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## Divalent metal-ion transporter DMT1 mediates both H<sup>+</sup>-coupled Fe<sup>2+</sup> transport and uncoupled fluxes

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**Abstract** The H<sup>+</sup>-coupled divalent metal-ion transporter DMT1 serves as both the primary entry point for iron into the body (intestinal brush-border uptake) and the route by which transferrin-associated iron is mobilized from endosomes to cytosol in erythroid precursors and other cells. Elucidating the molecular mechanisms of DMT1 will therefore increase our understanding of iron metabolism and the etiology of iron overload disorders. We expressed wild type and mutant DMT1 in *Xenopus* oocytes and monitored metal-ion uptake, currents and intracellular pH. DMT1 was activated in the presence of an inwardly directed H<sup>+</sup> electrochemical gradient. At low extracellular pH (pH<sub>o</sub>), H<sup>+</sup> binding preceded binding of Fe<sup>2+</sup> and its simultaneous translocation. However, DMT1 did not behave like a typical ion-coupled transporter at higher pH<sub>o</sub>, and at pH<sub>o</sub> 7.4 we observed Fe<sup>2+</sup> transport that was not associated with H<sup>+</sup> influx. His<sup>272</sup> → Ala substitution uncoupled the

Fe<sup>2+</sup> and H<sup>+</sup> fluxes. At low pH<sub>o</sub>, H272A mediated H<sup>+</sup> uniport that was inhibited by Fe<sup>2+</sup>. Meanwhile H272A-mediated Fe<sup>2+</sup> transport was independent of pH<sub>o</sub>. Our data indicate (i) that H<sup>+</sup> coupling in DMT1 serves to increase affinity for Fe<sup>2+</sup> and provide a thermodynamic driving force for Fe<sup>2+</sup> transport and (ii) that His-272 is critical in transducing the effects of H<sup>+</sup> coupling. Notably, our data also indicate that DMT1 can mediate facilitative Fe<sup>2+</sup> transport in the absence of a H<sup>+</sup> gradient. Since plasma membrane expression of DMT1 is upregulated in liver of hemochromatosis patients, this H<sup>+</sup>-uncoupled facilitative Fe<sup>2+</sup> transport via DMT1 can account for the uptake of nontransferrin-bound plasma iron characteristic of iron overload disorders.

**Keywords** Iron transport · Metal-ion transport · Proton-coupled transport · Cotransporters · Zinc transport · Oocyte · *Xenopus laevis* · Iron overload

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### Introduction

The DMT1 is a widely expressed, mammalian ferrous-ion (Fe<sup>2+</sup>) transporter that is energized by the H<sup>+</sup> electrochemical potential gradient [19, 33]. Its importance as a principal mechanism of intestinal Fe<sup>2+</sup> absorption and erythroid iron utilization is highlighted by the severe microcytic anemia characteristic of the *mk* mouse and Belgrade (*b*) rat, inbred rodent strains that bear an identical (G185R) mutation in DMT1 [12, 13, 16]. Analyses of DMT1 mRNA and protein distribution, as well as metal-ion transport assays in isolated cells, cell lines, or in vivo, suggest that DMT1 mediates not only apical iron uptake in the intestine and kidney [4, 5, 53, 56], but also the recovery of iron from recycling endosomes during transferrin receptor (TfR)-associated cellular uptake in erythroid precursor cells and most other cell types [5, 12, 15, 45].

In the small intestine, DMT1 mRNA and protein are expressed in enterocytes throughout the small intestine,

most strongly in the proximal duodenum, where the expression of DMT1 is tightly regulated by body iron status [5, 19, 23]. The acidic microclimate of the intestinal brush border [37] is thought to drive  $H^+$ -coupled  $Fe^{2+}$  uptake via DMT1 into enterocytes. In erythroid cells, DMT1 colocalizes with transferrin and TfR in recycling endosomes [6, 18, 50, 52]. Acidification of the endosomal lumen by the V-type  $H^+$ -ATPase [52] permits the dissociation of  $Fe^{3+}$  from transferrin, accelerates its reduction to  $Fe^{2+}$  [41], and provides the  $H^+$  gradient to energize DMT1-mediated  $Fe^{2+}$  transport from endosome to cytosol.

When expressed in *Xenopus* oocytes, rat DMT1 exhibited moderately high apparent affinity for  $Fe^{2+}$  and several other transition metal ions [19]. The  $Fe^{2+}$  transport was voltage-dependent and  $H^+$ -coupled [19]. Cotransport with  $H^+$  was also demonstrated in the intestinal Caco-2 cell line [51], which is known to express DMT1 [2, 51]. In the present study—using the two-microelectrode voltage clamp, radiotracer uptake assays, and intracellular pH-sensing microelectrodes—we have performed a kinetic analysis of DMT1 and present a model that describes  $H^+$  and  $Fe^{2+}$  transport mediated by DMT1. Analysis of the impact of mutations at two histidyl residues residing within transmembrane region 6 (TM6) both supports our model for DMT1 and constitutes an important step in structure-function analysis of this transport protein. Elucidating the molecular mechanisms of DMT1 will lead to a better understanding of the contribution of DMT1 to iron metabolism and the etiology of iron overload disorders.

## Methods

### Site-directed mutagenesis of rat DMT1

Rat wild type DMT1 (wtDMT1) is the product of the *Slc11a2* gene (and is also known as DCT1 or Nramp2). The wtDMT1 cDNA sequence [19] was excised from pSPORT1 between *SalI* (in the multiple cloning region) and *EcoRI* (at base pair 2111 of the DMT1 cDNA sequence), then subcloned into pBluescript II KS(+) to generate a construct (pBSKmrDMT1) for site-directed mutagenesis. Mutants were generated by PCR amplification of wtDMT1 using sense primers (Table 1)

designed to introduce single amino-acid substitutions at His-267 and His-272. The sense primers also spanned a unique, native *BclI* restriction site (T|GATCA) at base pair 892 of the DMT1 cDNA. Included in each PCR reaction was an antisense primer (5'-ATAGCA GCATGCTATTTGACAAAGACAG-3') identical to wtDMT1 cDNA and which contained a unique, native *SphI* restriction site (GCATG|C, *underlined*) at base pair 1988 of the DMT1 cDNA. The PCR products were gel-purified, double-digested with *BclI* and *SphI*, and ligated into pBSKmrDMT1 between the *BclI* and *SphI* sites. Competent DH5 $\alpha$  cells (Invitrogen) were electrotransformed with the mutant plasmids and selected on LB/agar plates containing 100–200  $\mu\text{g}\mu\text{l}^{-1}$  ampicillin. To restore the 3'-UTR and poly(A) tail and maximize expression in oocytes, the mutated DMT1 sequences were subcloned back into pSPORT1-DMT1. To do so, pSPORT1-DMT1 was first digested with *SmaI* and *Acc65I*, Klenow end-filled and re-ligated, thus removing the *SmaI*-to-*KpnI* fragment (containing an *EcoRI* site) of the multiple cloning region. A clone with only a single *EcoRI* restriction site (within the DMT1 3'-UTR) was selected. Meanwhile, mutant DMT1 sequences were excised from pBSKmrDMT1 between *SalI* and *EcoRI* of the multiple cloning region and ligated into the modified pSPORT1-DMT1 between the *SalI* and *EcoRI* sites (swapping out the wild type sequence). The DH5 $\alpha$  cells were electrotransformed with the modified pSPORT1 containing mutant DMT1 sequences and selected on LB/agar-ampicillin plates as before. Mutations at His-267 and His-272 were verified by DNA sequencing of the final constructs (at the Sequencing Facility of Beth Israel Deaconess Medical Center, Boston, MA, USA) using the sense primer 5'-ATC CTGTTTCAGGCTGCCA-CACCCC-3' (base pairs 833–856 of the DMT1 sequence) and the antisense primer 5'-GGTCA GCATGGGGGCTGCTGC-3' (base pairs 1134–1114).

### Expression of wild type and mutant rat DMT1 in *Xenopus* oocytes

The pSPORT1 vector containing wild type or mutant rat DMT1 under the T7 promoter was linearized with *NotI*. The cRNA was synthesized in vitro with the use of the mMESSAGE mMACHINE kit (Ambion) with T7 RNA

**Table 1** Oligonucleotide sense primers used for site-directed mutagenesis of rat DMT1

Mutation	Oligonucleotide primer sequence
H267A	5'-GAGCTG TGATCATGCCAGCTAACATGTACCTGCACTCTGCC-3'
H267D	5'-GGGAGCTG TGATCATGCCAGATAACATGTACCTGCACTCTGCC-3'
H267N	5'-GGGAGCTG TGATCATGCCAAACAACATGTACCTGCACTCTGCC-3'
H272A	5'-GAGCTG TGATCATGCCACACAACATGTACCTGGCTTCTGCC-3'
H272R	5'-GGGAGCTG TGATCATGCCACACAACATGTACCTGAGATCTGCCTTAGTC-3'

Sense primers flanked a unique, native *BclI* restriction site (T|GATCA, *underlined*) in the wtDMT1 cDNA sequence and the mutated triplet is shown in boldface. The antisense primer used in each PCR reaction was identical to a fragment of the wtDMT1 nucleotide sequence and contained a unique, native *SphI* restriction site (see text)

polymerase. We performed laparotomy and ovariectomy on adult female *Xenopus laevis* frogs under 2-aminoethylbenzoate anesthesia (0.1% in 1:1 water/ice, by immersion), in compliance with the Harvard Medical Area Standing Committee on Animals. Ovarian tissue was isolated and treated with collagenase A (Roche Diagnostics), and oocytes were isolated and stored at 18°C in modified Barths' medium [32]. Oocytes were injected with ≈50 ng of cRNA and incubated 3–5 days before functional assays were performed.

#### Media used for transport assays in oocytes

Functional assays in control oocytes and oocytes expressing wild type or mutant rat DMT1 were performed using low-calcium transport media containing L-ascorbic acid (to maintain the iron in its reduced form,  $\text{Fe}^{2+}$ ) and buffered using either (i) MES, HEPES and Tris base, or (ii) MES and piperazine-1,4-bis(2-propanesulfonic acid) (PIPES) as indicated. PIPES, obtained from GFS Chemicals, is a non-complexing buffer with  $pK_{a2}^m \approx 8.0$  [60], whereas the commonly used buffers Tris and HEPES are known to complex metal ions [1, 11, 60].

Transport media comprised 100 mM NaCl, 1 mM KCl, 0.6 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  L-ascorbic acid (or 1 mM L-ascorbic acid for radiotracer experiments), 0–5 mM MES, and either (i) 0–5 mM PIPES and 0–6 mM NaOH, or (ii) 0–5 mM HEPES and 0–5 mM Tris base. To prepare transport media in the pH range 5.2–7.0, a low-pH medium buffered with 5 mM MES was mixed with appropriate volumes of pH 7.5 media containing either (i) 5 mM PIPES, adjusted to pH 7.5 with NaOH, or (ii) 5 mM HEPES, adjusted to pH 7.5 with Tris base. All experiments were performed at ambient temperature (21–24°C) except where noted (Figs. 1, 6g).

