic (~42% increase in males, 35% in females) $Dmt1^{int/int}$ mice. Study 2 showed that, with adequate copper intakes, serum ceruloplasmin (Cp) activity was decreased (by ~29% in males and 20% in females) and spleens were enlarged (by 3-fold in both sexes) in $Dmt1^{int/int}$ mice. Higher dietary copper increased hepatic copper concentrations (by ~3.3-fold in males and 1.5-fold in females), restored serum Cp activity, and mitigated the noted splenomegaly in $Dmt1^{int/int}$ mice.

Conclusions: Copper homeostasis was disrupted in $Dmt1^{int/int}$ mice, particularly during IDA, despite the noted increases in intestinal copper transport. This was exemplified by the fact that extra dietary copper was required to restore serum Cp activity (a biomarker of copper status) and reduce the severity of the noted splenomegaly (which could reflect changes in erythropoietic demand) in $Dmt1^{int/int}$ mice. Collectively, these observations show that intestinal DMT1 is essential for the assimilation of sufficient quantities of dietary copper to maintain systemic copper homeostasis during IDA. *J Nutr* 2018;148:1244–1252.

Keywords: intestine-specific Dmt1 knockout mice, copper absorption, *Slc11a2*, ceruloplasmin, iron-deficiency anemia, manganese, zinc

Introduction

Previous studies over the past several decades have shown that iron deficiency is associated with alterations in copper homeostasis (1–5). For example, during iron depletion, copper accumulates in the duodenal epithelium (3), and expression of an intestinal copper exporter, copper-transporting ATPase 1 (Atp7a), is strongly induced (3, 4, 6). Moreover, copper accumulates in the liver (5), and increases in serum copper are associated with enhanced ceruloplasmin (Cp) ferroxidase activity (7). Copper is thus likely redistributed during iron deficiency to tissues important for iron metabolism, exemplifying a physiologically-relevant relationship between iron and copper. What is unknown, however, is what protein (i.e., transporter) mediates increased copper uptake into duodenal enterocytes during low-iron conditions. Possible mechanisms of copper import would logically include copper transporter 1 (CTR1) (8, 9) and divalent metal-ion transporter 1 (DMT1), which may also transport dietary copper (10, 11). Given the strong induction of DMT1 expression during iron deficiency (3, 12, 13), it is a likely candidate for enhancing the assimilation of dietary copper. Conceivably, intestinal DMT1 could also transport copper during physiologic conditions, but one recent study

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suggests that this is not the case (14). Further experimentation is, however, warranted to establish definitively whether intestinal DMT1 transports copper or otherwise influences copper homeostasis.

DMT1 is a widely expressed ferrous iron/proton cotransporter that has emerged as a critical player in iron metabolism in humans and other mammals (15). In the intestine, DMT1 is the principal importer of diet-derived, nonheme iron (16, 17). In other body cells—for example, developing erythrocytes—DMT1 functions intracellularly in endosomes where it mediates ferrous iron transport into the cytosol after endocytosis of diferric transferrin (18). Although earlier studies showed that DMT1 could transport a variety of divalent cations (19), or even monovalent cations (e.g., Cu^{+}) (11), more recent evidence suggests that iron is its main physiologic substrate, at least in mice (14). Despite this, however, there is also support for manganese transport by DMT1 in some tissues (20–22). Accumulating evidence also suggests that DMT1 may transport copper (10, 11, 23–26), but definitive in vivo studies are lacking. The current investigation was thus undertaken to test the hypothesis that lack of intestinal DMT1 will decrease intestinal copper transport and disrupt systemic copper homeostasis. The experimental approach used genetically-engineered mice lacking DMT1 only in the intestinal epithelium and control (normal) littermates. Notably, the lack of intestinal DMT1 activity led to compensatory increases in intestinal copper absorption, but systemic copper metabolism was nonetheless disturbed, showing that intestinal DMT1 is required for optimal assimilation of dietary copper.

Methods

Animal experiments. All animal studies were approved by the University of Florida Institutional Animal Care and Use Committee. Intestine-specific Dmt1 knockout ($Dmt1^{int/int}$) mice and phenotypically normal ($Dmt1^{fl/fl}$) control littermates on the 129S6 genetic background were used for this investigation. These mice have loxP sites flanking part of the *Slc11a2* gene and express the CRE recombinase under the control of the intestinal epithelium-specific villin promoter (42). Breeders were obtained from Dr. Bryan Mackenzie, University of Cincinnati, and the breeding strategy was identical to what was previously reported (14). Genomic DNA was isolated from tail clips, and genotype was determined by PCR (14). Mice were killed by carbon dioxide exposure followed by thoracotomy. Blood was collected by cardiac puncture after carbon dioxide exposure (but before thoracotomy), and various organs were removed and weighed. Tissue samples and serum were preserved at −80°C for mineral analyses and other experiments.

