Multicopper ferroxidases play a vital role in iron metabolism in bacteria, fungi, algae, and mammals. *Saccharomyces cerevisiae* utilizes a channeling mechanism to couple the ferroxidase activity of Fet3p to Fe\(^{3+}\) transport into the cell by Ftr1p. In contrast, the mechanisms by which mammals couple the ferroxidase reaction to iron trafficking is unclear. The human ferroxidases ceruloplasmin and hephaestin are twice the size of Fet3p and interact with proteins that are not expressed in fungi. *Chlamydomonas reinhardtii* FOX1 is a homolog of the human ferroxidases but likely supports iron uptake in a manner similar to that of yeast, since *Chlamydomonas reinhardtii* expresses a ferric iron permease homolog, FTR1. The results presented support this hypothesis. We show that FOX1 is trafficked to the plasma membrane and is oriented with its multicopper oxidase/ferroxidase domain in the exocyttoplasmic space. Our analysis of FTR1 indicates its topology is similar to that of *S. cerevisiae* Ftr1p, with a potential exocyttoplasmic iron channeling motif and two potential iron permeation motifs in membrane-spanning regions. We demonstrate that high-affinity iron uptake is dependent on FOX1 and the copper status of the cell. Kinetic inhibition of high-affinity iron uptake by a ferric iron chelator does not reflect the strength of the chelator, supporting a ferric iron channeling mechanism for high-affinity iron uptake in *Chlamydomonas*. Last, recombinant FOX1 (rFOX1) has been isolated in a partially holo form that exhibits the UV-visible absorbance spectrum of a multicopper oxidase and the catalytic activity of a ferroxidase.

Iron is essential to all cells, but its aqueous and redox chemistry poses a barrier to its cellular metabolism. The ferric species is the most abundant form of iron in the environment, but the insolubility of Fe\(^{3+}\) makes it relatively bio unavailable. Metallochaperones overcome this problem by presenting the cell with Fe\(^{2+}\), which has a far more tractable aqueous chemistry (1, 14, 26). The expression of iron binding proteins involved in assimilation, mobilization, and storage then serves to manage this Fe\(^{2+}\) (3, 39). Metallochaperones and iron-binding proteins have been identified in most organisms; however, the mechanism by which iron is trafficked between these proteins remains largely uncharacterized.

Detailed studies of the high-affinity iron uptake system in *Saccharomyces cerevisiae* have demonstrated that iron trafficking is coupled to iron redox chemistry (9, 27–28, 45–46). The soluble Fe\(^{2+}\) produced by the yeast metallochaperones Fre1p and Fre2p is reoxidized by the plasma membrane multicopper oxidase (MCO) Fet3p; with its MCO domain on the exocytoplasmic surface, Fet3p is a type Ia membrane protein (25). The Fe\(^{3+}\) produced by Fet3p serves as a substrate for a permease of the cell by the ferric permease Ftr1p (46). A key feature of this uptake system is that Fe\(^{3+}\) alone cannot serve as a substrate for uptake by Ftr1p. Iron permeation is coupled to ferroxidation via a substrate channeling mechanism in which the Fe\(^{3+}\) substrate of Ftr1p is handed off directly from Fet3p (27). Residues involved in iron channeling from Fet3p to Ftr1p have been identified in both proteins (27–28, 45). In Fet3p, E185 and D409 participate in Fe\(^{2+}\) binding and electron transfer to the type I copper site in this copper oxidase and are required for Fe trafficking to Ftr1p (27, 41, 47, 50). Ftr1p is a type III membrane protein with seven membrane-spanning helices; a DASE motif has been identified in extracellular loop 6 of Ftr1p that is required for trafficking of Fe\(^{3+}\) from Fet3p to Ftr1p (45).