#### Voltage-clamp experiments

A two-microelectrode voltage clamp (Dagan CA-1B) was used to measure currents associated with wild type or mutant DMT1 in oocytes. Microelectrodes (resistance 0.5–5 M $\Omega$ ) were filled with 3 M KCl. Voltage-clamp experiments comprised four protocols: (i) Continuous current recordings were made at holding potentials ( $V_h$ ) of –50 mV, or –70 mV, low-pass filtered at 1 Hz, and digitized at 10 Hz (except for  $\text{pH}_i$  experiments, see below). (ii) Oocytes were clamped at  $V_h = -50$  mV, and step-changes in membrane potential ( $V_m$ ) were applied from +50 to –150 mV (in 20 mV increments) each for a duration of 200 ms, before and after the addition of  $\text{Fe}^{2+}$ . Current was low-pass filtered at 500 Hz and digitized at 5 kHz. Steady-state data were obtained by averaging the points over the final 16.7 ms at each  $V_m$  step. (iii) Presteady-state currents were obtained at 23 or 28°C using protocol ii modified such that step-changes

were applied from +90 to –130 mV. (iv) Oocytes were clamped at  $V_h = -50$  mV, stepped to –150 mV for 13.6 ms (to allow for settling of the capacitive transient currents), and a 1 s ramp applied from –150 to +50 mV. Current was low-pass filtered at 500 Hz and digitized at 5 kHz. Steady-state data from protocols i or ii were fit to a modified 3-parameter Hill relationship (Eq. 1) for which  $I$  is the evoked current,  $I_{\max}$  the derived current maximum,  $S$  the concentration of substrate  $S$  ( $\text{H}^+$  or metal ion),  $K_{0.5}^S$  the substrate concentration at which current was half-maximal, and  $n_H$  the Hill coefficient for  $S$ .

$$I = \frac{I_{\max} S^{n_H}}{(K_{0.5}^S)^{n_H} + S^{n_H}} \quad (1)$$

To account for the putative  $\text{H}^+$ -uncoupled, facilitative  $\text{Fe}^{2+}$  transport activity ( $i^U$ ), two alternative functions were tested. In the first, a 4-parameter Hill function (Eq. 2),  $i^U$  is a static term (i.e. it does not vary with  $[\text{H}^+]_o$ ) describing the  $y$ -intercept, the  $\text{Fe}^{2+}$ -evoked current at nominally zero  $\text{H}^+$  concentration. In the second alternative function (Eq. 3),  $i^U$  is not fixed, since increasing  $[\text{H}^+]_o$  is assumed to inhibit  $i^U$  in favor of  $\text{H}^+/\text{Fe}^{2+}$  cotransport. We modified  $i^U$  by an exponential decay, the most empirical way of expressing inhibition by  $\text{H}^+$ . The  $[\text{H}^+]_o$  at which  $i^U$  is inhibited 50% is expressed as  $\ln(0.5)/-b$ .

$$I = i^U + \frac{I_{\max} S^{n_H}}{(K_{0.5}^S)^{n_H} + S^{n_H}} \quad (2)$$

$$I = i^U \exp^{-bS} + \frac{I_{\max} S^{n_H}}{(K_{0.5}^S)^{n_H} + S^{n_H}} \quad (3)$$

Currents obtained over the temperature range 18–31°C (Fig. 6g) were fit with an integrated Arrhenius function (Eq. 4), for which  $E_a$  is the Arrhenius activation energy,  $\ln A$  the  $y$ -intercept,  $R$  the universal gas constant (1.987 cal mol $^{-1}$  K),  $T$  the absolute temperature, and  $I$  the current induced by switching from pH 7.5 to 5.7 ( $I^{\Delta \text{pH}}$ ) or the 50  $\mu\text{M}$   $\text{Fe}^{2+}$ -evoked current at pH 5.7 ( $I^{\text{Fe}}$ ).

$$\ln(-I) = \ln A - \frac{E_a}{RT} \quad (4)$$

Following step-changes in  $V_m$  using protocol iii, we obtained presteady-state currents in oocytes expressing wild type or mutant DMT1. These were isolated from capacitive transient currents (which decayed with half times of 0.5–0.8 ms) and steady-state currents by the fitted method [21, 32]. Briefly, the first ten points after reaching maximal decay rate were fit using an exponential decay to describe the capacitive transients; these and the final steady-state currents were subtracted to obtain the compensated currents. The compensated currents were integrated with time to obtain charge movement ( $Q$ ) and fit using the Boltzmann relationship (Eq. 5) for which maximal charge  $Q_{\max} = Q_{\text{dep}} - Q_{\text{hyp}}$

(where  $Q_{\text{dep}}$  and  $Q_{\text{hyp}}$  represent the charge at depolarizing and hyperpolarizing limits),  $V_{0.5}$  is the  $V_m$  at the midpoint of charge transfer,  $z$  is the apparent valence of the movable charge, and  $F$ ,  $R$ , and  $T$  have their usual thermodynamic meanings.

$$\frac{Q - Q_{\text{hyp}}}{Q_{\text{max}}} = \frac{1}{1 + \exp(z[V_m - V_{0.5}]F/RT)} \quad (5)$$

Transporter-mediated presteady-state currents can be used to estimate transporter density [61]. We estimated the number of functional wild type or mutant DMT1 transporters ( $N_T$ ) per oocyte using Eq. 6, in which  $e$  is the elementary charge ( $1.6 \times 10^{-19}$  C).

$$N_T = \frac{Q_{\text{max}}}{ze} \quad (6)$$

### Radiotracer iron and zinc uptake

We obtained radiochemicals from Perkin-Elmer Life Science Products. The  $^{55}\text{Fe}^{2+}$  was used at specific activity 658 MBq  $\text{mg}^{-1}$  and  $^{65}\text{Zn}^{2+}$  at specific activity 110 MBq  $\text{mg}^{-1}$ . Radiotracer uptake was measured over 10, 20 or 30 min, with up to 15 oocytes in 2 ml transport medium. At the end of the uptake period, oocytes were rinsed twice with ice-cold pH 5.5 medium containing 500  $\mu\text{M}$  unlabeled  $\text{Fe}^{2+}$  and 1 mM L-ascorbic acid, then solubilized with 5% SDS before  $^{55}\text{Fe}$  or  $^{65}\text{Zn}$  content was assayed by liquid scintillation counting. Saturation kinetics of  $^{55}\text{Fe}^{2+}$  and  $^{65}\text{Zn}^{2+}$  uptake were determined using a modified Eq. 1, in which  $I$  was replaced with the radiotracer uptake velocity,  $V$ .

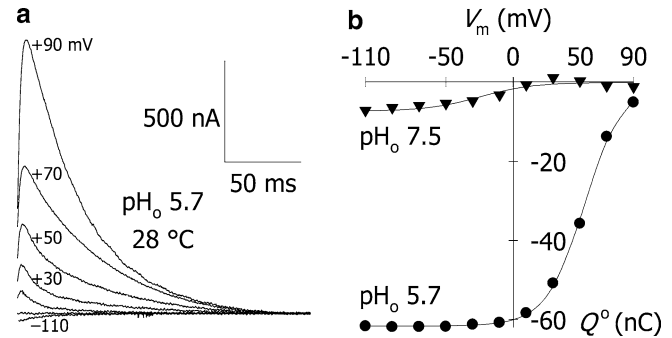
### Intracellular pH recordings

Intracellular pH ( $\text{pH}_i$ ) was measured under voltage clamp using ion-selective microelectrodes as described [3, 10]. Briefly, we used silanized borosilicate micropipettes backfilled with phosphate buffer at pH 7.0, and tips filled with hydrogen ionophore I-cocktail B (Fluka). Electrodes with response  $> -55$  mV/pH unit were selected. The signal from the  $\text{pH}_i$  electrode was subtracted from the output of the voltage clamp amplifier to yield the responses specifically due to pH. The  $\text{pH}_i$  and  $I_m$  signals were digitized at 0.5 Hz and filtered at 0.05 Hz.

## Results

### Presteady-state currents associated with wtDMT1 expression in oocytes

Following step changes in membrane potential ( $V_m$ ) in the *absence* of metal ion, we observed presteady-state currents in oocytes expressing wtDMT1. We did not observe these currents in the *presence* of  $\text{Fe}^{2+}$ , nor in



**Fig. 1** Presteady-state currents associated with the expression of rat wild type DMT1 (wtDMT1) in *Xenopus* oocytes. **a** Compensated records (from 10 ms after step-changes in  $V_m$  from  $-50$  mV to between  $-110$  mV and  $+90$  mV were applied) for one oocyte expressing wtDMT1 at  $\text{pH}_o$  5.7 and  $28^\circ\text{C}$ . For clarity, we show only records at  $-110$ ,  $-50$ ,  $+10$ ,  $+30$ ,  $+50$ ,  $+70$  and  $+90$  mV, omitting records at  $-90$ ,  $-70$ ,  $-30$ , and  $-10$  mV. **b** Presteady-state currents were integrated with time to obtain charge,  $Q$ . The  $Q/V_m$  relationship at  $\text{pH}_o$  5.7 was fit by a single Boltzmann relationship (Eq. 5) with  $Q_{\text{max}}$   $61.7 \pm 1.2$  nC,  $V_{0.5}$   $+53.5 \pm 0.9$  mV, and  $z$   $-1.8 \pm 0.1$  ( $r^2 = 0.975$ ). Data obtained at  $\text{pH}_o$  7.5 fit a Boltzmann function with  $Q_{\text{max}}$   $7.3 \pm 0.8$  nC,  $V_{0.5}$   $-24.5 \pm 8.5$  mV, and  $z$   $-1.3 \pm 0.5$  ( $r^2 = 0.759$ ). For display, data for  $Q$  were adjusted to  $Q^\circ$  by offsetting to zero the depolarizing limits of  $Q$  ( $Q_{\text{dep}}$ ) for each Boltzmann fit ( $Q_{\text{dep}}$  for  $\text{pH}_o$  5.7 was  $+61.9 \pm 1.2$  nC, and for  $\text{pH}_o$  7.5,  $+5.3 \pm 0.6$  nC).

control oocytes. Presteady-state currents were isolated from capacitive transients and steady-state currents (as described in Methods), and are illustrated for a single oocyte superfused at  $\text{pH}_o$  5.7, at  $28^\circ\text{C}$  (Fig. 1a). Presteady-state currents at  $\text{pH}_o$  5.7 decayed with time constants ( $\tau$ ) of 9–31 ms, when fit with a single exponential decay. The relationship of  $\tau$  to  $V_m$  fit a bell-shaped curve with a peak ( $\tau_{\text{max}}$ ) of  $32.3 \pm 0.4$  ms at  $V_{\tau_{\text{max}}}$  of  $+61.1 \pm 0.7$  mV (*not shown*). Presteady-state currents were integrated with time to obtain charge ( $Q$ ). The relationship of  $Q$  to  $V_m$  at  $\text{pH}_o$  5.7 could be described by a single Boltzmann function (Fig. 1b) with maximal charge movement ( $Q_{\text{max}}$ ) of 62 nC, apparent valence ( $z$ ) of  $\approx -2$ , and midpoint of charge transfer ( $V_{0.5}$ ) of  $+54$  mV, close to the value obtained for  $V_{\tau_{\text{max}}}$ . Charge movements were conserved for the onset and offset of step changes in  $V_m$  (*not shown*). We observed significantly less charge movement at  $\text{pH}_o$  7.5 ( $Q_{\text{max}}$  was 12 nC) and  $V_{0.5}$  was shifted to  $-25$  mV (Fig. 1b).

With the aid of computer simulation, presteady-state currents observed for other  $\text{H}^+$ -coupled transporters have been attributed to two steps within the transport cycle, namely (i) reorientation of the empty, charged transporter within the membrane plane and (ii) binding/dissociation of  $\text{H}^+$  within the membrane electric field (“ion-well”) [28, 34]. Similar observations have been made in modeling several  $\text{Na}^+$ -coupled transporters also [21, 30, 35, 55]. The dependence of both  $Q_{\text{max}}$  and  $V_{0.5}$  upon  $\text{pH}_o$  suggests that both transporter reorientation and ion-well binding of  $\text{H}^+$  contribute to the overall presteady-state charge observed for DMT1. If



$H^+$  binding were the only transition contributing charge, we would expect that  $pH_o$  alter  $V_{0.5}$  without effect on  $Q_{max}$  (see Ref. [28] for justification). That  $H^+$  may bind to DMT1 in the absence of metal-ion substrate is consistent with observations of a  $H^+$  “leak” (uniport) in the absence of metal ion (see below and Figs. 4, 6) [19, 59].

#### Iron transport mediated by DMT1: complexation in certain buffers

The uptake of  $2 \mu M$   $^{55}Fe^{2+}$  in medium buffered at pH 5.5 using MES and piperazine-1,4-bis(2-propane-sulfonic acid) (PIPES) was stimulated over 1500-fold in *Xenopus* oocytes expressing wtDMT1 compared with control oocytes (Fig. 2a). Since Tris is known to complex certain metal ions (most notably Cu) [1, 11], we

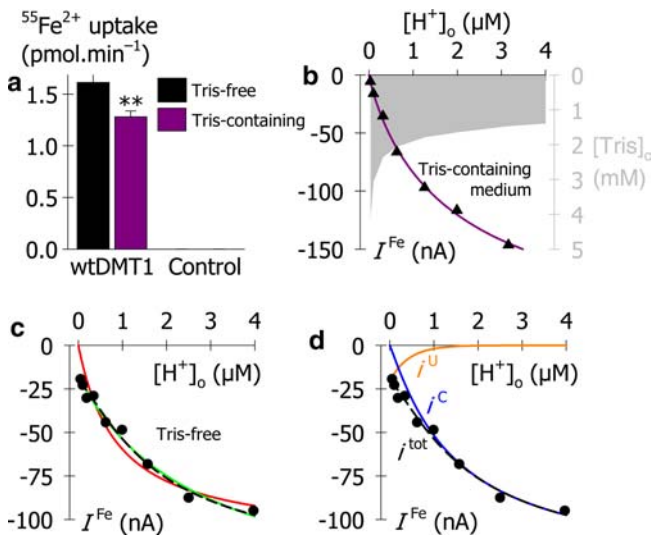
assessed its impact in our experimental system by comparing  $^{55}Fe^{2+}$  uptake in MES/PIPES-buffered and MES/HEPES/Tris-buffered media. Uptake of  $2 \mu M$   $^{55}Fe^{2+}$  in oocytes expressing wtDMT1 was 20% lower in Tris-containing medium at pH 5.5 (Fig. 2a), presumably as a result of complexation of  $Fe^{2+}$ . The HEPES (one of several Good’s buffers containing hydroxyalkyl or secondary amine groups) also may complex metal ions [1, 60].