Quantification of intestinal copper absorption (study 1). Mice were housed in stainless steel, overhanging, wire-mesh-bottom cages with ad libitum access to food and water. Newly weaned mice were fed AIN-93G–based diets with adequate copper (AdCu) content and variable iron content (low, adequate, or high). In experiment 1 ("normal"), $Dmt1^{fl/fl}$ mice were fed an adequate-iron (AdFe) diet (50 ppm Fe; TD.130018; Envigo) for 4 wk, and $Dmt1^{int/int}$ mice were fed a high-iron diet [10,000 ppm (carbonyl) Fe; TD.130015; Envigo] for 1 wk followed by the AdFe diet for 3 wk (the experimental design is depicted in **Figure 1**A). This approach was designed to normalize hemoglobin concentrations in the $Dmt1^{int/int}$ mice to control concentrations. In experiment 2 ["iron-deficiency anemia" (IDA)], $Dmt1^{fl/fl}$ mice were fed a low-iron (LFe) diet (~3 ppm Fe; TD.120105; Envigo) and $Dmt1^{int/int}$ mice were fed the AdFe diet for 4 wk. This experimental approach ensured that both groups of mice were iron deficient and anemic. The entire experiment was repeated twice. After the 4-wk dietary interventions, mice were feed-deprived overnight (but given free access to water) and then administered 20 μCi $^{64}CuCl_2$ (394 mCi/μg; Washington University, St. Louis, Missouri) diluted into PBS containing 0.1 N HCl by oral, intragastric gavage (27). Immediately after gavage feeding, mice were fed the AdFe diet and water, and then killed 9 h later. This time point was selected because intestinal transit time is ~11 h in mice (28) and excess copper can be excreted in the bile; thus, only minimal amounts of unabsorbed copper should have been lost in the feces. Moreover, biliary copper excretion should have been minimal because the mice were fed a diet with adequate copper content. In addition, the short half-life of ^{64}Cu (12.7 h) necessitated a more brief experimental period. Whole-carcass, blood, and tissue radioactivity were measured using a WIZARD2 automatic gamma counter (Perkin Elmer). The ^{64}Cu radioactive counts were normalized on the basis of the half-life of ^{64}Cu. Intestinal copper absorption was calculated as follows: radioactivity in the carcass minus radioactivity in the entire gut (esophagus to anus) divided by the amount of radioactivity in the oral gavage solution (×100). Blood and tissue radioactive counts were normalized by volume or weight, respectively.

Copper dietary study (study 2). Two-month-old mice of both sexes and genotypes, housed in standard shoebox cages, were fed either an AdCu diet (~9 ppm Cu) or a high-copper (HCu) diet (~183 ppm Cu) [both with AdFe content (~72 ppm)] for 4 wk (the experimental design is depicted in Figure 1A). Diets were fabricated on the basis of the AIN-93G formulation (Dyets, Inc.; the diet compositions are shown in **Supplemental Table 1**) (29–31). Mice had ad libitum access to food and water. The entire experiment was repeated 3 times. Mice were weighed weekly, and after being killed, blood and tissues were harvested. Serum hemoglobin and hematocrit concentrations were measured by standard methods (6). Serum Cp activity was assessed by a para-phenylenediamine assay, which quantifies the amine oxidase activity of Cp, as previously described (7). All experiments outlined below were performed with the mice described here in this section (i.e., as part of study 2).

Spleen histology. Spleen tissue samples were rinsed in PBS, fixed with 4% (wt:vol) paraformaldehyde, and embedded in paraffin. Sections were cut and then stained with hematoxylin and eosin and analyzed by light microscopy. The proportion of red compared with white pulp was assessed (by a blinded observer) by quantifying the area of periarterial lymphatic sheaths, representing white pulp, and comparing it to total area of whole-organ cross-sections in three 200× fields of view per mouse (32). Images were captured and areas calculated using cellSens Standard 1.11 software.

Quantification of serum and tissue mineral concentrations. Serum and liver, spleen, heart and kidney tissue samples were digested with HNO_3 at 90°C for 3 h. Digested tissues were diluted in MilliQ water before measurement by inductively coupled plasma–MS (NexION 300X; Perkin Elmer) (27). Serum and tissue iron, copper, manganese, and zinc concentrations were normalized by volume or mass, respectively.

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Supplemental Tables 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
Present address for J-HH: Department of Food and Nutrition, Chosun University, Gwangju, Korea.
Present address for CD: Nutrition and Dietetics Department, Namik Kemal University, Tekirdag, Turkey.
Address correspondence to JFC (e-mail: jfcollins@ufl.edu).
Abbreviations used: AdCu, adequate copper; AdFe, adequate iron; Cp, ceruloplasmin; DMT1, divalent metal-ion transporter 1; $Dmt1^{fl/fl}$ mice, phenotypically normal mice with the *Slc11a2* gene "floxed"; $Dmt1^{int/int}$ mice, intestine-specific Dmt1 knockout mice; *Epo*, erythropoietin; HCu, high copper; IDA, iron-deficiency anemia.

