Retromer-mediated direct sorting is required for proper endosomal recycling of the mammalian iron transporter DMT1

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Summary
Endosomal recycling of the mammalian iron transporter DMT1 is assumed to be important for efficient and rapid uptake of iron across the endosomal membrane in the transferrin cycle. Here, we show that the retromer, a complex that mediates retrograde transport of transmembrane cargoes from endosomes to the trans-Golgi network, is required for endosomal recycling of DMT1-II, an alternative splicing isoform of DMT1. Bacterially expressed Vps26-Vsp29-Vsp35 trimers, a retromer cargo recognition complex, specifically binds to the cytoplasmic tail domain of DMT1-II in vitro. In particular, this binding is dependent on a specific hydrophobic motif of DMT1-II, which is required for its endosomal recycling. DMT1-II colocalizes with the Vps35 subunit of the retromer in TR-positive endosomes. Depletion of the retromer by siRNA against Vps35 leads to mis-sorting of DMT1-II to LAMP2-positive structures, and expression of siRNA-resistant Vps35 can rescue this effect. These findings demonstrate that the retromer recognizes the recycling signal of DMT1-II and ensures its proper endosomal recycling.

Key words: Endosome, Retromer, Recycling, DMT1, Iron metabolism, Transferrin cycle

Introduction
The steady-state localization of transmembrane (TM) proteins in the endocytic system is a consequence of many sorting events that occur at various points throughout the endosomal pathway. The sorting decisions are governed by a complex system of sorting signals in the itinerant proteins and molecular machineries that recognize those signals and deliver the proteins to their intended destinations (Bonifacino and Traub, 2003; Seaman, 2008). In general, sorting signals are located in the cytoplasmic domain of TM proteins, and some TM proteins have two or more sorting signals in their individual cytoplasmic domains, which allow them a more complex and unique itinerary in the endosomal pathway. For instance, the cation-independent mannose-6-phosphate receptor (CI-MPR), which sorts lysosomal hydrolase precursors to lysosomes, shuttles between the trans-Golgi network (TGN) and endosomes by the actions of several different sorting mechanisms (Ghosh et al., 2003). Thus, intracellular traffic of CI-MPR is regulated by several different sorting signals coupled with each sorting mechanism, which ensures its proper recycling between the TGN and the endosomes. Unlike single-pass TM proteins such as CI-MPR, the multipass TM proteins, such as nutrient transporters, have several cytoplasmic domains that make it more difficult to understand the molecular mechanisms for their sorting events in the endosomal pathway.

Divalent metal transporter 1 (DMT1 or SLC11A2, formerly known as NRAMP2/DCT1) is an integral membrane protein consisting of 12 predicted TM domains with two potential N-glycosylation sites; both of its N- and C-terminal tail domains face the cytosol (Gunshin et al., 1997). DMT1 has been shown to transport a number of divalent metals including Fe²⁺, Mn²⁺, Co²⁺, Cu²⁺, Ni²⁺, Pb²⁺, Zn²⁺ and Cd²⁺ by a proton cotransport mechanism (Gunshin et al., 1997). A mutation in Dmt1 (G185R) has been identified in microcytic anemia (mk) mice and Belgrade (b) rats (Fleming et al., 1997; Fleming et al., 1998), which have significant defects in intestinal iron absorption and assimilation of iron by erythroid precursor cells, indicating that the protein has lost the iron transport function in those tissues of the mutant animals. Indeed, DMT1 localizes at the brush border of duodenal enterocytes (Canonne-Hergaux et al., 1999), where it is responsible for dietary iron absorption across the apical plasma membrane, and in the endocytic compartments of cells of peripheral tissues (Su et al., 1998; Gruenheid et al., 1999; Tabuchi et al., 2000; Tabuchi et al., 2002), which have significant defects in intestinal iron absorption and assimilation of iron by erythroid precursor cells, indicating that the protein has lost the iron transport function in those tissues of the mutant animals. Indeed, DMT1 localizes at the brush border of duodenal enterocytes (Canonne-Hergaux et al., 1999), where it is responsible for dietary iron absorption across the apical plasma membrane, and in the endocytic compartments of cells of peripheral tissues (Su et al., 1998; Gruenheid et al., 1999; Tabuchi et al., 2000; Tabuchi et al., 2002), where it is responsible for the transport across endosomal membranes of iron internalized via the transferrin (Tf)-transferrin receptor (TIR) complex.

Dmt1 produces at least two distinct mRNAs by alternative splicing of two 3’ exons encoding different 3’ untranslated regions
mediated sorting of DMT1-II to the recycling pathway and its role subsumed by the hematopoietic hormone erythropoietin (Canonne-Hergaux et al., 2001). Recently, additional isoforms of Dmt1 mRNA have been identified based on alternative promoter usage at exon 1 (exon1A vs 1B) (Hubert and Hentze, 2002). This alternative promoter usage is assumed to produce a DMT1 protein bearing an additional 29 amino acids (exon1A) upstream of the previously identified start codon of DMT1-I and II (exon1B) (Hubert and Hentze, 2002). The role of these additional residues in the function and targeting of DMT1 has not yet been studied. However, recently, it has been shown that DMT1-I expression from the exon1A promoter is activated by hypoxia-inducible factor signaling induced in the duodenum following an acute iron deficiency (Shah et al., 2009). This indicates that DMT1-I, with an N-terminus derived from exon1A, specifically functions in dietary iron absorption from the apical plasma membrane in the duodenum. These observations suggest that the cytoplasmic tail domains of the N- and C-termini of the DMT1 molecule are substituted by the alternative promoter usage and the alternative splicing, respectively, in a tissue-specific manner, and that these substitutions adapt the DMT1 localization to the tissue-specific iron acquisition (i.e. the dietary iron absorption from the apical plasma membrane in the duodenum and endosomal iron acquisition in the Tf cycle).