#### pH-dependence of the $Fe^{2+}$ -evoked currents for DMT1

Since we expected Tris-Fe complexation to be more pronounced at higher pH (as a result of the higher concentration of Tris base; Fig. 2b), we compared the pH-dependence of the  $Fe^{2+}$ -evoked currents in media with and without Tris buffer. The currents evoked by  $10 \mu M$   $Fe^{2+}$  at  $-70$  mV in the Tris-containing medium appeared to be strictly dependent upon  $[H^+]_o$  (Fig. 2b). The data satisfied a conventional 3-parameter Hill function (Eq. 1) with  $K_{0.5}^H$   $2.0 \pm 0.8 \mu M$  and Hill coefficient for  $H^+$ ,  $n_H^H \approx 1$ . In contrast, the currents evoked by  $10 \mu M$   $Fe^{2+}$  in the Tris-free medium displayed an incomplete dependence upon  $[H^+]_o$  (Fig. 2c). Although, the  $Fe^{2+}$ -evoked current was stimulated at lower  $pH_o$ , significant current remained at neutral  $pH_o$  and higher. For example, the  $Fe^{2+}$ -evoked current at  $pH_o$  7 ( $-20$  nA) was 21% that at  $pH_o$  5.2 ( $-95$  nA). This raised the possibility that DMT1 can also mediate  $Fe^{2+}$  transport that is independent of, or uncoupled from,  $H^+$ , and our subsequent data support this preliminary conclusion.

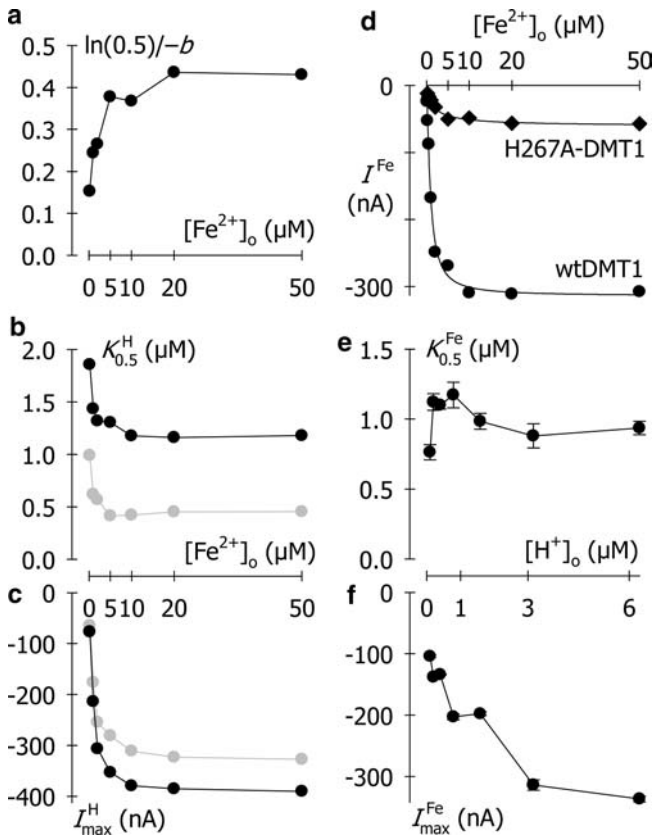
Why might not the  $H^+$ -independent component of the  $Fe^{2+}$ -evoked currents have been apparent when using Tris-containing medium? Presumably this resulted from significant complexation of  $Fe^{2+}$  with the higher Tris concentrations necessary to obtain higher  $pH_o$  (Fig. 2b). Therefore, we suggest that Tris should be avoided if possible in DMT1 metal-ion transport experiments, and certainly when examining pH-dependence. Yu et al. [60] identified a series of noncomplexing tertiary amine compounds, including the Good’s buffer MES as well as PIPES (which is now commercially available) that do not complex metal ions. In our subsequent experiments,  $Fe^{2+}$  was always presented in Tris-free, MES/PIPES-buffered media.

The  $H^+$ -dependence data obtained using Tris-free, MES/PIPES-buffered media could not satisfy a 3-parameter Hill function (Eq. 1) except by constraining the Hill coefficient for  $H^+$  ( $n_H^H$ ) at 1, yielding  $K_{0.5}^H$  of  $0.9 \mu M$  and  $I_{max}^H$  of  $-113$  nA ( $r^2=0.90$ ) (Fig. 2c, red line). (In initial fits to Eq. 2 or 3,  $n_H^H$  was  $\approx 1$ . Subsequent fits were standardized by constraining  $n_H^H$  at 1.) To account for the  $Fe^{2+}$ -evoked currents persisting at neutral  $pH_o$  or above, we first added a static term, the  $H^+$ -uncoupled current ( $i^U$ ), to form a 4-parameter Hill function (Eq. 2) that better satisfied the  $H^+$ -dependence



**Fig. 2** Effect of buffer composition on iron uptake and  $H^+$  saturation kinetics in oocytes expressing wtDMT1. **a** Uptake of  $2 \mu M$   $^{55}Fe$  was measured over 30 min at pH 5.5 and  $21^\circ C$  in control oocytes and oocytes expressing wtDMT1, media buffered using MES and piperazine-1,4-bis(2-propanesulfonic acid) (PIPES) (black bars) or MES, HEPES, and Tris (purple bars). Data are mean  $\pm$  SEM for 7–15 oocytes in each group.  $^{**}P < 0.01$ , significantly different from uptake in MES/PIPES buffer. **b, c**  $H^+$  saturation kinetics for wtDMT1 determined from currents evoked by  $10 \mu M$   $Fe^{2+}$  at  $-70$  mV, measured in separate wtDMT1-expressing oocytes in the MES/HEPES/Tris buffer system (**b**) or in MES/PIPES (**c**). Data in **b** were fit with a 3-parameter Hill function (Eq. 1) with  $K_{0.5}^H$   $2.0 \pm 0.8 \mu M$ ,  $I_{max}^H$   $-243 \pm 40$  nA, and  $n_H^H$   $0.9 \pm 0.1$  ( $r^2=0.998$ ). The concentration of Tris as a function of  $H^+$  concentration is indicated by the gray shading (right y-axis). Data in **c** were fit with a 3-parameter Hill function (Eq. 1, red line), a 4-parameter Hill function (Eq. 2, green line), and with a function (Eq. 4) combining an exponential decay and a 3-parameter Hill (black dashed line, see **d**). **d** The fit generated by the combined function (Eq. 4, black dashed line,  $i^{tot}$ ) is separated to display the exponential decay (orange line) describing the currents ( $i^U$ ) arising from  $H^+$ -uncoupled, facilitative  $Fe^{2+}$  transport, and the compensated Hill function (blue line) representing only  $H^+/Fe^{2+}$  cotransport currents ( $i^C$ ). See text for details of derived parameters and fits

data ( $r^2=0.98$ ) and predicted that  $H^+$ -coupled  $Fe^{2+}$  transport proceed with  $K_{0.5}^H$  of  $2.7 \mu M$  and maximal current the sum of  $I_{max}^H$  ( $-136$  nA) and the  $H^+$ -uncoupled component  $i^U$  ( $-17$  nA) (Fig. 2c, green line). However, we expected that increasing  $[H^+]_o$  would accelerate  $H^+/Fe^{2+}$  cotransport at the expense of  $H^+$ -uncoupled  $Fe^{2+}$  transport. We attempted to describe

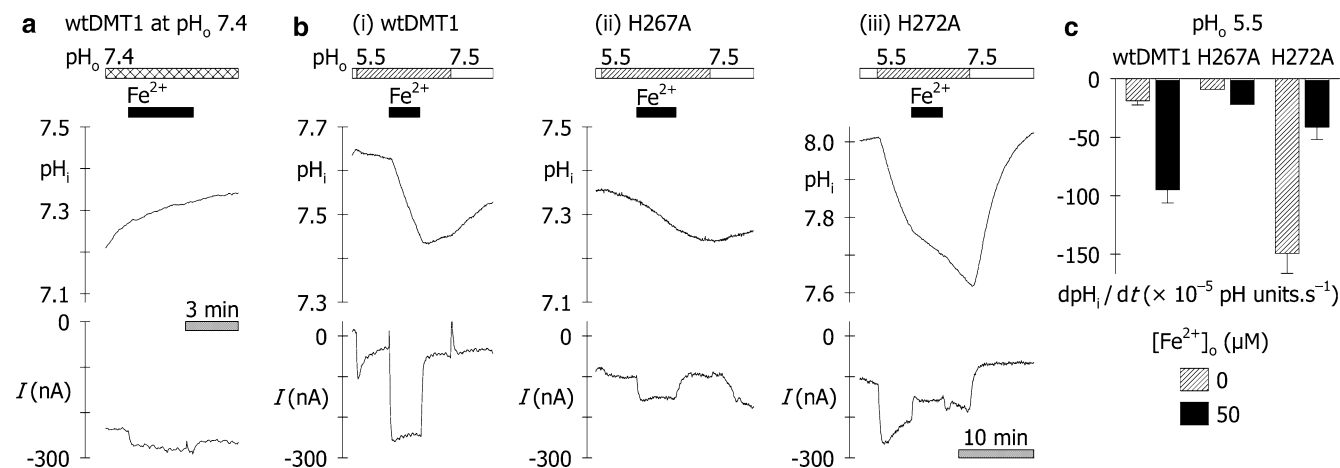


**Fig. 3**  $Fe^{2+}$  and  $H^+$  saturation kinetics of wtDMT1 as functions of cosubstrate concentrations. Kinetic data for wtDMT1 were derived from the currents evoked by  $0.1$ – $50 \mu M$   $Fe^{2+}$  at  $pH_o$   $5.2$ – $7.0$  in a single oocyte clamped at  $V_h$   $-70$  mV. The  $H^+$  kinetic parameters as a function of  $[Fe^{2+}]_o$  were determined by fitting data with Eq. 3 and as shown in Fig. 2c, d (however data in Fig. 2c are from an independent experiment). **a** The half-maximal  $H^+$  concentration for inhibition of the  $H^+$ -uncoupled, facilitative  $Fe^{2+}$  current is given by  $\ln(0.5)/-b$ , (Eq. 3) and shown as a function of  $[Fe^{2+}]_o$ . **b, c** Compensated  $H^+$  saturation kinetics ( $K_{0.5}$ ,  $I_{max}$ ) describing the cotransport currents as a function of  $[Fe^{2+}]_o$  (black). For comparison, the parameters derived using a conventional 3-parameter Hill function (Eq. 1) are also indicated (gray). Meanwhile, the pre-exponential value ( $a$ ) in Eq. 3 can be taken to represent the  $H^+$ -uncoupled, facilitative  $Fe^{2+}$  current ( $i^U$ ) in the theoretical absence of  $H^+$ . These data were plotted as a function of  $[Fe^{2+}]_o$  (not shown) and fit to Eq. 1, yielding the following parameters for  $i^U$  at  $[H^+]_o = 0$ :  $i_{max}^U$  of  $-99 \pm 2$  nA,  $K_{0.5}^U$  for  $Fe^{2+}$  of  $0.61 \pm 0.06 \mu M$ ,  $n_H^U$  for  $Fe^{2+}$  of  $1.0 \pm 0.1$  ( $r^2 = 0.993$ ). **d**  $Fe^{2+}$  saturation kinetics for wtDMT1 (filled circles) and H267A-DMT1 (filled diamonds) at  $pH_o$   $5.5$ ,  $-70$  mV. Half-maximal  $Fe^{2+}$  concentration ( $K_{0.5}^{Fe}$ ) for wtDMT1 was  $0.9 \pm 0.1 \mu M$ ; maximal current ( $I_{max}^{Fe}$ ) was  $-314 \pm 9$  nA; Hill coefficient for  $Fe^{2+}$  ( $n_{Fe}^{Fe}$ ) was  $1.3 \pm 0.1$  ( $r^2 = 0.991$ ). Kinetic parameters for H267A-DMT1 were  $K_{0.5}^{Fe}$   $1.5 \pm 0.4 \mu M$ ,  $I_{max}^{Fe}$   $-61 \pm 4$  nA, and  $n_H^{Fe}$   $0.9 \pm 0.2$  ( $r^2 = 0.975$ ). **e, f**  $Fe^{2+}$  saturation kinetics ( $K_{0.5}$ ,  $I_{max}$ ) as a function of  $pH_o$ . Error bars represent the standard error of regression

this effect by modifying  $i^U$  with an exponential decay which, when combined with the Hill function (Eq. 3), resulted in a further increase in the regression coefficient ( $r^2=0.99$ ) (Fig. 2c, dashed black line). Similar findings were obtained in four independent preparations (not shown). Whereas the fit resembled that for Eq. 2, the alternative prediction of the behavior of the  $H^+$ -uncoupled component resulted in different values for the  $H^+$  saturation kinetics of the coupled  $Fe^{2+}$  currents (Fig. 2d, blue line),  $K_{0.5}^H$  of  $1.6 \mu M$  and  $I_{max}^H$  of  $-139$  nA. Meanwhile, the  $H^+$ -uncoupled  $Fe^{2+}$  current (Fig. 2d, orange line) was described by its pre-exponential factor ( $a$ ) of  $-19$  nA, and the midpoint ( $\ln(0.5)/-b$ ) of its inhibition by  $H^+$  at  $[H^+]_o = 0.28 \mu M$  (i.e.  $pH_o$   $6.5$ ). On the basis of the improved regressions, and the theoretical interpretation of these and other data presented in support of DMT1-mediated facilitative  $Fe^{2+}$  transport that is uncoupled from  $H^+$  (see model in Fig. 8), we suggest that Eq. 3 may provide the most reliable estimates of  $H^+$  saturation kinetic parameters.