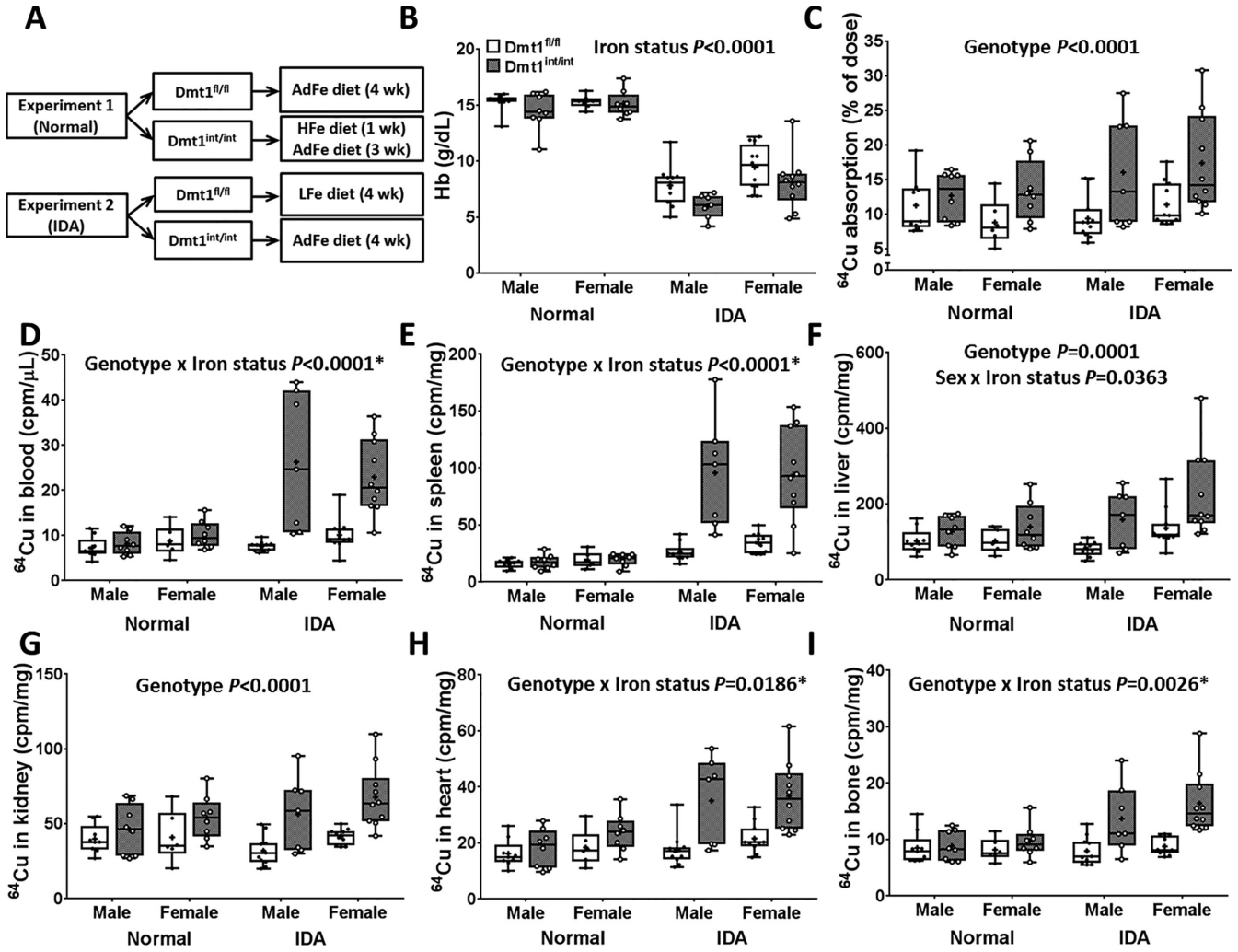

FIGURE 1 Study design (A), blood hemoglobin concentrations (B), quantification of intestinal copper absorption (C), and radioactive counts in blood (D), spleen (E), liver (F), kidney (G), heart (H), and bone (I) of male and female 7-wk-old anemic and nonanemic Dmt1$^{int/int}$ and Dmt1$^{fl/fl}$ mice (study 1). Results are depicted as box plots and represent $n = 9$ (serum hemoglobin concentrations), $n = 8$ (^{64}Cu absorption), $n = 6$ (^{64}Cu radioactivity in blood and spleen), $n = 11$ (in liver and heart), $n = 7$ (in kidney), or $n = 10$ (in bone) mice/group. No significant 3-way interactions were noted. Significant 2-way interactions and some main effects are denoted in each panel. Genotype main effects: $P < 0.0001$ (panels D, E, H, and I).*Slice test results (panels D, E, H, and I): normal, genotype not significant; IDA, genotype $P < 0.0001$. AdFe, adequate iron; cpm, counts per minute; Dmt1, divalent metal-ion transporter 1; Dmt1$^{fl/fl}$ mice, phenotypically normal mice with the *Slc11a2* gene "floxed"; Dmt1$^{int/int}$ mice, intestine-specific Dmt1 knockout mice; Hb, hemoglobin; HFe, high iron; IDA, iron-deficiency anemia; LFe, low iron; Normal, normal hematologic variables.

Renal erythropoietin mRNA expression analysis. Total RNA was isolated from mouse kidney with RNAzol RT reagent (Molecular Research Center, Inc.) following the manufacturer's protocol. RNA concentration was measured with a Nanodrop spectrophotometer, and RNA integrity was assessed by agarose gel electrophoresis. SYBR-Green qRT-PCR was performed according to a well-established protocol (10, 27). The $2^{-\Delta\Delta Ct}$ analysis method was used to calculate fold changes in erythropoietin (Epo) mRNA expression, which was normalized to expression of cyclophilin (which did not vary significantly between samples). Primer sequences are listed in **Supplemental Table 2**.

Statistical analyses. Results are depicted as box plots displaying the minimum, the lower (25th percentile), the median (50th percentile), the upper (75th percentile), and the maximum ranked sample. The mean value is indicated by a "+" sign. Data were analyzed by 2- or 3-factor ANOVA using JMP (version 12.2) software. Levene's test was used to test for equal variance. Some data sets (Figure 1C–I; **Figure 2**B, C, E; **Figure 3**A, C; **Figure 4**A, B, D, E) were transformed as a $\log_{10}$ scale to adjust for unequal variance. Data were then tested again to ensure equal variance before analysis by 3-factor ANOVA. Similarly, for 2-factor ANOVA, some data sets, including kidney/body weight, heart/body weight, and liver, spleen, heart, and renal iron concentrations, were $\log_{10}$ transformed to adjust for unequal variance. Again, data were retested to ensure equal variance before analysis by 2-factor ANOVA. Hemoglobin and hematocrit data, which were not equally distributed even after $\log_{10}$ transformation, were analyzed by a mixed-model test, for which hemoglobin concentrations and hematocrit levels (percentage) were allowed to vary by genotype. Even if data sets were $\log_{10}$ transformed before conducting statistical analyses, the nontransformed data are shown in the figures for ease of interpretation. If significant 2-factor (if 2-factor ANOVA was used) or 3-factor (if 3-factor ANOVA was used) interactions were noted, multiple pairwise comparisons were made by Tukey's post hoc analysis, and differences between individual groups are denoted in the figures and tables. In addition, simple effects analyses were made by slice tests (i.e., multiple-comparison F tests), which compare the levels of one factor for each level of the other factor. In this