Previously, we demonstrated that isoforms DMT1-I and DMT1-II, with their N-termini derived from exon1B, are targeted to distinct endosomal compartments. We also identified critical amino acids in the cytoplasmic tail domain of DMT1-II as a determinant for the isoform-specific localization (Tabuchi et al., 2002). It has recently been shown that this signal is required for endosomal recycling of DMT1-II, and sorting of DMT1-II to the recycling pathway occurs at the endosomes (Touret et al., 2003; Lam-Yuk-Tseung et al., 2005). In contrast to DMT1-II, DMT1-I is not efficiently recycled but is subsequently targeted to LAMP2-positive structures (Tabuchi et al., 2000; Tabuchi et al., 2002; Lam-Yuk-Tseung and Gros, 2006). These data indicate that a putative sorting receptor specifically recognizes the recycling signal of DMT1-II and ensures its endosomal recycling. However, the molecular basis for the sorting of signal-dependent endosomal recycling of DMT1-II has yet to be elucidated.

In this study, we report a molecular mechanism underlying the sorting signal-dependent endosomal recycling of DMT1-II. We found that the retromer is required for proper endosomal recycling of DMT1-II. We discuss the molecular mechanism for retromer-mediated sorting of DMT1-II to the recycling pathway and its role on iron acquisition in the Tf cycle.

Results

Structural requirements for endosomal recycling of DMT1-II

To identify the structural requirements for the recycling signal of DMT1-II, we performed a detailed mutational analysis of the cytoplasmic tail sequence. We expected that this approach might reveal a conserved sequence motif by comparison with known sorting signal sequences and then allow us to identify the molecule(s) required for endosomal recycling of DMT1-II. We constructed various GFP-tagged mutants of DMT1-II, which had deletions or amino acid substitutions within the 25-amino acid sequence specific for DMT1-II, to narrow the region required for endosomal recycling of DMT1-II, and to identify the critical amino acids and the structural requirements (Fig. 1A and listed in Fig. 1G). These constructs were expressed in HEp-2 cells that were immunostained with antibodies against GFP and LAMP2, a marker for organelles of the late endocytic pathway. Their localizations were analyzed by confocal microscopy. To measure the colocalization of DMT1-II mutants with LAMP2, pixel-by-pixel analysis using Pearson's correlation coefficient ($P_r$) was used and $P_c$ with LAMP2 ($P_{c\mathrm{LAMP}2}$) was calculated. A $P_r$ value of 1 indicates perfect colocalization. During the course of this analysis, we noticed that the localization of these mutants could be divided into three classes based on the comparison between their images and $P_{c\mathrm{LAMP}2}$ counts (typical patterns of their localization and $P_{c\mathrm{LAMP}2}$ counts are shown in Fig. 1B,C): 

- **Class A’ mutants**, similarly to DMT1-I, displayed a predominant localization in the perinuclear region of transfected cells and significantly colocalized with LAMP2 ($P_{c\mathrm{LAMP}2}$<0.8), indicating a severe defect of endosomal recycling of DMT1-II, as typified by the DMT1-II Y555A mutant. 
- **Class A** mutants displayed a dual localization in both the perinuclear region and in punctate structures diffusely scattered throughout the cytoplasm, and a partial colocalization of GFP signals with LAMP2 was observed exclusively in the perinuclear region ($0.5>P_{c\mathrm{LAMP}2}$<0.8), indicating a partial defect of endosomal recycling of DMT1-II, as typified by the DMT1-II Y555A mutant. 

- **Class C** mutants displayed a predominant localization in punctate structures scattered throughout the cytoplasm, and almost no colocalization of GFP signals with LAMP2 ($P_{c\mathrm{LAMP}2}$<0.5) was observed, indicating normal endosomal recycling of DMT1-II, as typified by the DMT1-II wild-type. Thirty cells for each sample were examined and their localizations were scored by the above criteria (Fig. 1D-F).

Deletion analysis of the cytoplasmic tail domain of DMT1-II mapped the most important element for endosomal recycling within the eight amino acid stretch 551-558 of DMT1-II (exon1A). Alanine-scanning analysis in this region confirmed that Tyr555 and Leu557 are the crucial amino acids for endosomal recycling of DMT1-II, which is in good agreement with our previous report (Tabuchi et al., 2002). Notably, the L557A mutant displayed a severe mis-sorting of DMT1-II to LAMP2-positive structures, whereas the Y555A mutant displayed a modest mis-sorting ($P_{c\mathrm{LAMP}2}$ L557A vs Y555A = 0.86±0.024; $n=30$ vs 0.71±0.126; $n=30$, $P=0.0001$, Fig. 1E), indicating that Leu557 is a more important residue for proper endosomal recycling of DMT1-II than Tyr555. Deletion of the Y555LL sequence led to a complete mis-sorting ($P_{c\mathrm{LAMP}2}$ L557A vs Y555A = 0.86±0.024; $n=30$ vs 0.71±0.126; $n=30$, $P<0.0001$, Fig. 1E), indicating that Tyr555 is a more important residue for proper endosomal recycling of DMT1-II than Tyr555. Deletion of the Y555LL sequence led to a complete mis-sorting ($P_{c\mathrm{LAMP}2}$ L557A vs Y555A = 0.86±0.024; $n=30$ vs 0.71±0.126; $n=30$, $P<0.0001$, Fig. 1E), indicating that Ty}
of Tyr555 with Ser led to a significant mis-sorting of DMT1-II to LAMP2-positive structures ($P_{\text{cLAMP2}} = 0.86\pm0.043$; $n=30$, Fig. 1F), whereas substitution by Phe, Trp or Leu did not affect their localizations (Fig. 1F,G), suggesting that any hydrophobic amino acid at position 555, either aromatic or aliphatic, is sufficient for endosomal recycling of DMT1-II. Substitution of Leu557 by any
hydrophobic amino acid except for Met led to a striking mis-sorting of DMT1-II to LAMP2-positive structures (Fig. 1F,G). On the basis of the detailed mutational analysis of the cytoplasmic tail domain of DMT1-II in this study, we conclude that a Ox(Leu/Met)x motif (where Ø can be any hydrophobic amino acid and x can be any amino acid) is the recycling signal responsible for proper endosomal recycling of DMT1-II.