### Binding order and transport mechanism of DMT1

In order to explore the substrate binding order and transport mechanism of DMT1, we examined both the  $H^+$  and  $Fe^{2+}$  saturation kinetics as a function of cosubstrate concentrations at  $-70$  mV (Fig. 3). The  $H^+$ -dependence data were fit with Eq. 3 (as in Fig. 2c, d) at each  $Fe^{2+}$  concentration from  $0.2$  to  $50 \mu M$ . The  $H^+$  concentration at which the uncoupled  $Fe^{2+}$  current was inhibited  $50\%$  (i.e.  $\ln(0.5)/-b$ ) increased with increasing  $Fe^{2+}$  concentration (Fig. 3a), evidence that  $H^+$  and  $Fe^{2+}$  may compete for the unloaded transporter. The  $K_{0.5}^H$  describing  $H^+/Fe^{2+}$  cotransport was lowest ( $1.2 \mu M$ ) at high  $Fe^{2+}$  concentration and rose to  $1.9 \mu M$  at low  $Fe^{2+}$  concentration, consistent with a simultaneous transport mechanism [25, 28, 35, 36, 49]. That is,  $H^+$  and  $Fe^{2+}$  are translocated within the same transport cycle when both substrates are present. In contrast,  $K_{0.5}^H$  would be expected to increase at higher  $Fe^{2+}$  concentrations if  $H^+$  and  $Fe^{2+}$  were transported consecutively [25, 49]. The  $I_{max}^H$  was maximal when  $Fe^{2+}$  concentrations were saturating (Fig. 3c), but was significantly reduced at lower  $Fe^{2+}$  concentrations. Thus,  $Fe^{2+}$  concentration limited the maximal rate of cotransport, consistent with ordered binding of  $H^+$  then  $Fe^{2+}$  [25, 35, 36, 49]. Fitting our data using the combined function (Eq. 3) resulted in quantitative differences  $K_{0.5}^H$  and  $I_{max}^H$ —but no qualitative difference in their behavior as a function of  $[Fe^{2+}]_o$ —relative to the values generated from the conventional 3-parameter Hill function (Eq. 1) (Fig. 2b, c). However, since Eq. 3 also accounts for the facilitative component of the  $Fe^{2+}$ -evoked currents, we anticipate that fitting to Eq. 3 provides more appropriate  $K_{0.5}^H$  and  $I_{max}^H$  values. When  $n_H^H$  was not constrained in Eq. 3,  $n_H^H$  was  $0.9$ – $1.3$  and did not vary with  $Fe^{2+}$  concentration, indicating that the binding of only one  $H^+$  is required to activate the



**Fig. 4** Changes in intracellular pH ( $pH_i$ ) associated with DMT1 activity in oocytes expressing wild type and mutant DMT1. **a**, **b**  $pH_i$  changes (upper panels) and currents (lower panels) were recorded simultaneously in individual oocytes voltage-clamped at  $V_h = -90$  mV. **a** An oocyte expressing wtDMT1 was superfused at  $pH_o$  7.4 (cross-hatched bar) and 50  $\mu$ M  $Fe^{2+}$  added for the period shown by the filled box. The  $Fe^{2+}$  evoked a small inward current that was not associated with intracellular acidification. **b** Oocytes were superfused with pH 7.5 medium (blank boxes), then

pH 5.5 medium (hatched boxes), and 50  $\mu$ M  $Fe^{2+}$  at  $pH_o$  5.5 (filled boxes). Typical records are shown for oocytes expressing (i) wtDMT1, (ii) H267A-DMT1, and (iii) H272A-DMT1. (The 10 min scale bar refers to all panels in **b**). **c** Summary of acidification rates for all oocytes tested at  $pH_o$  5.5. The rate of intracellular acidification ( $dpH_i/dt$ ) was calculated after switching from  $pH_o$  7.5 to 5.5 (hatched bars) and upon adding 50  $\mu$ M  $Fe^{2+}$  at  $pH_o$  5.5 (solid bars). Data are mean  $\pm$  SEM for wtDMT1 ( $n = 7$ ) and H272A ( $n = 3$ ). Data for H267A are from a single oocyte

cotransport cycle, or that there is no cooperativity between multiple binding sites. However, the Hill coefficient is not a direct index of coupling stoichiometry in transporters, and the  $H^+/Fe^{2+}$  stoichiometry of DMT1 is not fixed. Significant slippage is evident, with  $H^+$  fluxes generally exceeding the fluxes of  $Fe^{2+}$  [7, 59].

The  $Fe^{2+}$ -evoked currents also were saturable. In a typical example, at  $V_h = -70$  mV and  $pH_o$  5.5, the half-maximal  $Fe^{2+}$  concentration ( $K_{0.5}^{Fe}$ ) was  $0.9 \pm 0.1$   $\mu$ M (Fig. 3d). The Hill coefficient for  $Fe^{2+}$  ( $n_H^{Fe}$ ) was close to 1 and was independent of  $pH_o$  (not shown), suggesting that only one  $Fe^{2+}$  is involved in each transport cycle. The relationship of  $K_{0.5}^{Fe}$  to  $H^+$  concentration was biphasic (Fig. 3e): at low  $H^+$  concentrations,  $K_{0.5}^{Fe}$  rose significantly from 0.8  $\mu$ M at

neutral  $pH_o$  to 1.2  $\mu$ M at  $pH_o$  6.1, further evidence that  $Fe^{2+}$  and  $H^+$  compete at higher  $pH_o$ . However,  $K_{0.5}^{Fe}$  fell again (to 0.9  $\mu$ M) at higher  $H^+$  concentrations, consistent with simultaneous translocation of substrates at low  $pH_o$ .

We would expect  $I_{max}^{Fe}$  to be independent of  $H^+$  concentration if  $H^+$  binds first in a strict cotransport model, so that saturating  $Fe^{2+}$  should always drive the transporter at maximal velocity regardless of  $H^+$  concentration [25, 35, 36, 49]. However,  $I_{max}^{Fe}$  was markedly dependent upon  $H^+$  concentration (Fig. 3f), consistent with a  $H^+$ -uncoupled, facilitative  $Fe^{2+}$  transport pathway short-circuiting cotransport at high  $pH_o$ . These data indicate that, at low  $pH_o$ ,  $H^+$  binding precedes  $Fe^{2+}$  binding and its simultaneous transport

**Table 2** Summary of analysis of rat wild type DMT1, and the His-267 and His-272 mutants

Protein	$Q_{max}$ (nC) <sup>a</sup>	$I_{max}^{Fe}$ (nA) <sup>b</sup>	Turnover rate (s <sup>-1</sup> ) <sup>c</sup>	Transporter density ( $N_T$ ) <sup>d</sup>	$K_{0.5}^{Fe}$ ( $\mu$ M) <sup>e</sup>	Substrate preference	$K_{0.5}^H$ of cotransport ( $\mu$ M) <sup>f</sup>	$K_{0.5}^H$ of leak ( $\mu$ M) <sup>g</sup>
WtDMT1	$48 \pm 3$	-1002	21	$3.2 \times 10^{11}$ /oocyte	$0.9 \pm 0.1$	$Fe^{2+} \gg Zn^{2+}$	$1.4 \pm 0.2$	—
H267A	$23 \pm 1$	-515	23	$1.3 \times 10^{11}$ /oocyte	$1.5 \pm 0.4$	—	—	—
H267D	< 2	-6	—	—	—	—	—	—
H267N	$6 \pm 1$	-74	12	$0.4 \times 10^{11}$ /oocyte	< 5	—	—	—
H272A	None detected	+83	—	—	$0.36 \pm 0.06$ ( $K_i^{Fe}$ )	$Fe^{2+} = Zn^{2+}$	NA	$2.2 \pm 0.3$
H272R	None detected	+186	—	—	—	—	NA	—

<sup>a</sup> Total presteady-state charge ( $Q_{max}$ ) determined using Eq. 4, at  $pH_o$  5.5 and 23°C (data are from a different preparation than that used in Fig. 1). In the case of H267D, presteady-state currents were visible but smaller than could be isolated satisfactorily from capacitive currents. In the case of H272A and H272R, no presteady-state currents were visible

<sup>b</sup> Current evoked by 50  $\mu$ M  $Fe^{2+}$  at  $pH_o$  5.5, 23°C, and -150 mV (Fig. 5a) in the same oocytes in which we determined  $Q_{max}$

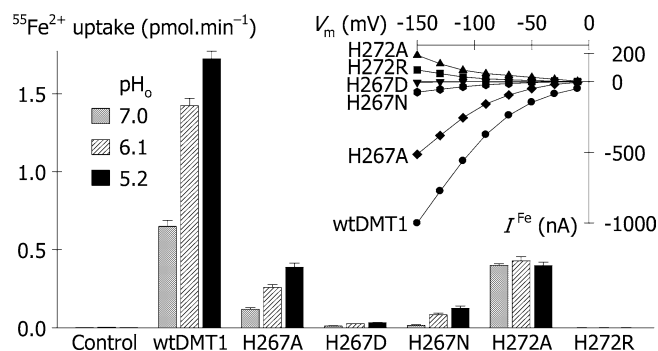
<sup>c</sup> Turnover rate determined as  $I_{max}/Q_{max}$  (under the conditions defined here separately for  $Q_{max}$  and  $I_{max}^{a,b}$ )

<sup>d</sup> Transporter density determined according to Eq. 6

<sup>e</sup>  $K_{0.5}^{Fe}$  determined at  $pH_o$  5.5 and -70 mV; in the case of H272A, the value given is  $K_i^{Fe}$ , the  $Fe^{2+}$  concentration at which the  $H^+$  leak was inhibited by 50%

<sup>f</sup>  $K_{0.5}^H$  determined at 10  $\mu$ M  $Fe^{2+}$  and -50 mV, mean of three oocytes<sup>g</sup>  $K_{0.5}^H$  of the  $H^+$  leak pathway determined at -50 mV/NA not applicable