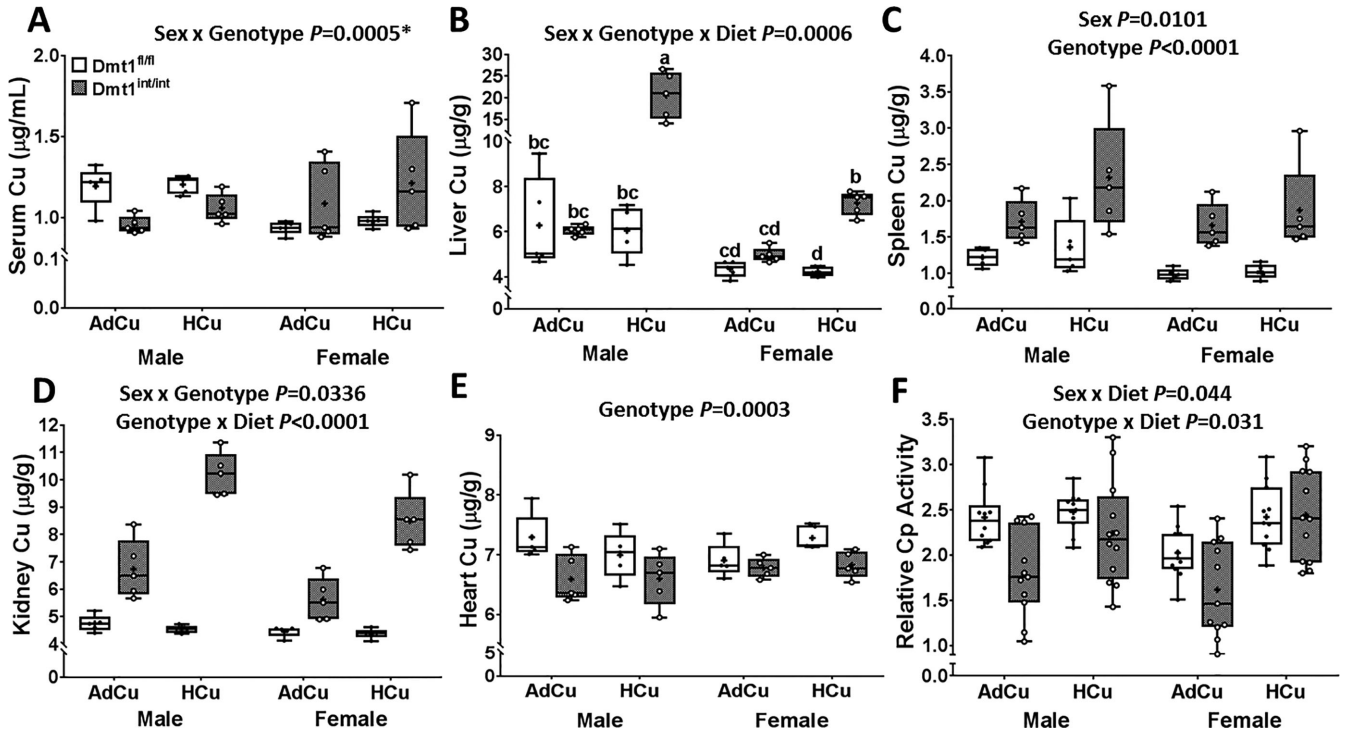

FIGURE 2 Copper content in serum (A), liver (B), spleen (C), kidney (D), and heart (E) and relative serum Cp activity (F) of 2-mo-old mice of both genotypes and sexes fed diets with AdFe and either AdCu or HCu for 4 wk (study 2). Results are depicted as box plots and represent $n = 5$ (A–E) or $n = 10$–12 (F) mice/group. A significant 3-way interaction was noted for liver copper concentrations (B); different letters above whiskers indicate differences between groups. Significant 2-way interactions and/or main effects are also denoted in each panel. Genotype main effects: $P < 0.0001$ (D), $P = 0.0005$ (F). *Slice test results (panel A): female, genotype $P = 0.0095$; male, genotype $P = 0.0093$; wild-type, sex $P = 0.0015$; knockout, sex $P = 0.0489$. AdCu, adequate copper; AdFe, adequate iron; Cp, ceruloplasmin; Dmt1, divalent metal-ion transporter 1; $Dmt1^{fl/fl}$ mice, phenotypically normal mice with the *Slc11a2* gene "floxed"; $Dmt1^{int/int}$ mice, intestine-specific Dmt1 knockout mice; HCu, high copper.

case, this approach allowed us to independently compare the influence of genotype in the normal and IDA groups in the copper-absorption experiments, and the influence of genotype and sex on serum copper concentrations. The use of slice tests is indicated by "*" in Figure 1 (panels D, E, H, and I) and Figure 2A, and individual effect P values are presented in the figure legends. Differences between groups were considered significant at $P < 0.05$. Significant 2-way interactions (when 3-factor ANOVA was used) and main effects (for both 2- and 3-factor ANOVA) are also indicated where appropriate.