**Structural similarity between the recycling signal of DMT1-II and the retromer recognition sequence of CI-MPR**

To find sequence motif(s) that are similar to the recycling signal of DMT1-II, we investigated the known sorting motifs and found a structurally similar sequence motif WLM, which has been recently identified in the CI-MPR tail as a sorting motif required for retromer-mediated endosome-to-TGN retrieval (Seaman, 2007). The retromer was originally identified as an essential protein complex in yeast for the proper retrieval of the vacuolar hydrolase receptor Vps10, a functional analog of mammalian CI-MPR, from endosomes back to the TGN (Seaman et al., 1998), and the mammalian retromer has also been shown to function in endosome-to-TGN retrieval of CI-MPR (Argihi et al., 2004; Carlton et al., 2004; Seaman, 2004). The mammalian retromer comprises a sorting nexin (SNX) dimer composed of four possible combinations of SNX1-SNX5, SNX1-SNX6, SNX2-SNX5 and SNX2-SNX6 (Wassmer et al., 2009), and a cargo-recognition complex composed of Vps26, Vps29 and Vps35 (Bonifacino and Hurley, 2008). Intriguingly, recent reports showed that the retromer also functions in the endosomal recycling of several TM proteins including the yeast iron transporter Fet3-Ftr1 complex (Strochlic et al., 2007) and Wntless, a membrane protein dedicated to the secretion of the morphogen Wnt proteins (Eaton, 2008). The sequence similarity and recent findings of the retromer function in membrane protein recycling prompted us to examine the potential involvement of the retromer in endosomal recycling of DMT1-II. We first swapped the YLL motif in the cytoplasmic tail domain of DMT1-II for WLM and analyzed the localization of this mutant by immunofluorescence confocal microscopy. This substitution did not affect the proper localization of DMT1-II, indicating that the WLM motif is sufficient for endosomal recycling, even in the case of DMT1-II (Fig. 1F,G). Seaman (Seaman, 2007) has also reported the FLV motif in sortilin as a retromer recognition sequence. However, the FLV motif is not sufficient for endosomal recycling in the case of DMT1-II (Fig. 1F,G).

**Retromer directly interacts with the recycling signal of DMT1-II in vitro**

We next examined whether the recycling signal of DMT1-II could directly bind to the retromer complex. Bacterially expressed glutathione S-transferase (GST)-DMT1-II tail domain (Fig. 2A) was used to pull down a bacterially expressed FLAG-tagged retromer cargo-recognition complex, which consists of 3×FLAG-Vps26-Vps29-Vps35-Hiø6 (Fig. 2B). Since a previous study showed that the cytoplasmic tail domain of CI-MPR interacts with Vps35 using a yeast two-hybrid assay (Argihi et al., 2004), we used the GST-CI-MPR tail (Fig. 2A) as a positive control. Immunoblot analysis showed that both GST-CI-MPR and GST-DMT1-II tails, but not GST alone, efficiently pulled down the recombinant retromer in a dose-dependent manner (Fig. 2C), showing that the cytoplasmic tail domain of DMT1-II can directly bind to the retromer. Strikingly, this interaction was almost completely abrogated by deletion of the YLL sequence from the DMT1-II tail domain (Fig. 2C), which leads to a complete mis-sorting of DMT1-II to LAMP2-positive structures in HEP-2 cells (Fig. 1E,G).

Using the results of the in vivo mutagenesis experiments (Fig. 1G), we next expressed GST-DMT1-II tail fusion proteins with nonfunctional mutations Y555A or L557A, and GST-DMT1-II tail fusion proteins with the functional mutation L557M. These proteins were used for pull-down assays to investigate the binding specificity of the DMT1-II-cytoplasmic tail domain to the retromer. As shown in Fig. 2D, both the GST-DMT1-II tail Y555A and L557A mutant pulled down the retromer less efficiently than the GST-DMT1-II tail wild type (reduced to <65% compared with that of the wild type). Notably, the GST-DMT1-II tail L557M mutant pulled down the retromer more efficiently (increased to more than 125% compared with the wild type), indicating that this L557M mutant has higher affinity to the retromer than the wild type. The binding specificity of the DMT1-II tail domain to the retromer in vitro was well correlated with the structural requirements for the recycling signal of DMT1-II in cultured cells.

**DMT1-II colocalization with the retromer in TfR-positive endosomes**

We next determined the subcellular localization of DMT1-II and the retromer. HEP-2 cells stably expressing 3×HA-tagged DMT1-II were immunostained with antibodies against HA epitope Vps35 and various organelar markers. A triple-staining experiment showed that DMT1-II colocalized with Vps35 in relatively large puncta, where TfR, a marker for organelles of the early endocytic and recycling pathways, also colocalized with Vps35 (Fig. 3Ae-h, arrows). To clarify the localization of DMT1-II and Vps35 double-positive punctate-structures, antibody against EEA1, a marker for organelles of the early endocytic pathway, was used for triple staining instead of TfR (Fig. 3B). Only the small patches of the DMT1-II and Vps35 double-positive punctate structures overlapped with EEA1, whereas the majority of the double-positive punctate structures were juxtaposed to EEA1 and not colocalized with EEA1 (Fig. 3Be-h, arrows). Furthermore, these double-positive punctate structures colocalized particularly with CI-MPR in the perinuclear region (supplementary material Fig. S1Ae-h, arrows) but not with TGN marker p230 or recycling endosome marker Rab11 (supplementary material Fig. S1B,C). These data showed that DMT1-II colocalizes with the retromer in TfR-positive endosomes, whereas EEA1 is mostly excluded from this configuration. These results support the hypothesis that the retromer might regulate endosomal recycling of DMT1-II at maturing endosomes (see Discussion).