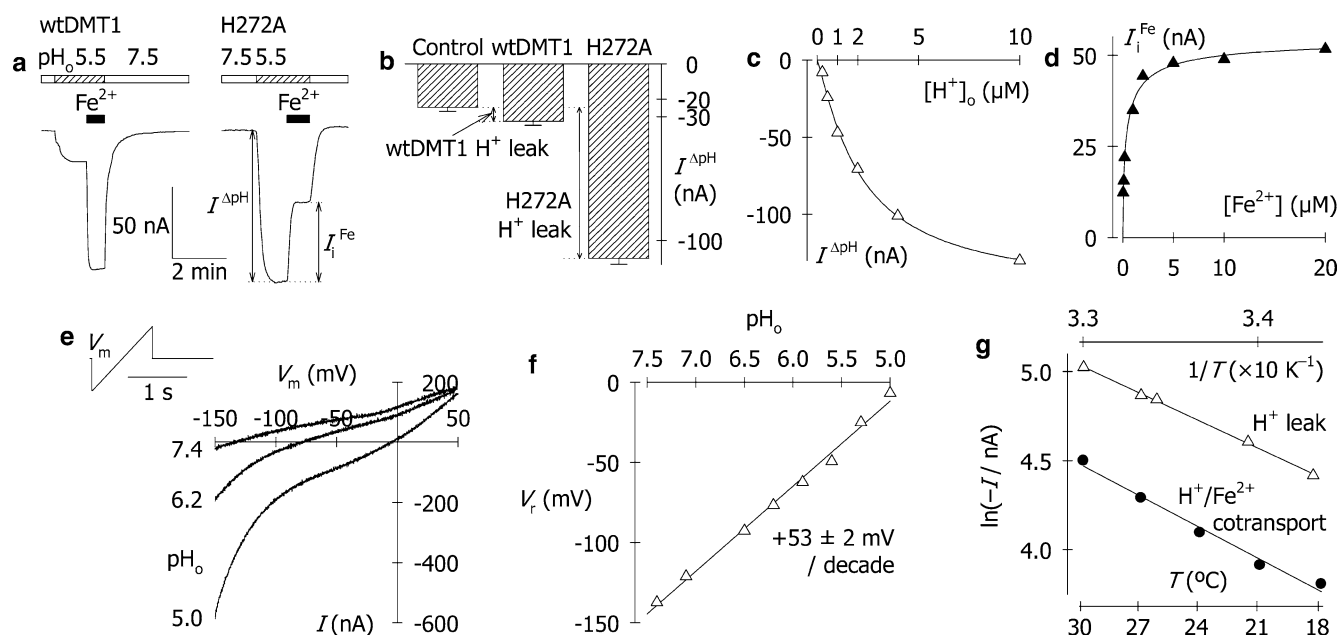


**Fig. 5**  $\text{Fe}^{2+}$  transport mediated by wtDMT1 and histidyl mutants: effects of alternative substitutions. The pH-dependence of  $2 \mu\text{M}$   $^{55}\text{Fe}^{2+}$  uptake, determined over 30 min. Data are mean  $\pm$  SEM for 7–15 oocytes in each group. *Inset* Current/voltage relationships for  $50 \mu\text{M}$   $\text{Fe}^{2+}$  superfused at pH<sub>o</sub> 5.5. Inward currents were observed for wtDMT1 (circles), H267A (diamonds), H267N (hexagons), and H267D (inverted triangles). Outward currents, presumably representing inhibition of the  $\text{H}^+$  leak by  $\text{Fe}^{2+}$ , were observed for H272A (triangles) and H272R (squares). Currents derive from a separate oocyte preparation from that in which we measured  $^{55}\text{Fe}^{2+}$  uptake (main figure)

across the membrane. However, around neutral pH<sub>o</sub>, wtDMT1 binds and transports  $\text{Fe}^{2+}$  independent of  $\text{H}^+$ . Additionally,  $\text{H}^+$  binding and translocation may proceed at low pH<sub>o</sub> in the complete absence of metal ion (i.e.  $\text{H}^+$  leak), as evidenced by the modest intracellular acidification and small inward current detected upon switching from pH<sub>o</sub> 7.5 to 5.5 prior to adding metal ion (see Fig. 4b(i)).

$\text{Fe}^{2+}$  transport at pH<sub>o</sub> 7.4 is not accompanied by  $\text{H}^+$  influx

We showed previously that the  $\text{Fe}^{2+}$ -evoked current in DMT1-expressing oocytes at pH<sub>o</sub> 5.5 was associated with a rapid intracellular acidification, consistent with  $\text{H}^+$ -coupled  $\text{Fe}^{2+}$  transport [19]. Here, we superfused DMT1-expressing oocytes in the absence of an inwardly directed  $\text{H}^+$  chemical gradient (pH<sub>o</sub> was 7.4), we found that  $50 \mu\text{M}$   $\text{Fe}^{2+}$  evoked a modest but significant inward current that was not accompanied by a  $\text{H}^+$  influx.



**Fig. 6** Properties of a leak current mediated by H272A-DMT1. **a** Typical current records at  $-50 \text{ mV}$  for wtDMT1 and H272A-DMT1 first superfused with pH 7.5 medium (blank boxes), then pH 5.5 medium (hatched boxes), and the effect of adding  $50 \mu\text{M}$   $\text{Fe}^{2+}$  (black boxes). Indicated are the magnitudes of the inward current ( $I^{\Delta\text{pH}}$ ) resulting from switching from pH<sub>o</sub> 7.5–5.5, and the  $\text{Fe}^{2+}$ -induced inhibition current ( $I^{\text{Fe}}$ ) in H272A. **b** Leak currents associated with expression of wtDMT1 and H272A-DMT1 in oocytes, quantified as  $I^{\Delta\text{pH}}$  (see **a**) less that observed for control oocytes. Data are mean  $\pm$  SEM for 7–12 oocytes. **c**  $\text{H}^+$  saturation of the leak current mediated by H272A-DMT1. Data were fit with Eq. 2, from which we obtained  $K_{0.5}^{\text{H}}$   $2.2 \pm 0.3 \mu\text{M}$ ,  $I_{\text{max}}^{\text{H}}$   $-152 \pm 9 \text{ nA}$ , and  $n_{\text{H}}^{\text{H}}$   $1.2 \pm 0.1$  ( $r^2 = 0.998$ ). **d**  $\text{Fe}^{2+}$ -induced inhibition current ( $I^{\text{Fe}}$ ) in H272A (see **a**). The  $\text{Fe}^{2+}$ -induced inhibition current mediated by H272A-DMT1 was inhibited by  $\text{Fe}^{2+}$  with  $K_i^{\text{Fe}}$  of  $0.2 \pm 0.1 \mu\text{M}$  (other parameters from the fit to Eq. 2 were  $I_{\text{max}}^{\text{Fe}}$   $+55 \pm 2 \text{ nA}$ ,  $n_{\text{H}}^{\text{Fe}}$   $0.7 \pm 0.1$ ,  $r^2 = 0.994$ ). **e** A 1 s voltage ramp protocol (*inset*) was applied in an oocyte expressing H272A-DMT1 to determine reversal potential ( $V_r$ ) as a function of pH<sub>o</sub>, plotted in

**f**. For clarity, only the records at pH<sub>o</sub> 7.4, 6.2, and 5.0 are displayed. **f**  $V_r$  of the leak currents for H272A-DMT1 as a function of pH<sub>o</sub>. The  $V_r$  shifted to depolarized  $V_m$  with increasing  $[\text{H}^+]_o$ , with a slope of  $+53 \pm 2 \text{ mV}$  per decade ( $r^2 = 0.994$ ). **g** The temperature dependence of the  $\text{H}^+$  leak was determined as  $I^{\Delta\text{pH}}$  mediated by H272A-DMT1 (filled triangles) at pH<sub>o</sub> 5.7 and  $-50 \text{ mV}$ , after subtracting the endogenous  $\text{H}^+$  currents determined in a control oocyte from the same batch (not shown, these were 12–15% the magnitude of the  $\text{H}^+$  currents in the oocyte expressing H272A-DMT1). The temperature dependence of  $\text{H}^+$ /Fe<sup>2+</sup> cotransport was determined as the current evoked by  $50 \mu\text{M}$   $\text{Fe}^{2+}$  in an oocyte expressing wtDMT1 (filled circles) at pH<sub>o</sub> 5.7 and  $-50 \text{ mV}$ . Arrhenius transformation (Eq. 4) yielded activation energy ( $E_a$ ) of  $10.3 \pm 0.2 \text{ kcal mol}^{-1}$  for wild type  $\text{H}^+$ /Fe<sup>2+</sup> cotransport ( $\ln A = 21.6 \pm 1.3$ ;  $r^2 = 0.985$ ).  $E_a$  for the  $\text{H}^+$  leak mediated by H272A-DMT1 was  $9.1 \pm 0.2 \text{ kcal mol}^{-1}$  ( $\ln A = 20.1 \pm 0.3$ ;  $r^2 = 0.999$ ). (Endogenous  $\text{H}^+$  currents in a control oocyte had  $E_a = 5.9 \pm 0.4 \text{ kcal mol}^{-1}$ ,  $\ln A = 13.0 \pm 0.6$ ;  $r^2 = 0.989$ )



We confirmed this observation for three individual oocytes at  $\text{pH}_o$  7.4, and a representative record is shown in Fig. 4a. In contrast, when we imposed an inwardly directed  $\text{H}^+$  chemical gradient ( $\text{pH}_o$  5.5), the larger  $\text{Fe}^{2+}$ -evoked current was associated with significant intracellular acidification (Fig. 4b(i)). These observations support our conclusion that  $\text{Fe}^{2+}$  transport at neutral  $\text{pH}_o$  or above is predominantly uncoupled from  $\text{H}^+$ .

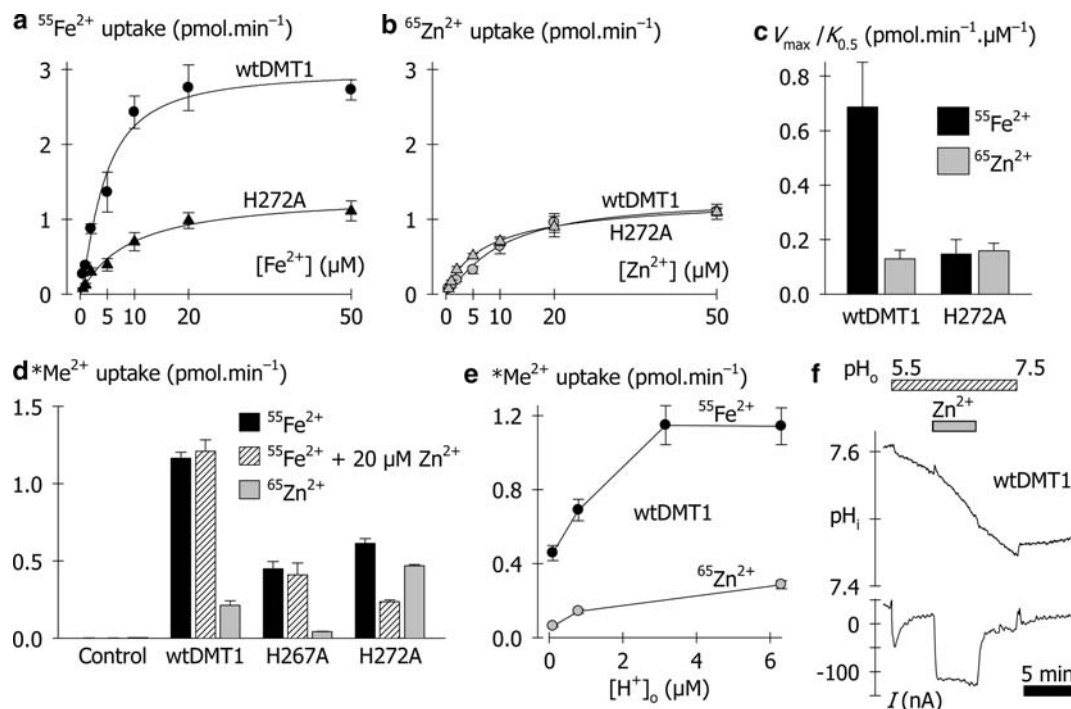
#### Impact of mutations in DMT1 at His-267 and His-272

We then evaluated the impact of mutations in DMT1 at His-267 and His-272 in putative TM6 [33]. The remaining seven histidyl residues in DMT1 are thought to reside in the intracellular *N*-terminal region, or in intracellular or extracellular loops. Histidyl residues are common targets for transporter mutagenesis, and we considered the possibilities that His-267 and His-272 may interact with  $\text{H}^+$  or—as a pair—form a metal-

binding site. The H267A-DMT1, when expressed in oocytes, displayed properties similar to those of wtDMT1 (summarized in Table 2), but at a lower level. However, the H272A mutation resulted in striking changes in the activity of the protein.