Results

Phenotypical characterization of experimental mice. The $Dmt1^{int/int}$ mice were challenging to breed because litter sizes were small (2–4 pups), and only 50% of pups were the correct genotype for use here, making it difficult to obtain enough mice of both sexes for various experiments. The number of mice used for individual experiments thus varied. It was previously reported that male adult $Dmt1^{int/int}$ mice had severe IDA and

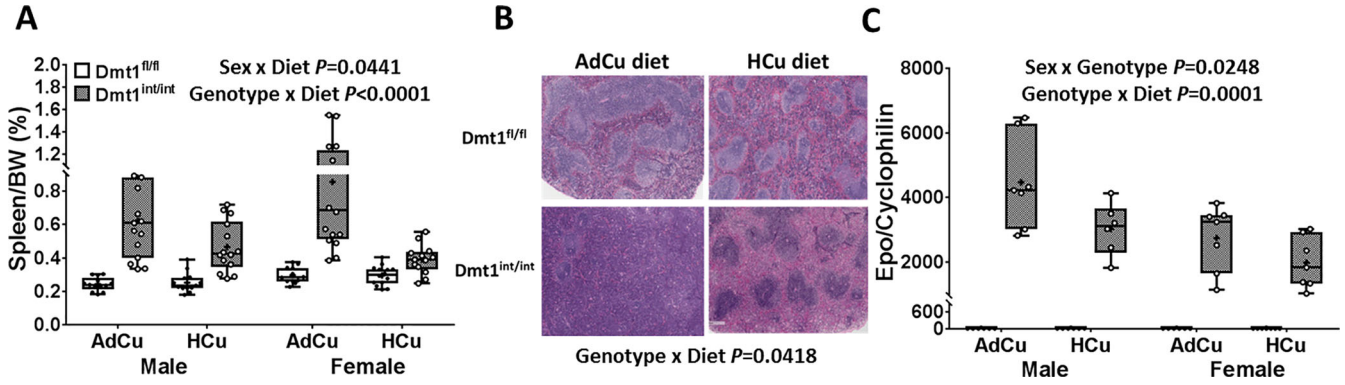

FIGURE 3 Relative spleen weights (A), example images of spleen histology (B), and renal Epo mRNA expression (C) in 2-mo-old mice of both genotypes and sexes fed diets with AdFe and either AdCu or HCu for 4 wk (study 2). Relative proportions of red and white pulp in the spleen were calculated by mean periarterial lymphatic sheath area/total area (B) ($n = 3$ mice/group). The significant 2-way interaction shown (panel B) refers to the relative proportions of white compared with red pulp. Results are depicted as box plots for $n = 12$–16 (A) or $n = 5$–8 (C) mice/group. Genotype main effects: $P < 0.0001$ (panels A and C). AdCu, adequate copper; AdFe, adequate iron; BW, body weight; Dmt1, divalent metal-ion transporter 1; $Dmt1^{fl/fl}$ mice, phenotypically normal mice with the *Slc11a2* gene "floxed"; $Dmt1^{int/int}$ mice, intestine-specific Dmt1 knockout mice; Epo, erythropoietin; HCu, high copper.

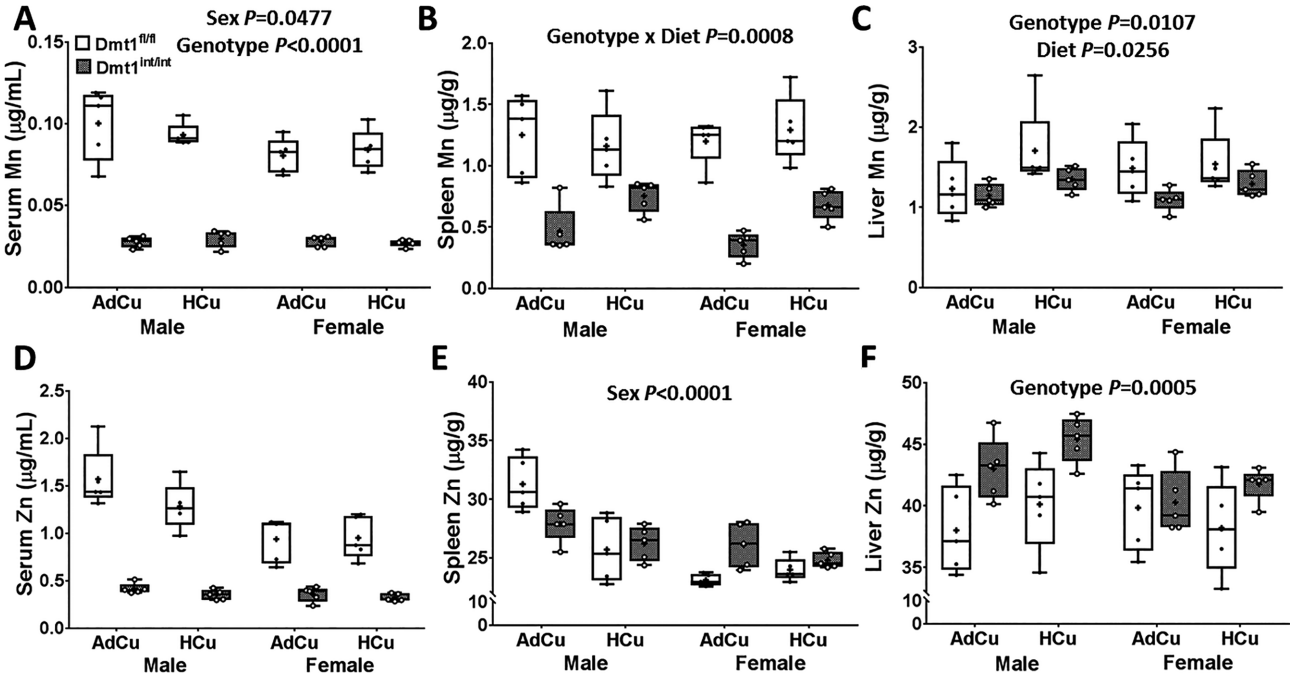

FIGURE 4 Manganese concentrations in serum (A), spleen (B), and liver (C), and zinc concentrations in serum (D), spleen (E), and liver (F) of 2-mo-old mice of both genotypes and sexes fed diets with AdFe and either AdCu or HCu for 4 wk (study 2). Results are depicted as box plots representing $n = 5$ mice/group. No significant 3-way interactions were noted. Significant 2-way interactions and/or main effects are denoted in each panel. Genotype main effects: $P < 0.0001$ (panels A, B, and D). AdCu, adequate copper; AdFe, adequate iron; Dmt1, divalent metal-ion transporter 1; $Dmt1^{fl/fl}$ mice, phenotypically normal mice with the *Slc11a2* gene "floxed"; $Dmt1^{int/int}$ mice, intestine-specific Dmt1 knockout mice; HCu, high copper.