**Retromer depletion by RNAi leads to mis-sorting of DMT1-II to LAMP2-positive structures**

To examine directly whether the retromer is required for endosomal recycling of DMT1-II, we knocked down VPS35 mRNA using small interfering RNA (siRNA). HEP-2 cells stably expressing 3×HA-tagged DMT1-II were transfected with two types of siRNA oligonucleotide pairs against VPS35: VPS35 siRNA#1 and #2. After two rounds of transfection at 72 hour intervals with each of these siRNAs, the protein expression of Vps35 decreased to undetectable levels in both samples of siRNA-treated cells, which was confirmed by immunoblot analysis using anti-Vps35 antibody (Fig. 4A). The protein expression of DMT1-II was not significantly affected by depletion of Vps35 (Fig. 4A). Notably, an additional band at approximately 40 kDa was observed in Vps35-depleted cells but not in control siRNA-treated cells (Fig. 4A, indicated with a single asterisk).
We next examined the localization of DMT1-II in Vps35-depleted cells. We observed that DMT1-II colocalized with TfR but not with LAMP2 in control siRNA-treated cells (Fig. 4Ba-d and i-l, respectively). However, upon depletion of Vps35 by treatment with VPS35 siRNA#1, localization of DMT1-II changed dramatically. We observed almost no colocalization of DMT1-II with TfR in Vps35-depleted cells (Fig. 4Be-h). Instead, DMT1-II colocalized well with LAMP2 (Fig. 4Bm-p). To quantify this effect, we performed pixel-by-pixel colocalization analysis and calculated $P_c$. Depletion of Vps35 caused a significant reduction of $P_{c,TfR}$ ($P_c$ with TfR) (control siRNA vs VPS35 siRNA=0.61±0.09; $n=50$ vs 0.28±0.14; $n=50$, $P<0.0001$; Fig. 4C), and conversely a significant increase of $P_{c,LAMP2}$ (control siRNA vs VPS35 siRNA=0.12±0.10; $n=50$ vs 0.77±0.06; $n=50$, $P<0.0001$; Fig. 4D). This analysis statistically confirmed that DMT1-II was mis-sorted to LAMP2-positive structures in Vps35-depleted cells, indicating that endosomal recycling of DMT1-II was perturbed by the loss of retromer function.

Phenotypic rescue of Vps35-depleted cells by expression of siRNA-resistant Vps35

To further confirm the retromer function in endosomal recycling of DMT1-II, we monitored the rescue of this phenotype by...
transfecting Vps35-depleted cells with GFP alone, or GFP-tagged siRNA-resistant Vps35 wild-type or GFP-tagged nonfunctional mutant Vps35 R107A (Gokool et al., 2007). The expression of the siRNA-resistant Vps35 wild-type in Vps35-depleted cells well restored the mis-sorting of DMT1-II. GFP-Vps35-positive cells in Vps35-depleted cells displayed almost no colocalization of DMT1-II with LAMP2 (Fig. 5Aa-e), but significant colocalization of DMT1-II with TfR (supplementary material Fig. S2Aa-e). To measure the colocalization of DMT1-II with LAMP2 in both GFP-Vps35-positive and -negative cells, pixel-by-pixel analysis was used and $P_c$ with LAMP2 was calculated. This analysis revealed almost no colocalization of DMT1-II with LAMP2 in GFP-Vps35-positive cells ($P_c$LAMP2 = 0.24±0.16; n=50; Fig. 5Aa-e), but did show significant colocalization in GFP-Vps35-negative cells ($P_c$LAMP2 = 0.62±0.10; n=50; Fig. 5Aa-e). These results confirmed that expression of GFP-Vps35 rescued the defect of endosomal recycling of DMT1-II in endogenous Vps35-depleted cells.

In contrast to the wild-type GFP-Vps35, neither GFP alone nor GFP-tagged nonfunctional mutant Vps35 R107A could rescue this phenotype (supplementary material Fig. S2B,C). More than 90% of cells displayed colocalization of DMT1-II with LAMP2 in cells positive for GFP alone or GFP-Vps35 R107A, whereas less than 3% of cells displayed colocalization of DMT1-II with LAMP2 in cells positive for wild-type GFP-Vps35 (Fig. 5B; supplementary material Fig. S2C,D), confirming that the rescue of DMT1-II localization by ectopic expression of siRNA-resistant Vps35 in Vps35-depleted cells is due to the restoration of normal retromer function. Thus, these results demonstrate that the defect of endosomal recycling of DMT1-II in Vps35-depleted cells is a direct consequence of loss of retromer function.

In conclusion, these results strongly suggest that DMT1-II is recognized by a retromer cargo-recognition complex via its recycling signal at maturing endosomes, thereby becoming incorporated into tubular vesicles formed by the retromer and sorting DMT1-II to the recycling pathway.

**Discussion**

In this study, we report a molecular mechanism underlying the sorting-signal-dependent endosomal recycling of DMT1-II. First, we identified the structural requirements for the recycling signal of DMT1-II and found the retromer to be a candidate molecule required for endosomal recycling of DMT1-II. Second, we demonstrated that the retromer cargo-recognition complex could directly bind to the cytoplasmic tail domain of DMT1-II in vitro in a recycling-signal-dependent manner. Third, we showed that DMT1-II colocalized with a Vps35 subunit of the retromer in TfR-positive endosomes. Finally, we showed that depletion of Vps35 by RNAi led to a complete mis-sorting of DMT1-II to LAMP2-positive structures, and ectopic expression of siRNA-resistant Vps35 rescued this effect.
Cumulatively, these data argue that the retromer can directly bind and sort the DMT1-II molecule at maturing endosomes (Fig. 6B), thereby ensuring proper endosomal recycling of this molecule.