#### Analysis of H267A-DMT1

The  $\text{Fe}^{2+}$  evoked currents of up to  $-500$  nA at  $\text{pH}_o$  5.5 in oocytes expressing H267A-DMT1, and the current/voltage relationship resembled that of wtDMT1 (Fig. 5, inset). The smaller  $\text{Fe}^{2+}$ -evoked currents observed for H267A-DMT1 compared with wtDMT1 correlated with the reduced transporter density (estimated from pre-steady-state currents) for H267A-DMT1 in the oocyte membrane (Table 2). The turnover rate for H267A-DMT1 was  $23 \text{ s}^{-1}$ , and that for wtDMT1 was  $21 \text{ s}^{-1}$ , indicating that the smaller currents observed for H267A-DMT1 did not result from reduced transporter effi-



**Fig. 7** Comparison of  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$  transport mediated by wtDMT1 and DMT1 mutants. **a** Concentration dependence of  $^{55}\text{Fe}^{2+}$  uptake, measured over 10 min at  $\text{pH}_o$  5.5. Data for wtDMT1 (black circles) were fit with Eq. 1 to determine  $\text{Fe}^{2+}$  transport kinetic parameters:  $V_{\text{max}}^{\text{Fe}}$   $3.0 \pm 0.3 \text{ pmol min}^{-1}$ ,  $K_{0.5}^{\text{Fe}}$   $4.3 \pm 0.9 \text{ }\mu\text{M}$ , and Hill coefficient for  $\text{Fe}^{2+}$  ( $n_{\text{H}}^{\text{Fe}}$ )  $1.3 \pm 0.3$  ( $r^2 = 0.977$ ). For H272A-DMT1 (black triangles):  $V_{\text{max}}^{\text{Fe}}$   $1.3 \pm 0.2 \text{ pmol min}^{-1}$ ,  $K_{0.5}^{\text{Fe}}$   $9.1 \pm 3.1 \text{ }\mu\text{M}$ , and  $n_{\text{H}}^{\text{Fe}}$   $1.0 \pm 0.2$  ( $r^2 = 0.985$ ). **b** Concentration dependence of  $^{65}\text{Zn}^{2+}$  uptake over 10 min under the same experimental conditions, set to the same scaling, and in oocytes from the same preparation as in (a). Data were fit with Eq. 1, for wtDMT1 (gray circles):  $V_{\text{max}}^{\text{Zn}}$   $1.3 \pm 0.1 \text{ pmol min}^{-1}$ ,  $K_{0.5}^{\text{Zn}}$   $9.9 \pm 2.3 \text{ }\mu\text{M}$ , and  $n_{\text{H}}^{\text{Zn}}$   $1.2 \pm 0.2$  ( $r^2 = 0.989$ ), and for H272A-DMT1 (gray triangles):  $V_{\text{max}}^{\text{Zn}}$   $1.3 \pm 0.1 \text{ pmol min}^{-1}$ ,  $K_{0.5}^{\text{Zn}}$   $8.3 \pm 1.4 \text{ }\mu\text{M}$ ,  $n_{\text{H}}^{\text{Zn}}$   $0.9 \pm 0.1$  ( $r^2 = 0.998$ ). Each data point in A and B represents mean

$\pm$  SEM for 6–11 oocytes. **c**  $I_{\text{max}}/K_{0.5}$  as an index of transport efficiency, using kinetic parameters from  $^{55}\text{Fe}^{2+}$  and  $^{65}\text{Zn}^{2+}$  uptake data in A and B, for wtDMT1 and H272A-DMT1. Error bars were propagated from the standard errors of regression. **d** Radiotracer metal-ion ( $^*\text{Me}^{2+}$ ) uptake mediated by wtDMT1, and the H267A and H272A mutants. Uptake of  $2 \text{ }\mu\text{M}$   $^{55}\text{Fe}^{2+}$  in the absence (black bars) or presence (hatched bars) of  $20 \text{ }\mu\text{M}$   $\text{Zn}^{2+}$ , and uptake of  $2 \text{ }\mu\text{M}$   $^{65}\text{Zn}^{2+}$  (gray bars), were measured over 10 min at  $\text{pH}_o$  5.5. Data are mean  $\pm$  SEM for 7–14 oocytes. **e** Wildtype DMT1-mediated uptake of  $2 \text{ }\mu\text{M}$   $^{55}\text{Fe}^{2+}$  (black circles) and  $2 \text{ }\mu\text{M}$   $^{65}\text{Zn}^{2+}$  (gray circles) over 10 min as a function of extracellular pH ( $\text{pH}_o$ ). Data are mean  $\pm$  SEM for 6–13 oocytes. **f** Effect of  $\text{Zn}^{2+}$  on intracellular pH ( $\text{pH}_i$ ) in an oocyte expressing wtDMT1, superfused with pH 7.5 medium (blank boxes), then pH 5.5 (hatched boxes).  $50 \text{ }\mu\text{M}$   $\text{Zn}^{2+}$  was superfused (at  $\text{pH}_o$  5.5) for the period shown by the gray box

ciency. The  $\text{Fe}^{2+}$ -evoked currents in oocytes expressing H267A-DMT1 were saturable, with  $K_{0.5}^{\text{Fe}}$  of  $1.5 \pm 0.4 \mu\text{M}$  (Fig. 3d), close to that observed for wtDMT1 ( $\approx 1 \mu\text{M}$ ). The  $\text{Fe}^{2+}$  transport mediated by H267A-DMT1 was associated with a modest  $\text{H}^+$  influx (Fig. 4b(ii)), and  $^{55}\text{Fe}^{2+}$  uptake mediated by H267A-DMT1 displayed a pH-dependence similar to that of wtDMT1 (Fig. 5). Therefore, although the H267A mutant is expressed at lower levels in the oocyte plasma membrane, the properties of H267A-DMT1 closely resemble those of wtDMT1. Nevertheless, additional effects may be expected when both His-267 and His-272 are mutated together [29].

### Analysis of H272A-DMT1

As previously observed [19, 59], wtDMT1 mediated a modest, 'leak' current upon switching from  $\text{pH}_o$  7.5 to 5.5 in the absence of metal ion (Figs. 4b(i), c, 6a). The resulting  $\text{H}^+$ -induced inward current ( $I^{\text{APH}}$ ) exceeded by about 10 nA that observed in control oocytes (Fig. 6b) and was associated with a modest intracellular acidification (Fig. 4b(i)). Addition of  $\text{Fe}^{2+}$  resulted in a much more rapid acidification and a much larger inward current (Figs. 4b(i), c, 6a). In contrast, H272A-DMT1 mediated a significant leak current (approximately tenfold that observed for wtDMT1) and this leak current was inhibited by  $\text{Fe}^{2+}$  (Figs. 4b(iii), c, 6a, b). The leak in H272A-DMT1 was associated with a rapid intracellular acidification (Fig. 4b(iii)) that was significantly slowed by  $72 \pm 20\%$  upon the addition of  $\text{Fe}^{2+}$  (Fig. 4c). The slowing of acidification rate with  $\text{Fe}^{2+}$  was not the result of intracellular buffering in the oocyte, since removal of the  $\text{Fe}^{2+}$  again accelerated the intracellular acidification at  $\text{pH}_o$  5.5 (Fig. 4b(iii)). As for wtDMT1, the inward currents and changes in intracellular pH observed for H272A-DMT1 were reversed upon returning to  $\text{pH}_o$  7.5 (Fig. 4b(i), (iii)).

The H272A-DMT1-mediated leak current was inhibited by  $\text{Fe}^{2+}$  with apparent  $K_i^{\text{Fe}} \approx 0.4 \mu\text{M}$ . This value may be a better reflection of the affinity at which DMT1 binds  $\text{Fe}^{2+}$  than is the  $\text{Fe}^{2+}$  concentration at which wtDMT1-mediated transport is half-maximal (i.e.  $K_{0.5}^{\text{Fe}}$ ), since  $K_{0.5}^{\text{Fe}}$ —but not  $K_i^{\text{Fe}}$ —additionally describes the complete transport cycle. Supersaturating  $\text{Fe}^{2+}$  ( $50 \mu\text{M}$ ) did not fully reverse the inward leak current associated with H272A-DMT1 (Figs. 4, 6a, c, d), indicating either that inhibition of the leak by  $\text{Fe}^{2+}$  is incomplete, or that  $\text{Fe}^{2+}$  is itself transported by H272A-DMT1, resulting in a residual inward current, a conclusion supported by the significant  $^{55}\text{Fe}^{2+}$  uptake we observed in oocytes expressing H272A-DMT1 (Fig. 5). Notably, however, the  $^{55}\text{Fe}^{2+}$  uptake mediated by H272A-DMT1 was independent of  $\text{pH}_o$  (7.0, 6.1, or 5.2), whereas wtDMT1-mediated  $^{55}\text{Fe}^{2+}$  uptake was markedly stimulated at low  $\text{pH}_o$  (Figs. 5, 7e). Since H272A-DMT1 mediated a substantially increased leak current as well as pH-independent  $\text{Fe}^{2+}$  transport, we conclude

that the major impact of the H272A mutation was an uncoupling of  $\text{Fe}^{2+}$  transport from the  $\text{H}^+$  flux. The  $K_{0.5}^{\text{Fe}}$  for H272A-DMT1 at  $\text{pH}_o$  5.5 was  $\approx 9 \mu\text{M}$  (Fig. 7b), similar to the  $K_{0.5}^{\text{Fe}}$  of  $7.2 \mu\text{M}$  derived for wtDMT1 at  $\text{pH}_o$  7.0 (*not shown*). These observations provide evidence that the DMT1 protein possesses the machinery for facilitative  $\text{Fe}^{2+}$  transport, uncoupled from  $\text{H}^+$ .

### Properties of the leak current mediated by H272A-DMT1

Given its substantially larger leak current and the uncoupling of  $\text{Fe}^{2+}$  and  $\text{H}^+$  fluxes, H272A-DMT1 can serve as a model system in which to study the DMT1-mediated leak. In oocytes expressing H272A-DMT1,  $I^{\text{APH}}$  (which largely represents the H272A-DMT1-mediated leak since  $I^{\text{APH}}$  in control oocytes was around  $-20 \text{ nA}$ ) was dependent on the final  $\text{pH}_o$ . The relationship of  $I^{\text{APH}}$  to extracellular  $\text{H}^+$  concentration fit a Hill function (Eq. 1) with Hill coefficient ( $n_H$ )  $\approx 1$  (Fig. 6c), as for cotransport. The  $K_{0.5}^{\text{H}}$  of  $\approx 2 \mu\text{M}$  for the leak is similar to the  $K_{0.5}^{\text{H}}$  ( $1\text{--}2 \mu\text{M}$ ) for wtDMT1  $\text{Fe}^{2+}$ -evoked currents (Figs. 3b, 6c). We applied a voltage-ramp protocol to determine the reversal potential ( $V_r$ ) as a function of  $\text{pH}_o$  in oocytes expressing H272A-DMT1 (Fig. 6e, f). The  $V_r$  varied from  $-137 \text{ mV}$  at  $\text{pH}_o$  7.4 to  $-6 \text{ mV}$  at  $\text{pH}_o$  5.0 (Fig. 6f). The slope of  $V_r$  as a function of  $\text{pH}_o$  was  $+53 \text{ mV per pH}_o$  unit, close to the slope of  $+58 \text{ mV per pH}_o$  unit predicted from the Nernst equation (at  $22^\circ\text{C}$ ), indicating that the H272A-DMT1-mediated leak current is carried solely by  $\text{H}^+$ . This observation is consistent with the intracellular acidification observed upon switching from  $\text{pH}_o$  7.5 to 5.5 for H272A-DMT1 (Fig. 5) and, although less markedly, also for wtDMT1 (Figs. 5, 7f). The wtDMT1-mediated  $\text{Fe}^{2+}$ -evoked currents were temperature-dependent, with Arrhenius activation energy ( $E_a$ ) of  $\approx 10 \text{ kcal mol}^{-1}$  (Fig. 6g). The H272A-DMT1-mediated  $\text{H}^+$  leak currents displayed a similar temperature dependence with  $E_a \approx 9 \text{ kcal mol}^{-1}$ , suggesting that  $\text{H}^+$  uniport (leak)—like wild type cotransport—is carrier-mediated and involves substantial conformational changes. In contrast, if the leak were channel-mediated, we would expect the  $\text{H}^+$  leak to have activation energy lower than that for cotransport. That the parameters  $n_H$  and  $K_{0.5}^{\text{H}}$  do not differ for the  $\text{H}^+$  leak and cotransport pathways suggests a common  $\text{H}^+$  binding site.