other pathologic perturbations associated with systemic iron depletion (14). Here (as part of study 2), we fed mice of both genotypes and sexes defined diets with AdFe contents in combination with adequate or HCu content for 4 wk, and then assessed various iron-related variables. With adequate iron and copper intake, $Dmt1^{int/int}$ mice of both sexes were smaller, severely anemic, and had smaller livers and larger kidneys and hearts than their control littermates (**Table 1**). Serum iron was also lower in $Dmt1^{int/int}$ mice of both sexes, and liver, spleen, heart, and kidney iron concentrations were also significantly decreased (**Table 2**). These observations confirm the findings reported earlier (14), and show that intestinal DMT1 is important for adequate assimilation of dietary iron in mice. Furthermore, none of these physiologic variables changed when mice were fed an AdFe diet with HCu content (data not shown).

Copper-absorption studies. The dietary feeding regimens, intended to normalize hematologic status between genotypes or to induce IDA in control mice, are shown in Figure 1A. In experiment 1 ("normal"), blood hemoglobin concentrations were similar in all mice and close to normal values, whereas in experiment 2 ("IDA"), all mice were iron deficient and anemic (Figure 1B). Copper absorption and tissue ^{64}Cu accumulation were higher in $Dmt1^{int/int}$ mice in both experiments (Figure 1C–I). The increases in copper absorption and tissue accumulation, however, tended to be more robust in mice with IDA (as indicated by significant genotype × iron status interactions; Figure 1D, E, H, I). In fact, slice tests showed that genotype effects were only significant in the IDA groups (individual *P* values are presented in Figure 1 legend).

TABLE 1 Hematologic variables, BWs, and relative tissue weights of 3-mo-old male and female mice fed an adequate-iron diet with adequate copper content for 4 wk (study 2)[1]

Sex, genotype	Hemoglobin, g/dL	Hematocrit, %	BW, g	Liver/BW, %	Kidney/BW, %	Heart/BW, %
Male, $Dmt1^{fl/fl}$	15.3 ± 1.0	59.5 ± 6.6	29.2 ± 3.7[a]	4.07 ± 0.39	1.26 ± 0.14	0.47 ± 0.08[c]
Male, $Dmt1^{int/int}$	1.5 ± 0.2	7.4 ± 1.7	19.4 ± 1.7[c]	3.41 ± 0.26	1.58 ± 0.12	1.82 ± 0.28[a]
Female, $Dmt1^{fl/fl}$	15.4 ± 0.8	60.4 ± 3.7	22.1 ± 2.3[b]	3.71 ± 0.38	1.04 ± 0.06	0.45 ± 0.03[c]
Female, $Dmt1^{int/int}$	1.8 ± 0.4	10.4 ± 2.9	21.1 ± 1.9[b,c]	3.16 ± 0.27	1.31 ± 0.07	1.16 ± 0.33[b]
Two-way interactions and main effects, *P*						
Sex	0.30	0.10	0.0001	0.0009	<0.0001	<0.0001
Genotype	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	<0.0001
Sex × genotype	0.39	0.36	<0.0001	0.52	0.82	<0.0001

[1] Values are means ± SDs, $n = 13$–16/group. Data were analyzed by 2-factor ANOVA. Labeled means without a common superscript letter differ, $P < 0.05$. None of the physiological variables assessed here changed when mice were fed an adequate-iron diet with high copper content (data not shown). BW, body weight; Dmt1, divalent metal-ion transporter 1; $Dmt1^{fl/fl}$ mice, phenotypically normal mice with the *Slc11a2* gene "floxed"; $Dmt1^{int/int}$ mice, intestine-specific Dmt1 knockout mice.

TABLE 2 Serum and tissue iron concentrations of 3-mo-old male and female mice fed an adequate-iron diet with adequate copper content for 4 wk (study 2)[1]

	Iron				
Sex, genotype	Serum, μg/mL	Liver, μg/g	Spleen, mg/g	Heart, μg/g	Kidney, μg/g
Male, Dmt1$^{fl/fl}$	9.70 ± 2.83	157 ± 24.8^{b}	1.44 ± 0.217	110 ± 11.2	100 ± 8.79^{a}
Male, Dmt1$^{int/int}$	3.80 ± 2.68	22.9 ± 1.59^{c}	0.036 ± 0.007	44.8 ± 3.36	28.9 ± 2.83^{b}
Female, Dmt1$^{fl/fl}$	5.07 ± 1.37	269 ± 57.7^{a}	1.70 ± 0.258	117 ± 11.7	117 ± 10.1^{a}
Female, Dmt1$^{int/int}$	2.31 ± 2.51	21.3 ± 2.46^{c}	0.036 ± 0.008	63.5 ± 35.7	25.9 ± 2.63^{b}
Two-way interactions and main effects, *P*					
Sex	0.0120	0.0023	0.51	0.15	0.58
Genotype	0.0010	<0.0001	<0.0001	<0.0001	<0.0001
Sex × genotype	0.17	0.0002	0.31	0.37	0.0057

[1] Values are means ± SDs, $n = 5$/group. Data were analyzed by 2-factor ANOVA. Labeled means without a common superscript letter differ, $P < 0.05$. None of the physiological variables assessed here changed when mice were fed an adequate-iron diet with high copper content (data not shown). Dmt1, divalent metal-ion transporter 1; Dmt1$^{fl/fl}$ mice, phenotypically normal mice with the *Slc11a2* gene "floxed"; Dmt1$^{int/int}$ mice, intestine-specific Dmt1 knockout mice.