**Retromer cargo-recognition mechanism**

Detailed mutational analysis of the cytoplasmic tail domain of DMT1-II revealed the structural requirements for the recycling signal of DMT1-II and identified the Ox(Leu/Met)x motif as its recycling signal. This motif is also required for the binding of the DMT1-II-cytoplasmic tail domain to the retromer in vitro. These data suggest that this motif represents a novel retromer-binding motif. So far, no strong consensus retromer-binding motifs have been identified, and none have been characterized at a structural level (Bonifacino and Hurley, 2008; Collins, 2008). The WLM motif of C1-MPR (Seaman, 2007) fits the structural requirement for the recycling signal of DMT1-II and the swapping of the YLL motif in DMT1-II for the WLM motif did not affect its proper localization in HEp-2 cells, showing that the WLM motif is sufficient for endosomal recycling of DMT1-II. Other known retromer-recognition sequences, such as FLV in sortilin (Seaman, 2007) or FxFxD in yeast DPAP-A (Nothwehr et al., 2000), are structurally similar, but the residues Val and Phe at amino acid position 2 in these sequences do not fit the structural requirement for the recycling signal of DMT1-II (Fig. 6A). Furthermore, the FLV motif is not sufficient for endosomal recycling in the case of DMT1-II. We found that the upstream and downstream sequences of the crucial amino acids Y555LL were also important for the proper recycling of DMT1-II (Fig. 6D,E,G). The upstream and downstream sequences of the FLV motif in sortilin might also be important for the efficient binding of this motif to the retromer.
In addition to the known retromer-binding sequences, we found structurally similar sequences F746LLQ and Y532KLT in the cytoplasmic tail domains of human polymeric immunoglobulin receptor (pIgR) and human Wntless, respectively, both of which are known to be retromer cargo proteins (Verges et al., 2004; Yang et al., 2008). Mostov and colleagues have shown that the retromer directly binds to the cytoplasmic tail domain of pIgR and regulates transcytosis of pIgR from the basolateral to the apical surface (Verges et al., 2004). Interestingly, they showed that a pIgR mutant lacking the 30 C-terminal residues of its cytoplasmic domain, which completely lacks the F746LLQ sequence, impaired its retromer-binding ability. Furthermore, the same group recently found that the F746LLQ sequence of pIgR is sufficient to transcytose the pIgR from the basolateral to the apical surface (Luton et al., 2009). These data suggest that the F746LLQ sequence functions as a retromer-binding sequence, which is in good agreement with our prediction. The relative contribution of the Y532KLT to retromer-dependent recycling of human Wntless remains to be determined. Further experiments are needed to define the consensus retromer-binding motif.

**Endosomal sorting pathways for DMT1 isoforms**

Endosomes are highly dynamic organelles that comprise distinct subdomains occupied by different Rab proteins within the same continuous membrane. For example, during endocytosis of degradative cargos, such as low-density lipoproteins to lysosomes, endosomes containing the cargos gradually mature from early to late stages with a continuous conversion of Rab5 to Rab7 (Rink et al., 2005). It has recently been reported that the retromer cargo-recognition complex is recruited onto the endosomal membrane by the action of GTP-bound Rab7 (Rojas et al., 2008; Seaman et al., 2009). Based on our present results and these recent findings, we assume that the endosomal recycling pathway for DMT1-II is as follows: Rab5-positive endosomes containing DMT1-II gradually mature by recruiting the GTP-bound Rab7, which further recruits the retromer cargo-recognition complex onto its endosomal membrane (Fig. 6B). DMT1-II is subsequently recognized by the retromer cargo-recognition complex via its recycling signal and incorporated into the tubular vesicle formed by the retromer cargo-recognition complex and the BAR-domain-containing proteins SNXs, which stimulate membrane-curvature formation (McMahon and Gallop, 2005). DMT1-II is then transported to the TGN before endosomes are completely matured. At the TGN, DMT1-II is transported via the recycling endosome en route to the plasma membrane. Since DMT1-I cannot be recognized by the retromer, this isoform cannot escape from maturing endosomes and is consequently transported to LAMP2-positive structures.

Although depletion of the retromer by treatment with VPS35 siRNA caused a complete mis-sorting of DMT1-II to LAMP2-positive structures, we did not observe any significant reduction of the DMT1-II protein level in Vps35-depleted cells. Nevertheless, we did observe a faint band at ~40 kDa, which presumably corresponds to a degradation product of DMT1-II (Fig. 3A). In addition, Vps35-depleted cells treated with cycloheximide for 12 hours displayed a DMT1-II protein level that was comparable with that in control cells treated with ethanol for 12 hours (supplementary
and endosomes (Andrews, 1999; Touret et al., 2003; Andrews, 2008). However, the molecular mechanisms underlying their recycling from endosomes to the plasma membrane are apparently distinct. DMT1-II is directly recognized by the retromer cargo-recognition complex and transported through the TGN en route to the recycling endosome, whereas TfR is directly transported to the recycling endosome by a retromer-independent mechanism (Traer et al., 2007), and subsequently both molecules are recycled back to the plasma membrane for another round of the Tf cycle (Fig. 6B). Since endosomes are highly dynamic organelles forming a continuum of maturing organelles from early to late stages (Bonifacino and Rojas, 2006), the retromediator-mediated dynamic recycling system must be important for retention of the iron transporter at TfR-positive endosomes to facilitate the endosomal iron uptake in the Tf cycle, which is particularly important for the production of hemoglobin in mitochondria in erythroid precursors (Fig. 6B). Further studies are required to gain a better understanding of the relative contribution of retromediator-mediated endosomal recycling of DMT1-II on endosomal iron uptake in the Tf cycle.