### Impact of alternative substitutions at His-267 and His-272

We examined the impact of additional substitutions at histidyl residues 267 and 272. Substitution of His-267 with asparagine (N) or aspartic acid (D) resulted in activities that were qualitatively similar to wtDMT1 and H267A-DMT1, but with significantly reduced expres-

sion of functional units at the plasma membrane (Table 2). Based on  $^{55}\text{Fe}^{2+}$  uptake and  $\text{Fe}^{2+}$ -evoked currents (Fig. 5),  $\text{Fe}^{2+}$  transport in oocytes expressing H267N-DMT1 was dependent on both  $\text{pH}_o$  and membrane potential.  $K_{0.5}^{\text{Fe}}$  was  $<5\ \mu\text{M}$  (since 5, 50 and  $500\ \mu\text{M}\ \text{Fe}^{2+}$  evoked similar currents  $-20$ ,  $-17\ \text{nA}$ , and  $-19\ \text{nA}$ , respectively, at  $-50\ \text{mV}$  and  $\text{pH}_o\ 5.5$ , *not shown*). The H267N-DMT1 also displayed presteady-state currents (*not shown*), with  $V_{0.5} = +30 \pm 2\ \text{mV}$ ,  $z = -1.0 \pm 0.1$  ( $r^2 = 0.921$ ). The  $Q_{\text{max}}$  of  $6 \pm 1\ \text{nC}$  was only 13% of that for wtDMT1 (Table 2). The H267N-DMT1 turnover rate was lower than for wtDMT1 (Table 2) but our confidence in this value for H267N-DMT1 is limited since both the presteady-state currents and  $\text{Fe}^{2+}$ -evoked currents were so small. The H267D-DMT1 exhibited even lower activity, in keeping with the tiny presteady-state currents observed for that mutant (too small to reliably isolate from capacitive currents). The H267D-DMT1 mediated pH-dependent  $^{55}\text{Fe}^{2+}$  uptake that exceeded that observed for control oocytes, in addition to  $\text{Fe}^{2+}$ -evoked inward currents that were barely discernible (Fig. 5).

We mutated His-272 to an arginine (R) residue (i.e. permanently cationic, rather than titratable within the physiological  $\text{pH}_o$  range). Like H272A, the H272R mutation abolished presteady-state currents (*not shown*) and mediated a small  $\text{Fe}^{2+}$ -inhibitable  $\text{H}^+$  leak (*not shown*). The inhibition of the  $\text{H}^+$  leak underlies the small outward currents observed in the presence of  $\text{Fe}^{2+}$  at  $\text{pH}_o\ 5.5$  (Fig. 5 inset). However, whereas H272A-DMT1 also mediated pH-independent  $\text{Fe}^{2+}$  transport, H272R-DMT1 did not mediate any  $^{55}\text{Fe}^{2+}$  transport activity (Fig. 5).

#### Comparison of zinc and iron transport mediated by DMT1

The wtDMT1 also transported  $\text{Zn}^{2+}$  but at much lower maximal velocity ( $V_{\text{max}}$ ) and with lower apparent affinity than wtDMT1-mediated  $\text{Fe}^{2+}$  transport. The  $V_{\text{max}}$  for  $^{65}\text{Zn}^{2+}$  uptake in oocytes expressing wtDMT1 was only 43% the  $V_{\text{max}}$  for  $^{55}\text{Fe}^{2+}$  uptake (Fig. 7a, b). Meanwhile, the zinc concentration at which uptake was half-maximal ( $K_{0.5}^{\text{Zn}} = 9.9\ \mu\text{M}$ ) was more than double the  $K_{0.5}^{\text{Fe}}$  ( $4.3\ \mu\text{M}$ ). Taking the ratio  $V_{\text{max}}/K_{0.5}$  as an index of the efficiency with which each metal ion is transported by wtDMT1, wtDMT1 displayed a fivefold preference for  $\text{Fe}^{2+}$  over  $\text{Zn}^{2+}$  (Fig. 7c). The H272A mutation significantly affected  $\text{Fe}^{2+}$  transport, both reducing  $I_{\text{max}}^{\text{Fe}}$  and increasing  $K_{0.5}^{\text{Fe}}$  compared with wtDMT1, without impact on zinc transport (Fig. 7a, b). Thus, the H272A mutation abolished the marked preference for  $\text{Fe}^{2+}$  over  $\text{Zn}^{2+}$  (Fig. 7c).

Subsaturating  $\text{Zn}^{2+}$  failed to inhibit the uptake of  $2\ \mu\text{M}\ ^{55}\text{Fe}^{2+}$  in oocytes expressing wtDMT1 (or H267A-DMT1), but significantly inhibited  $^{55}\text{Fe}^{2+}$  uptake in oocytes expressing H272A-DMT1 (Fig. 7d). The H272A-DMT1 transported  $2\ \mu\text{M}\ ^{65}\text{Zn}^{2+}$  and  $^{55}\text{Fe}^{2+}$

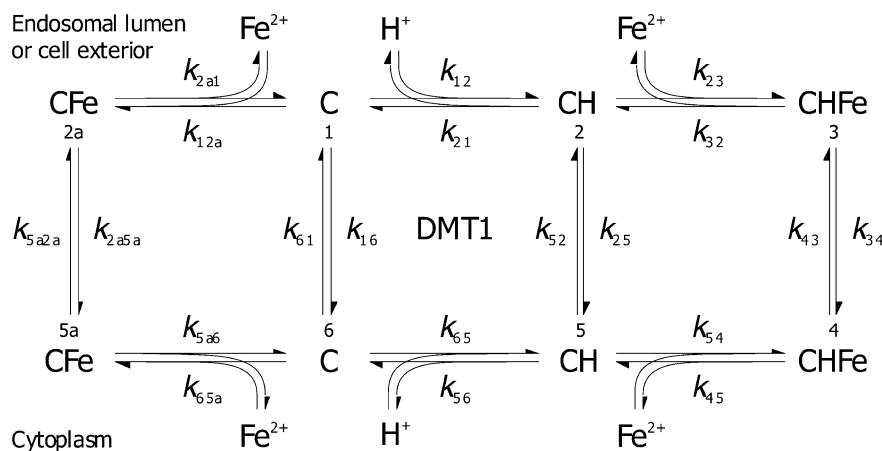
equally well, whereas uptake of  $2\ \mu\text{M}\ ^{55}\text{Fe}^{2+}$  greatly exceeded that of  $^{65}\text{Zn}^{2+}$  in oocytes expressing wtDMT1 or H267A-DMT1 (Fig. 7d). That H272A-DMT1 (in which metal-ion transport is uncoupled from  $\text{H}^+$ ) did not exhibit the marked preference for  $\text{Fe}^{2+}$  over  $\text{Zn}^{2+}$  displayed by wtDMT1 led us to consider the possibility that  $\text{Zn}^{2+}$  may be transported only by the  $\text{H}^+$ -uncoupled, facilitative metal-ion pathway in DMT1, and that the  $\text{H}^+$ -coupled and  $\text{H}^+$ -uncoupled pathways could differ in their metal-ion selectivity. However, examination of the pH-dependence and  $\text{H}^+$ -coupling of  $\text{Zn}^{2+}$  transport mediated by wtDMT1 revealed that uptake of  $2\ \mu\text{M}\ ^{65}\text{Zn}^{2+}$  was accelerated at low  $\text{pH}_o$ , as was  $^{55}\text{Fe}^{2+}$  uptake (Fig. 7e).

Consistent with  $\text{Fe}^{2+}$ -evoked currents (Fig. 2c), significant  $^{55}\text{Fe}^{2+}$  uptake persisted at  $\text{pH}_o\ 7.0$ , further supporting our conclusion that DMT1 can also mediate  $\text{H}^+$ -uncoupled facilitative  $\text{Fe}^{2+}$  transport. Meanwhile,  $\text{H}^+$ -coupled  $^{55}\text{Fe}^{2+}$  transport (at  $2\ \mu\text{M}\ \text{Fe}^{2+}$ ) proceeded with  $K_{0.5}^{\text{H}}$  of  $1\text{--}3\ \mu\text{M}$  (estimated from Fig. 7e), close to that determined from  $\text{Fe}^{2+}$ -evoked currents ( $K_{0.5}^{\text{H}} = 1\text{--}2\ \mu\text{M}$ ; see Fig. 3b). The  $K_{0.5}^{\text{H}}$  for  $^{65}\text{Zn}^{2+}$  transport was on the order of that for  $^{55}\text{Fe}^{2+}$  transport (Fig. 7e). In an oocyte expressing wtDMT1, superfusing  $50\ \mu\text{M}\ \text{Zn}^{2+}$  resulted in an inward current and a modest acceleration of intracellular acidification compared with that induced by low  $\text{pH}_o$  alone (Fig. 7f). Removing the  $\text{Zn}^{2+}$  slowed the intracellular acidification. The  $\text{Zn}^{2+}$ -induced acidification in this oocyte was much slower ( $-28 \times 10^{-5}\ \text{pH units s}^{-1}$ ) than that induced by  $\text{Fe}^{2+}$  ( $-95 \times 10^{-5} \pm 11 \times 10^{-5}\ \text{pH units s}^{-1}$ ,  $n = 7$ ; see Fig. 4c), but consistent with the lower uptake rates observed for  $^{65}\text{Zn}^{2+}$ . These data indicate that DMT1-mediated  $\text{Zn}^{2+}$  transport at low  $\text{pH}_o$  is  $\text{H}^+$ -coupled, like  $\text{Fe}^{2+}$  transport, and do not support the idea that  $\text{Zn}^{2+}$  transport is limited to the  $\text{H}^+$ -uncoupled, facilitative transport pathway in DMT1.

## Discussion

DMT1-mediated  $\text{Fe}^{2+}$  transport is stimulated by the  $\text{H}^+$  electrochemical potential gradient

The DMT1-mediated  $\text{Fe}^{2+}$  transport is  $\text{H}^+$ -coupled, driven by the  $\text{H}^+$  electrochemical potential gradient. However, coupling is not strict, and significant slippage may occur. Evidence for  $\text{H}^+$ -coupling includes our observations in oocytes expressing wtDMT1 that (i) the  $V_{0.5}$  (midpoint) of the presteady-state charge transfer in the absence of metal ion was  $\text{pH}_o$ -sensitive (Fig. 1), supporting binding/dissociation of  $\text{H}^+$ ; (ii)  $^{55}\text{Fe}^{2+}$  uptake and  $\text{Fe}^{2+}$ -evoked currents were stimulated at low  $\text{pH}_o$  (Figs. 2, 5, 7e); (iii)  $\text{Fe}^{2+}$  transport at low  $\text{pH}_o$  was associated with rapid intracellular acidification (Fig. 4b, c). The  $\text{Fe}^{2+}$ -evoked currents had no  $\text{pH}_o$  optimum, but followed Michaelis–Menten-type saturation kinetics (Fig. 3) and were not inhibited at lower  $\text{pH}_o$ . The half-maximal  $\text{H}^+$  concentration ( $K_{0.5}^{\text{H}}$ ) was  $\approx 1\ \mu\text{M}$ , i.e.  $\text{pH}_o$



**Fig. 8** Mechanisms of DMT1. We propose an eight-state model for the binding and transport of  $\text{H}^+$  and  $\text{Fe}^{2+}$  by rat DMT1. Each reaction step  $x \rightarrow y$  is described by its rate constant  $k_{xy}$ . We anticipate that at least  $k_{16}$ ,  $k_{61}$ ,  $k_{12}$ , and  $k_{21}$  (rates describing reorientation of the empty, charged carrier and binding/dissociation of  $\text{H}^+$ ) will contain voltage-dependent terms, and that rate constants describing binding/dissociation steps are modified by

extracellular or intracellular ligand ( $\text{H}^+$ ,  $\text{Fe}^{2+}$ ) concentrations. This model can be tested, and rate constants estimated, by computer simulation. In addition to  $\text{H}^+/\text{Fe}^{2+}$  cotransport (step  $3 \rightarrow 4$ ), we observed under certain conditions a  $\text{H}^+$  leak or uniport (step  $2 \rightarrow 5$ ) and facilitative ( $\text{H}^+$ -uncoupled)  $\text{Fe}^{2+}$  transport (step  $2a \rightarrow 5a$ )

6.0, which closely matches the extracellular pH within the acid microclimate of the mammalian small intestine brush border. Mucosal surface pH in the jejunum was measured at 6.0–6.2 in the rat in vivo [37, 47, 48] and in control human subjects in situ [38, 46]. Therefore, we anticipate that intestinal absorption of iron will be modulated by small pH changes in the mucosal surface acid microclimate.