Serum and tissue copper concentrations and serum Cp activity. Our next objective was to assess possible perturbations of copper homeostasis in Dmt1$^{int/int}$ mice fed diets with AdFe and AdCu contents. Serum and tissue copper concentrations were thus assessed, and serum Cp activity was quantified (because Cp is a well-established biomarker of copper status) (33–35). Under these (normal) dietary conditions, serum and hepatic copper concentrations did not vary significantly by genotype (Figure 2A, B). Splenic and renal copper concentrations were, however, higher in Dmt1$^{int/int}$ mice of both sexes (Figure 2C, D), perhaps reflecting increased intestinal copper absorption. Conversely, copper concentrations in the heart were lower in the knockout mice of both sexes (Figure 2E), as was serum Cp activity (Figure 2F). Given the noted cardiac copper depletion and decrements in serum Cp activity, we postulated that increasing copper intake would correct these abnormalities. Mice of both genotypes and sexes were thus fed a HCu diet (with adequate iron) for 4 wk and all of these physiological variables were assessed again. HCu feeding increased serum copper in Dmt1$^{int/int}$ mice of both sexes, with the increase being more robust in females (Figure 2A). In control mice, serum copper concentrations were, however, not affected by HCu intake. Moreover, no diet effect was noted for spleen or heart copper concentrations. Renal and hepatic copper concentrations were further increased by higher copper intake (Figure 2B, D), but no effect was noted in Dmt1$^{fl/fl}$ mice. Last, serum Cp activity, which was reduced in Dmt1$^{int/int}$ mice, was restored close to control values by HCu feeding in males and females. Overall, these data show that the lack of intestinal DMT1 disrupts copper metabolism in mice when dietary copper intake is at normal levels. When dietary copper was elevated, copper homeostasis was partially restored in Dmt1$^{int/int}$ mice, as reflected by increases in serum and liver copper concentrations and serum Cp activity.

Spleen weight and morphology, and renal Epo mRNA expression. With AdCu intakes, Dmt1$^{int/int}$ mice of both sexes had enlarged spleens and increased proportions of red pulp (likely reflecting extramedullary, splenic erythropoiesis) (32) (Figure 3A, B). Renal Epo expression was also strongly induced, reflecting enhanced erythroid demand (Figure 3C). These notable physiologic alterations could relate to iron depletion; however, because copper deficiency also causes splenomegaly (36–38) and an iron-deficiency–like anemia (1, 5, 33, 35), we postulated that copper depletion exacerbated the anemia in Dmt1$^{int/int}$ mice. If true, correcting the copper imbalance could reduce erythropoietic demand. Indeed, increasing dietary copper intake partially corrected the noted splenomegaly and re-established the balance between red and white pulp in both sexes of Dmt1$^{int/int}$ mice (Figure 3A, B). In control mice, HCu intake did not, however, influence spleen size or red-to-white pulp ratios. We also noted that copper supplementation blunted the induction of renal Epo mRNA expression (Figure 3C). Quantification of Epo mRNA levels is a useful proxy for circulating protein concentrations, because the *Epo* gene is transcriptionally upregulated when erythroid demand increases (21). These observations suggested that copper depletion (or impaired copper utilization) contributed to the enhanced erythropoietic demand in the Dmt1$^{int/int}$ mice.

Quantification of serum and tissue manganese and zinc concentrations. Interactions among dietary, essential minerals are common, given their similar physiochemical properties. Moreover, some published reports suggested that DMT1 can transport manganese (20–22) and one early report suggested that DMT1 could also transport zinc (19). Serum and tissue manganese and zinc concentrations were thus assessed in experimental mice. With AdCu intake, serum, spleen, and liver manganese concentrations were lower in Dmt1$^{int/int}$ mice (Figure 4A–C). The manganese concentration in the heart was not, however, different between genotypes (data not shown). Serum zinc concentrations were also dramatically reduced in Dmt1$^{int/int}$ mice (Figure 4D), whereas the splenic zinc concentration was not influenced by genotype (Figure 4E). Hepatic zinc concentrations, conversely, increased in the knockout mice (Figure 4F). Surprisingly, higher dietary copper intake also influenced manganese metabolism. For example, splenic manganese concentrations increased with HCu consumption in Dmt1$^{int/int}$ mice, but not in controls (Figure 4B). Liver manganese concentrations were lower in both sexes of Dmt1$^{int/int}$ mice, and surprisingly, higher copper intake increased hepatic manganese concentrations (Figure 4C). Manganese concentrations in the heart, however, did not change with higher copper intake (data not shown). Collectively, these observations show that lack of intestinal DMT1 impairs manganese and zinc absorption and/or utilization in mice.

Discussion

Copper transport by DMT1 remains enigmatic, because most studies on DMT1 function have been performed in various in

vitro model systems, with more physiologically relevant in vivo experimentation lacking. In the original DMT1 cloning study, copper transport was noted in *Xenopus* oocytes expressing rat DMT1 (19); however, this observation was later refuted (39). Several in vitro studies exemplify copper transport by DMT1 (10, 11, 23, 24, 26, 40, 41). One current study with relevance to this topic utilized Belgrade rats, which harbor an inactivating point mutation in the *Slc11a2* gene (encoding DMT1) (10). Copper transport experiments in isolated duodenal loops showed that copper absorption was significantly higher in iron-deprived control rats, as compared with (naturally) iron-deficient Belgrade rats. The potential physiologic significance of this observation was, however, limited by the ex vivo experimental approach. A more recent investigation addressed this issue using the same mice that were used in the current investigation (14). Before experimentation, the severe anemia in the $Dmt1^{int/int}$ mice was corrected by iron injections, and then copper absorption was assessed by in vivo gavage of ^{64}Cu. It was noted that ^{64}Cu accumulated in blood, duodenal enterocytes, and liver to the same extent in $Dmt1^{int/int}$ and $Dmt1^{fl/fl}$ mice at all time points tested, so these authors concluded that DMT1 is not required for intestinal absorption of copper under physiologic (i.e., normal) conditions.