Role for retromediator-mediated endosomal recycling of DMT1-II in the Tf cycle

In the Tf cycle, DMT1-II and TfR are functionally coupled to operate pH-dependent iron uptake across the endosomal membrane, and both molecules are recycled between the plasma membrane and endosomes (Andrews, 1999; Touret et al., 2003; Andrews, 2008). However, the molecular mechanisms underlying their recycling from endosomes to the plasma membrane are apparently distinct. DMT1-II is directly recognized by the retromer cargo-recognition complex and transported through the TGN en route to the recycling endosome, whereas TfR is directly transported to the recycling endosome by a retromer-independent mechanism (Traer et al., 2007), and subsequently both molecules are recycled back to the plasma membrane for another round of the Tf cycle (Fig. 6B). Since endosomes are highly dynamic organelles forming a continuum of maturing organelles from early to late stages (Bonifacino and Rojas, 2006), the retromediator-mediated dynamic recycling system must be important for retention of the iron transporter at TfR-positive endosomes to facilitate the endosomal iron uptake in the Tf cycle, which is particularly important for the production of hemoglobin in mitochondria in erythroid precursors (Fig. 6B). Further studies are required to gain a better understanding of the relative contribution of retromediator-mediated endosomal recycling of DMT1-II on endosomal iron uptake in the Tf cycle.

Materials and Methods

Antibodies and reagents

We used mouse or rat monoclonal antibodies (mAbs) against the following proteins or epitope tags: EAE1, p230 and Rab11 (BD Biosciences); Cl-MPR (2G11; Affinity Biosciences); LAMP2 (H4B4, Developmental Studies Hybridoma Bank); α-tubulin (GE Healthcare); HA tag (mouse mAb HA.11, Covance; rat mAb 3F10, Roche Applied Science); FLAG tag (M2, Sigma); TIR [N2, provided by Tamotsu Yoshimori, Osaka University, Osaka, Japan (Yoshimori et al., 1988)]. A rabbit polyclonal antibody (pAb) to the FLAG tag was obtained from Sigma, and one to the HA tag was obtained from MBL, Japan. A goat pAb to Vps35 was obtained from Immunex. We raised a rabbit pAb against the GFP. A rabbit was immunized with a maltose-binding protein-GFP fusion protein, and the anti-GFP antiseraum was affinity purified using a GST-GFP fusion-protein-immobilized column. Goat anti-rabbit IgG or donkey anti-mouse IgG conjugated with Alexa Fluor 488, goat anti-rat IgG or donkey anti-rabbit IgG conjugated with Alexa Fluor 555, and goat anti-mouse IgF or donkey anti-goat IgG conjugated with Alexa Fluor 633 were purchased from Invitrogen. Horseradish-peroxidase-conjugated anti-mouse and anti-rabbit IgG was purchased from Cell signaling technology. Horseradish-peroxidase-conjugated anti-goat IgG was purchased from R&D systems. Most of the other general reagents were from Wako chemicals, Nacalai Tesque or Sigma.

Recombinant DNA procedures

To introduce the mutations into the cytoplasmic domain of DMT1-II, the pUC13-DMT1-II plasmid (Tabuchi et al., 2002) was used for site-directed mutagenesis to change the deletion or alanine-scanning mutants. Site-directed mutagenesis was performed by the inverse PCR method (Imai et al., 1991). The plasmids carrying mutant constructs were prepared from the Escherichia coli strain SCS110 (Stratagene) and digested with BciI. The BciI fragments containing the mutated constructs of DMT1-II were ligated into the BamHI site of pIREs neo-GFP plasmid (Clontech) to generate the mammalian expression plasmids carrying GFP-tagged DMT1-II mutants.

To generate plasmids encoding GFP or FLAG-tagged VPS26, VPS29 and VPS35, DNA encoding full-length human Vps26, Vps29 and Vps35 was amplified by PCR using KOD plus DNA polymerase (ToyoBo). Each amplified fragment was cloned into pDONR221 by BP reaction using Gateway™ technology (Invitrogen) to generate entry clones for VPS26, VPS29, and VPS35. Each entry clone was transferred into pIREs neo-GFP or 3×FLAG-based Gateway vectors by LR reaction to generate pIREs neo-GFP or 3×FLAG-VPS26, VPS29 and VPS35 plasmids.

To generate a plasmid encoding VPS35 siRNA#1-resistant wild type of VPS35, pDONR221-siRNA#1-VPS35 WT, inverse PCR was performed with primers (the forward primer: 5′-GTA GGT AGC TTA CAA GTC TCT-3′; the reverse primer: 5′-GTC TTC ACT AGG CAT GTC TTG-3′; substituted nucleotides without an amino acid change are underlined) corresponding to the sequence of VPS35 siRNA#1 using the pDONR221-VPS35 plasmid as a template, and the PCR product was self-ligated with T4 DNA ligase and T4 poly nucleotide kinase (see supplementary material Fig. S4 for further details). The resulting clone was transferred into a pIREs neo-GFP-based Gateway vector by LR reaction to generate a pIREs neo-GFP-siRNA#1-VPS35 WT plasmid.

To generate a plasmid expressing the GST-cl-MPR tail fusion protein in E. coli, the fragment containing the entire encompassing residues 2328-2491 of the human Cl-MPR was amplified by PCR from a human skin fibroblast cDNA library. The amplified fragment was digested with BamHI and Xhol, and the BamHI/Xhol fragment was cloned into the BamHI/Xhol sites of pGEX-5X-1 (GE Healthcare) to generate a pGEX-5X-1-cl-MPR tail plasmid.
To generate plasmids expressing fusion proteins of GST-DMT1-II tail wild-type or various mutants, the human iron transporter cDNA encoding residues 533-568 of human DMT1-II was amplified by PCR from pUC13-DMT1-II plasmids containing a wild-type or mutant version of DMT1-II, as described above. The amplified fragments were digested with EcoRI and XhoI, and the EcoRI-XhoI fragments were cloned into the EcoRI-XhoI sites of pGEX 5X-1 to generate plasmids of pGEX-5X-1-DMT1-II tail wild type or various mutants. To generate a plasmid expressing the 3×FLAG-tagged retroroller complex in E. coli, DNA fragments encoding 3×FLAG-tagged full-length molecules of each Vps29, Vps35, and Vps35 with a Shine-Dalgarno sequence at 5′ upstream of each ORF, were amplified by PCR from pIRES neo-3/H11003 respectively. The amplified fragments were sequentially cloned into pET23d plasmid in the order of 3×FLAG-P26, 3×FLAG-P35 and then 3×FLAG-P35 to generate a pET23d-3×FLAG-Retromer plasmid. 3×FLAG-Vps35 was fused to a C-terminal His6 tag. The cloned regions of all plasmids were completely sequenced to ensure that unplanned mutations were not introduced during the cloning procedures.