Worthington et al. [57] questioned our conclusion [19] that the  $\text{H}^+$  electrochemical potential gradient drives  $\text{Fe}^{2+}$  transport via a saturable cotransport mechanism in DMT1. Finding that  $\text{Fe}^{2+}$  transport in COS-7 cells and Caco-2 cells expressing human DMT1 was nonsaturable and exhibited an optimum at pH 6.75, these authors concluded that the  $\text{Fe}^{2+}$ -evoked currents we observed in oocytes reflect only a  $\text{H}^+$  current and not  $\text{Fe}^{2+}$  transport per se, and that  $\text{Fe}^{2+}$  and  $\text{H}^+$  are uncoupled. Whereas it is known that the inward current exceeds that expected for strict stoichiometric  $\text{H}^+/\text{Fe}^{2+}$  cotransport in oocytes expressing DMT1 [7, 59], we demonstrate here that the pH-dependence of  $^{55}\text{Fe}^{2+}$  uptake is identical to that of the  $\text{Fe}^{2+}$ -evoked currents in oocytes expressing rat DMT1 (see Fig. 2c, c.f. Fig. 7e). The inhibition of  $\text{Fe}^{2+}$  uptake reported in COS-7 or Caco-2 cells at low  $\text{pH}_o$  [57] may have resulted from a loss of integrity of the cell-culture monolayers when exposed to low  $\text{pH}_o$  over the 2 h incubation period. In a separate study [51] using *fully differentiated* Caco-2 cells expressing human DMT1,  $^{55}\text{Fe}^{2+}$  uptake (1 h) displayed a pH dependence similar to that observed by us for DMT1-expressing oocytes. Likewise in CHO cells expressing DMT1 (Nramp2),  $\text{Fe}^{2+}$  or  $\text{Mn}^{2+}$  transport (measured by fluorescence quenching of metal-ion-sensitive dyes) was stimulated at low  $\text{pH}_o$ , and the existence of an optimal  $\text{pH}_o$  was not apparent within the  $\text{pH}_o$  range 7.0–5.0 [14, 29].

## Mechanisms of DMT1

From our present biophysical data, we have arrived at an eight-state model (Fig. 8) to describe the mechanisms of DMT1. We conclude that DMT1 mediates both simultaneous  $\text{H}^+$ -coupled  $\text{Fe}^{2+}$  transport and thermodynamically uncoupled fluxes of  $\text{H}^+$  or  $\text{Fe}^{2+}$  depending on prevailing conditions. Analysis of the partial activities retained by H272A-DMT1, for which  $\text{Fe}^{2+}$  transport is uncoupled from the  $\text{H}^+$  flux, demonstrated that the DMT1 protein possesses the machinery to catalyze facilitative  $\text{Fe}^{2+}$  transport without  $\text{H}^+$  coupling. Our evidence for  $\text{H}^+$ -uncoupled  $\text{Fe}^{2+}$  transport is also derived from the observations that (i) a fraction of the  $^{55}\text{Fe}^{2+}$  uptake and  $\text{Fe}^{2+}$ -evoked currents in DMT1-expressing oocytes persisted at neutral  $\text{pH}_o$ , (Figs. 2c, 7e) and (ii) mathematical fits of saturation kinetics data at  $\text{pH}_o > 6.1$  support competition between  $\text{Fe}^{2+}$  and  $\text{H}^+$  for the empty carrier (Fig. 3a, e). Our data are not explained by a *six-state consecutive transport* model (i.e. one in which only one of the two ligands is translocated in a single cycle), since there is evidence of competition between  $\text{Fe}^{2+}$  and  $\text{H}^+$  (and their subsequent uniport) only when  $[\text{H}^+]_o$  is very low. Instead, our data for  $K_{0.5}^{\text{H}}$  (Fig. 3b) and  $K_{0.5}^{\text{Fe}}$  at  $\text{pH}_o < 6.1$  (Fig. 3e) are consistent with a *simultaneous*  $\text{H}^+/\text{Fe}^{2+}$  cotransport model [25, 49].

Why are alternative six-state simultaneous transport models insufficient to explain our data? We first considered a *six-state ordered-binding simultaneous transport* model (i.e. excluding states 2a and 5a from our model, Fig. 8). The observation of  $\text{H}^+$ -sensitive presteady-state currents only in the absence of metal ion is consistent with  $\text{H}^+$  being the first ligand to bind to DMT1. Since  $\text{Fe}^{2+}$  inhibits the  $\text{H}^+$  leak mediated by H272A-DMT1, in such a model we should expect  $\text{Fe}^{2+}$  binding to lock



the mutant transporter in a nontransporting state (state 3, Fig. 8). This would be analogous with the way in which phlorizin inhibits the  $\text{Na}^+$  leak in the  $\text{Na}^+$ /glucose cotransporters by binding to the sugar-binding site without itself being transported [9, 31, 35, 43, 44]. However,  $\text{Fe}^{2+}$  binding to H272A-DMT1 instead resulted in  $\text{pH}_0$ -independent  $\text{Fe}^{2+}$  transport (Fig. 5).

Can our data satisfy a six-state, simultaneous transport model in which ligand binding is *random*? The dependence of  $I_{\text{max}}^{\text{Fe}}$  on  $[\text{H}^+]_0$  (Fig. 3f) appears to support such a mechanism, and the  $\text{pH}$ -independence of the H272A-DMT1-mediated  $^{55}\text{Fe}^{2+}$  uptake (Fig. 5) might be explained if the impact of the H272A mutation were to markedly increase affinity for  $\text{H}^+$  such that  $0.1 \mu\text{M}$   $\text{H}^+$  ( $\text{pH}_0$  7.0) were saturating. However, in a *random-binding simultaneous transport* mechanism, addition of  $\text{Fe}^{2+}$  to H272A-DMT1 should be expected to accelerate the  $\text{H}^+$  flux and current. Instead, H272A-mediated  $\text{Fe}^{2+}$  transport was associated with a significant inhibition of  $\text{H}^+$  influx (Fig. 4c) and overall current (Fig. 6a, d), indicating that  $\text{H}^+$  and  $\text{Fe}^{2+}$  compete for the empty mutant transporter and are translocated independently (i.e. H272A-DMT1 is described by the model in Fig. 8, excluding states 3 and 4).

A recent observation in the literature lends strong support to our conclusion that DMT1 mediates  $\text{H}^+$  -uncoupled facilitative metal-ion transport at higher  $\text{pH}_0$ . Xu and coworkers measured the reversal potential ( $V_r$  or  $E_{\text{rev}}$ ) of the  $\text{Mn}^{2+}$ -evoked currents in DMT1-transfected CHO cells [59]. The slope of  $V_r$  versus  $\text{pH}_0$  was close to the predicted Nernst potential for a  $\text{H}^+$  selective electrode at  $\text{pH}_0 < 5.8$ . However,  $V_r$  varied little at  $\text{pH}_0 > 5.8$ , indicating an increased permeability for  $\text{Mn}^{2+}$  relative to  $\text{H}^+$  at higher  $\text{pH}_0$ .

Future studies may be directed towards further testing our model (Fig. 8), deriving specific rate constants with the aid of computer simulation, and defining the rate-determining steps. The maximal turnover rate for wtDMT1 (or H267A-DMT1) was  $21\text{--}23 \text{ s}^{-1}$  at  $-150 \text{ mV}$  (Table 2) and much slower at depolarized  $V_m$ . The inverse of this rate provides us with the minimum time required to complete one transport cycle,  $43\text{--}48 \text{ ms}$ . Since most of the presteady-state charge transfer is complete within this time ( $\tau_{\text{max}}$  was  $32 \text{ ms}$  at  $+61 \text{ mV}$ ), we conclude that transporter reorientation (step  $6 \rightarrow 1$ ) and  $\text{H}^+$  binding (step  $1 \rightarrow 2$ ) are not rate-limiting at any  $V_m$ . Since the  $K_{0.5}^{\text{Fe}}$  (which reflects  $\text{Fe}^{2+}$  binding and transport) in wtDMT1 significantly exceeded the  $K_i^{\text{Fe}}$  (reflecting  $\text{Fe}^{2+}$  binding) in H272A-DMT1 (Table 2), we also conclude that  $\text{Fe}^{2+}$  binding (step  $2 \rightarrow 3$ ) is not rate limiting, at least at physiological  $V_m$  ( $-50$  to  $-70 \text{ mV}$ ).

### Transport of $\text{Zn}^{2+}$ by DMT1

The wtDMT1 exhibited a marked preference for  $\text{Fe}^{2+}$  over  $\text{Zn}^{2+}$ , resulting from both a lower maximal transport and a reduced apparent affinity for  $\text{Zn}^{2+}$  compared

with  $\text{Fe}^{2+}$ . This preference was abolished by the H272A mutation, which lowered  $I_{\text{max}}^{\text{Fe}}$  and increased  $K_{0.5}^{\text{Fe}}$ . These kinetic data provide further evidence that His-272 plays a structural role in the DMT1  $\text{H}^+$  -to- $\text{Fe}^{2+}$  coupling mechanism. His-272 also appears to be involved in transducing the effect of  $\text{H}^+$  binding (via a conformational change) to increase the affinity with which DMT1 binds  $\text{Fe}^{2+}$ . On the basis of our H272A data,  $\text{H}^+$  binding may not alter the affinity of DMT1 to bind  $\text{Zn}^{2+}$ . Evidence that  $\text{Zn}^{2+}$  transport is not limited to the facilitative metal-ion transport route comes from the observations that  $\text{Zn}^{2+}$  transport was (i)  $\text{pH}$ -dependent and (ii) associated with intracellular acidification (Fig. 7e, f). Although,  $\text{Zn}^{2+}$  is poorly transported relative to  $\text{Fe}^{2+}$ , we have shown previously that  $\text{Zn}^{2+}$  is one of several divalent metal ions that evoke currents of a similar magnitude to the  $\text{Fe}^{2+}$ -evoked currents in oocytes expressing wtDMT1 at  $-50 \text{ mV}$  [19]. Therefore, DMT1-mediated  $\text{Zn}^{2+}$  transport may be associated with significant slippage of  $\text{H}^+$ , or  $\text{Zn}^{2+}$  may induce in DMT1-expressing oocytes a conductance for another ion.

### Conclusions

We have identified His-272 as critical in the coupling of metal-ion transport to the  $\text{H}^+$  flux through DMT1. Histidyl residues may be titratable within the physiological  $\text{pH}$  range. Since mutating His-272 to an alanyl or arginyl residue disrupted coupling, transient protonation of His-272 may be a requirement for the  $\text{H}^+$  -coupling of DMT1.

DMT1 is responsible both for apical membrane  $\text{Fe}^{2+}$  transport in epithelial systems and for its cellular uptake via TfR-associated endocytosis, as in erythroid precursors. The acid microclimate of the intestinal brush border and the endosomal acidification in erythroid cells provide transmembrane  $\text{H}^+$  gradients that will allow DMT1 to be highly concentrative. However, our study reveals for the first time an uncoupled  $\text{Fe}^{2+}$  transport mode in DMT1, with  $\text{Fe}^{2+}$  transport driven by the electrochemical gradient for  $\text{Fe}^{2+}$  alone. Whereas  $\text{H}^+$ -coupling (and the  $\text{H}^+$  -dependent increase in affinity for  $\text{Fe}^{2+}$ ) in *acidified* endosomes will ensure that DMT1 is an effective scavenger of endosomal  $\text{Fe}^{2+}$ , DMT1-mediated facilitative  $\text{Fe}^{2+}$  transport may permit mobilization of  $\text{Fe}^{2+}$  from endosome to cytosol even before significant luminal acidification. Notably, in erythroid precursors,  $\text{Fe}^{3+}$  may be liberated from the transferrin-TfR complex following only a small drop in endosomal  $\text{pH}$  [40].

Our identification of facilitative  $\text{Fe}^{2+}$  transport at neutral  $\text{pH}$  raises the possibility that DMT1 may also be operational at the *plasma* membrane of cells facing a neutral- $\text{pH}$  environment. Whether this is of physiological relevance in various cell types will have to be determined, but it is likely to be of significant importance in iron overload conditions. For example, substantial increases in nontransferrin-bound plasma iron (NTBI) are common in the hemoglobinopathies and disorders

involving defective erythropoiesis, e.g. thalassemia (compounded by transfusional iron overload), and in hereditary hemochromatosis [8, 17, 22, 42]. Much of this NTBI will be cleared by the liver after it is reduced to  $\text{Fe}^{2+}$  [54, 58], and strong evidence exists for the presence of ferrireductase activity at the cell surface of hepatocytes [39] and other cell types [24, 26]. Notably, DMT1 is upregulated at the plasma membrane of hepatocytes in iron overload [53] and is also expressed in the heart [19, 23, 27], a major site of iron toxicity in overload [20]. Thus,  $\text{H}^+$ -uncoupled  $\text{Fe}^{2+}$  transport mediated by DMT1 expressed at the plasma membrane can account for the hepatic accumulation of NTBI and directly contribute to the etiology of hepatic and cardiac toxicity in iron overload disorders.

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