Here, we sought to extend this previous investigation to include mice of both sexes studied under physiologic conditions and during IDA. This approach was important because iron metabolism is notably different in males and females (43, 44), and moreover, because DMT1 could have different functional properties during iron depletion. Under both circumstances [normal (experiment 1) and IDA (experiment 2)] and in both sexes, intestinal copper (^{64}Cu) absorption was enhanced and copper accumulation in all tissues tested increased. Unexpectedly then, the lack of intestinal DMT1 increases intestinal copper absorption. Because previous studies in laboratory rodents (27, 34) and in humans (45, 46) have clearly established that dietary copper absorption responds to changes in body copper requirements, the most logical interpretation of these findings is that copper absorption was enhanced in response to disruption of copper homeostasis. Supporting this possibility, iron-deficient $Dmt1^{int/int}$ mice fed a diet with AdCu content had reduced serum Cp activity and enlarged spleens (compared with controls). Interestingly, there is ample evidence linking splenomegaly and reductions in serum Cp activity to copper depletion (33–38). Low cardiac copper concentrations were also documented in both sexes of $Dmt1^{int/int}$ mice when copper intake was adequate. Importantly, it was previously reported that depletion of cardiac copper induced production and secretion of a heart-specific, regulatory factor that enhanced intestinal copper absorption (47). This hormonal factor has never been identified, but nonetheless, this could be one trigger for increased intestinal copper transport in $Dmt1^{int/int}$ mice.

Given that $Dmt1^{int/int}$ mice with IDA showed signs of copper depletion with AdCu intakes (despite increased intestinal copper absorption), we hypothesized that increasing systemic copper concentrations would prevent (or reverse) some of the noted physiologic abnormalities. Supporting this postulate, HCu intake restored serum Cp activity close to control values in $Dmt1^{int/int}$ mice. This likely reflects re-establishment of copper homeostasis, because reductions in serum Cp activity typify moderate to severe copper deficiency (1, 5, 48–50). Restoration of serum Cp activity occurred concurrently with significant increases in serum and hepatic copper concentrations in the knockout mice fed the HCu diet. Higher liver copper content was thus necessary to allow normal Cp production (and activity), showing that lack of intestinal DMT1 in the setting of IDA impairs hepatic copper utilization. Moreover, higher dietary copper intake increased splenic copper concentrations and reduced spleen size and decreased the relative proportion of red pulp in $Dmt1^{int/int}$ mice. Increasing dietary copper amounts did not, however, restore cardiac copper concentrations to normal levels in $Dmt1^{int/int}$ mice. This investigation thus did not clarify whether cardiac copper depletion contributed to the noted cardiac hypertrophy, which is important because deficiencies of both iron and copper are associated with this notable physiologic perturbation (5, 33–35). Overall, these experimental observations suggested that knockout of intestinal DMT1 increased copper requirements during IDA, because copper supplementation corrected some of the noted copper-related pathologies. Specificity for copper was established since HCu feeding did not alter serum, hepatic, splenic, or cardiac iron concentrations (data not shown). Moreover, increases in copper absorption in $Dmt1^{int/int}$ mice were insufficient to prevent the development of notable copper-related abnormalities at normal (i.e., adequate) dietary copper intakes. DMT1 must thus be required for optimal intestinal copper transport during IDA.

Also notable were significant decreases in serum manganese and zinc concentrations in $Dmt1^{int/int}$ mice. Previous studies, mentioned above, support possible manganese transport by DMT1, but little evidence of zinc transport by DMT1 has been published to date. Decreases in serum manganese were associated with lower splenic and hepatic manganese concentrations, but manganese concentrations in other tissues were not remarkably different when comparing genotypes. Interestingly, increasing dietary copper intake was associated with increases in hepatic manganese concentrations. To our knowledge, such an interaction between copper and manganese has not been previously reported. Furthermore, hepatic zinc concentrations were elevated in $Dmt1^{int/int}$ mice. Lack of intestinal DMT1, or the severe IDA associated with loss of DMT1, thus disrupts manganese and zinc homeostasis in mice. Because the focus of this investigation is on alterations of copper metabolism in $Dmt1^{int/int}$ mice, assessment of the functional consequences of altered manganese and zinc metabolism must await future experimentation. Importantly, however, manganese and zinc are not known to directly relate to any of the copper- (or iron-) related physiologic variables assessed in the current study.

In summary, many of the pathologies noted in $DMT1^{int/int}$ mice, including decrements in serum and tissue iron concentrations, blood hemoglobin and hematocrit concentrations, body weight, and liver mass, as well as increases in heart mass, typify murine iron deficiency. Lack of intestinal DMT1 thus does not appear to have any particular iron-related influence that differs from iron deficiency more generally. What is, however, notable are disruptions of copper homeostasis in iron-deficient $Dmt1^{int/int}$ mice when dietary copper intake was at a typically adequate amount. Increasing dietary copper consumption corrected the noted copper deficiency, without altering serum or tissue iron concentrations, proving that copper depletion was the underlying cause of these physiologic perturbations. Ablation of intestinal DMT1 thus increases copper requirements during IDA. Collectively, these observations show that intestinal DMT1 is essential for optimal assimilation of dietary copper and normal systemic copper homeostasis.

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the technical assistance of SRLF, J-HH, CD, RRW, PX, and AG; XW: analyzed data and prepared figures; XW, AG, SV, and JFC: interpreted data and drafted the manuscript; and all authors: read and approved the final manuscript.

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