**Protein expression and purification**

For purification of the GST-fusion protein, Rosetta-gamiB (DE3) cells (EMD) transformed with protein expression plasmids were grown in Luria broth with 100 µg/ml ampicillin at 37°C to an OD594 of 0.8, and then protein expression was induced by the addition of isopropyl-thiogalactoside (IPTG) to 0.3 mM for 3 hours at 37°C. Cell pellets were resuspended in buffer A (0.5% Triton X-100 in PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM mg/lysozyme, and disrupted by sonication. Lysates were cleared by centrifugation at 20,000 g for 30 minutes and then 3×FLAG-tagged proteins were purified from the supernatant using glutathione-Sepharose 4B beads (GE Healthcare). After incubation for 2 hours with the beads, the beads were washed five times with buffer A, and then proteins were eluted with G buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 9.0). Proteins were subsequently dialyzed in PBS and protein concentrations were determined using a micro BCA protein assay kit (Pierce).

For the purification of the FLAG-tagged retroroller complex, Rosetta-gamiB (DE3) pLys cells (EMD) transformed with pET23d-3×FLAG-Retromer plasmid were grown in Terrific broth (Invitrogen) with 34 µg/ml chloramphenicol, 50 µg/ml streptomycin, 12.5 µg/ml tetracycline, and 100 µg/ml ampicillin at 37°C to an OD594 of 0.8. The bacteria were then centrifuged at 700 g for 30 minutes and protein expression was induced by the addition of IPTG to 0.3 mM for 19 hours. The cell pellet was resuspended in buffer B (50 mM NaH2PO4, 300 mM NaCl, 5 mM imidazole, pH 8.0) containing complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals), 1 mM PMSF and 1 mg/ml lysozyme, and disrupted by sonication. The lysate was cleared by centrifugation at 20,000 g for 30 minutes and then 3×FLAG-tagged proteins were purified from the supernatant using glutathione-Sepharose 4B beads (GE Healthcare). After incubation for 2 hours with the beads, the beads were washed five times with buffer A, and then proteins were eluted with G buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 9.0). Proteins were subsequently dialyzed in PBS and protein concentrations were determined using a micro BCA protein assay kit (Pierce).

**GST pull-down assay**

GST or GST fusion proteins (0.1 mg/ml) were incubated with recombinant FLAG-tagged retroroller cargo recognition complex (3×FLAG-Vps29, Vps35 and Vps35-His6) in 0.5 ml GST pull-down buffer [50 mM HEPES-KOH, pH 7.4, 50 mM KCl, 12.5 mM MgCl2, 10% (v/v) glycerol, 2 mM dithiothreitol, and 0.6 mg/ml bovine serum albumin] for 2 hours at 4°C. Glutathione-Sepharose 4B beads (50 µl) were added and the incubation was continued for an additional 30 minutes. The beads were washed with 1 ml GST pull-down buffer five times and the bound proteins were eluted with 50 µl of G buffer (10 mM glutathione, 50 mM Tris-HCl, pH 9.0). Samples were analyzed by immunoblot analysis. Immunoblot analysis was performed using mouse anti-FLAG M2 antibody or horseradish peroxidase-conjugated anti-mouse IgG. Signals were detected using Pierce SuperSignal West Pico chemiluminescent detection reagent (Pierce).

**Cell transfection and immunofluorescence microscopy**

Human HEp-2 epithelial cells were cultured on 12-well dishes at 37°C in DME high-glucose (4.5% C) and 4°C and GST-tagged proteins were purified from the supernatant using glutathione-Sepharose 4B beads (50 µl) and washed four times with G buffer (10 mM glutathione, 50 mM Tris-HCl, pH 9.0). Samples were analyzed by immunoblot analysis. Immunoblot analysis was performed using mouse anti-FLAG M2 antibody or horseradish peroxidase-conjugated anti-mouse IgG. Signals were detected using Pierce SuperSignal West Pico chemiluminescent detection reagent (Pierce).

To determine the subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulate the iron homeostasis.

**References**


Pixel-by-pixel colocalization analysis was performed using ImageJ (plug-in JACoP) to determine the fraction P, with n = 60-120 for each cell type and condition.


Legends for supplementary material Fig.

Fig. S1. Immunostaining of DMT1-II and Vps35 with various endosomal marker proteins in HEp-2 cells. HEp-2 cells stably expressing 3×HA-DMT1-II were fixed, permeabilized, immunostained with rabbit anti-HA pAb (A-a, B-a, C-a), mouse anti-cation-independent mannose 6-phosphate receptor (CI-MPR) mAb (B) or anti-trans-Golgi marker, p230 mAb (B-b) or anti-Rab11 mAb (C-b), and goat anti-human Vps35 pAb (A-c, B-c, C-c), followed by Alexa Fluor 555-conjugated donkey anti-rabbit IgG, Alexa 488-conjugated donkey anti-mouse IgG, and Alexa Fluor 633-conjugated donkey anti-goat IgG. Cells were examined by confocal microscopy. Bar, 5 µm.

Fig. S2. Expression of siRNA-resistant Vps35WT could rescue the missorting of DMT1-II upon the depletion of endogenous Vps35 by VPS35 siRNA#1 but not that of GFP alone; the siRNA-resistant Vps35R107A mutant could not. (A) Cells transfected with VPS35 siRNA#1 followed by transfection of plasmid encoding VPS35 siRNA#1-resistant GFP-tagged VPS35 wild-type were fixed, immunostained with rabbit anti-GFP pAb, rat anti-HA mAb and mouse anti-TfR mAb followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG, Alexa Fluor 555-conjugated goat anti-rat IgG and Alexa Fluor 633-conjugated goat anti-mouse IgG. Cells were inspected by confocal microscopy. DMT1-II colocalizes with TfR in GFP-Vps35-positive cells, but not in GFP-negative cells. (B) Cells transfected with VPS35 siRNA#1 followed by transfection of plasmid encoding GFP alone (C) or VPS35 siRNA#1-resistant Vps35R107A, nonfunctional Vps35 mutant, were fixed, immunostained with anti-GFP and -LAMP2 antibodies and analyzed by confocal microscopy. Bar, 10 µm.
**Fig. S3.** Stability of 3×HA-DMT-II protein in Vps35-depleted cells. Immunoblot analysis of 3×HA-DMT1-II half-life in control-siRNA and VPS35 siRNA#1-treated cells after treatment with 100 µg/ml cycloheximide for 0, 3, 6, 9, and 12 hours or with control ethanol for 12 hours. Tubulin was used as a control.

**Fig. S4.** Construction of siRNA-resistant Vps35 wild-type and R107A mutant. (A) Schematic representation of the construction of siRNA-resistant VPS35 and target sequences of two VPS35 siRNAs. To generate the siRNA-resistant VPS35 allele, the mutations in 10 of 25 nucleotides corresponding to the VPS35 siRNA#1 sequence without any amino acid changes were introduced by inverse PCR. (B) HEp-2 cells stably expressing 3×HA-DMT1-II were transfected with control or two different VPS35 siRNAs and subsequently transfected with plasmids encoding GFP alone, GFP-tagged siRNA-sensitive Vps35, siRNA-resistant Vps35 wild-type, or R107A mutant. At 72 hours after plasmid transfection, lysates were analyzed by immunoblotting using anti-GFP antibody. In lysates from the control siRNA-treated cells, the expression levels of GFP-tagged proteins were almost equivalent except for the R107A mutant of Vps35, which was not incorporated into the retromer complex and must be unstable. The expression of siRNA-sensitive Vps35 wild-type was significantly decreased by VPS35 siRNA#1 treatment (to less than 20% as compared to control siRNA), whereas those of the siRNA-resistant Vps35 wild-type and R107A mutant were unchanged, confirming that these constructs are entirely resistant to VPS35 siRNA#1 treatment. The expressions of all proteins except for GFP alone, were strikingly decreased by VPS35 siRNA#2 treatment, confirming the specificity of siRNA resistance of these constructs. Tubulin was used as a control. (C) HEp-2 cells were transfected with
control or two different VPS35 siRNAs and subsequently transfected with the indicated plasmids. At 72 hours after plasmid transfection, lysates were analyzed by immunoblotting using anti-GFP antibody. Expression of GFP-Vps26 and -Vps29 was significantly increased upon expression of the siRNA-resistant Vps35 compared to that of the siRNA-sensitive Vps35. Tubulin was used as a control.
A

DMT1-II + Vps35

Vps35 + CI-MPR

Merge

DMT1-II + Vps35

Vps35 + p230

Merge

DMT1-II + Vps35

Vps35 + Rab11

Merge

DMT1-II + Vps35

Vps35 + Rab11

Merge
**Fig. S3, Tabuchi et al.**

- **CHX chase (hrs)**: 0, 3, 6, 9, 12, 12
- **kD**: 55, 55
- **3xHA-DMT1-II**
- **Tubulin**
- **VPS35 siRNA#1 EtOH**
- **Control siRNA EtOH**

Longer exposure
A

VPS35 siRNA#2
5' -GC AUG AGU UGU UAU GUG CUU AAU-3'
3' -TAC GAG TCC AGA AAG AGC ATG AGT TGT TAT GTG CTT AGT AAT GTT CTG GAT TAT AAC

VPS35
796 a.a.

VPS35 siRNA#1
5' -GC AUG AGU UGU UAU GUG CUU AGU AA-3'
3' -CG UAC UCA ACA AUA CAC GAA UCA UU-5'

VPS35 siRNA#1-sensitive allele
VPS35 siRNA#1-resistant allele
Mutagenesis by inverse PCR

B

Control siRNA
VPS35#1 siRNA
VPS35#2 siRNA

kD
170
95
72
55
43
34
55

GFP-Vps35
GFP
Tubulin

GFP-Vps26
GFP-Vps29

C

Control siRNA
VSP35#1 siRNA
VSP35#2 siRNA

siRNA-GFP-VPS35WT
siRNA-GFP-VPS35R107A
GFP-Vps26
GFP-Vps29

Fig. S4, Tabuchi et al.
Legends for supplementary material Fig.

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Fig. S1, Tabuchi et al.
Fig. S2, Tabuchi et al.
Fig. S3, Tabuchi et al.
**A**

VPS35 siRNA#2

5' - GC AUG AGU UGU UAU GUG CUU AGU AA-3'  
Y E S R K S M S C Y V L S N V L D Y N

R107A

VPS35 796 a.a.

VPS35 siRNA#1

5' - G CCU UCA GAG GAU GUU GUA UCU UUA-3'  
3' - C GGA AGU CUC CUA CAU CAU AGA AAU-5'  
Q S R Q D M P S K D V S L Q V S L I

Mutagenesis by inverse PCR

VPS35 siRNA#1-sensitive allele

VPS35 siRNA#1-resistant allele

**B**

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GFP-Vps35

GFP

Tubulin

**C**

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GFP-Vps35

GFP-Vps26

GFP-Vps29

Tubulin

Fig. S4, Tabuchi et al.