Ferroxidases are also expressed by higher eukaryotes (19, 38). The mammalian ferroxidases ceruloplasmin (Cp) and hephaestin (Hp) are critical components of iron metabolism. For example, patients lacking functional human Cp (hCp) exhibit neurological disorders associated with malnutrition of systemic iron (17, 57). The role that hephaestin plays in iron metabolism is demonstrated by the severe iron deficiency phenotype of the *sla* mouse; this mouse strain expresses a truncated murine Cp (mHp) protein that fails to localize to the basolateral membrane in intestinal enterocytes, where this ferroxidase activity is required for iron release into circulation (55). That is, Hp (and Cp) appears to play an essential role in the trafficking of Fe from ferroportin (Fpn), an iron exporter at the plasma membrane, to transferrin (Tf) for systemic iron delivery (8, 35–36). The oxidation of Fe\(^{2+}\) by Cp (or Hp) is essential to the Fe\(^{3+}\) loading of Tf in plasma (35), but whether export of Fe\(^{2+}\) from Fpn or loading of Fe\(^{3+}\) onto Tf is coupled to ferroxidation by either of the two proteins remains unclear. Limited inferences can be drawn from the yeast iron-channeling studies in regard to iron-trafficking mechanisms in mammals for two reasons. First, structural differences between the mammalian and fungal ferroxidases suggest the catalytic ferroxidase mechanism may be different (25, 31, 49–50, 59). In all ferroxidases, the oxidation of 4Fe\(^{2+}\) is coupled to the reduction of O\(_2\) to H\(_2\)O via an electron transfer pathway that includes a T1 copper site and a trinuclear copper cluster (25). Fet3p is comprised of three cupredoxin domains and a single T1 Cu...
site; as noted, carboxylate side chains at this site directly contribute to the enzyme’s specificity toward Fe$^{3+}$ and to the trafficking of Fe$^{3+}$ to Ftr1p. The mammalian MCOs are double the size of Fet3p, containing six cupredoxin domains which support three T1 copper sites in addition to the trinuclear Cu cluster (49, 59). All of the T1 sites have carboxylate residues that likely contribute to Fe$^{3+}$ binding (31), and thus, Cp and Hp (a close Cp paralog) have the potential to oxidize Fe$^{3+}$ at more than one site. Consequently, Fet3p is inadequate as a model to suggest how Cp and Hp might “hand off” iron to another protein. Second, mammals do not express a ferric permease homologous to yeast Ftr1p. The potential Fe$^{3+}$-channeling partners to Cp and Hp in mammals likely include the transmembrane protein, Fp, and the serum-soluble protein, Tf (8, 35), neither of which has Fe-binding motifs comparable to those found in Ftr1 proteins.

However, a link between iron-trafficking mechanisms in yeast and mammals may be revealed through examination of iron uptake systems in algae. *Chlamydomonas reinhardtii* expresses FOX1, a homolog of the Cp and Hp ferroxidases, and FTR1, a homolog of the fungal Ftr1 permeases; these two proteins likely function together as a high-affinity iron import complex in this alga (20, 29). Therefore, in this system a “mammalian-like” ferroxidase potentially traffics iron in a “yeast-like” manner. In addition, the MCO produced in the halotolerant alga Dunalieilla salina interacts with a transferrin-like protein (TTf) to support iron uptake (12–13, 37); D-Fox is a FOX1 and hCp homolog. Thus, this “mammalian-like” algal ferroxidase appears to traffic iron to a “mammalian-like” ferric iron binding protein. A thorough examination of the iron uptake mechanisms in these algae may provide the bridge between yeast and humans in terms of our understanding of iron trafficking.

A structural homology model indicates FOX1 contains six cupredoxin domains and three T1 copper sites, each with potential iron binding residues within close proximity (51). The FOX1 gene is coordinate upregulated at the transcript level under iron deficiency along with *FTR1* and *FRE1*, genes so named for their homology to the yeast ferric permease and reductase, respectively (29). La Fontaine and colleagues showed also that FOX1 protein production increased under iron deficiency (29). Growth of a fox1 knockdown strain is slowed in iron-deficient media relative to that of a wild-type strain, indicating FOX1 plays a direct role in iron metabolism (5). Additionally, wild-type cells grown in copper-depleted media have diminished iron uptake activity, highlighting the link between copper and iron metabolism that is likely due to a ferroxidase (20).

Such evidence indirectly points to a role for FOX1 in high-affinity iron uptake, yet there is no evidence that its potential partner permease, FTR1, is involved in this pathway aside from the fact that the gene is also upregulated under low-iron conditions and has sequence homology to the fungal permeases. Alignment of FTR1 with fungal Ftr1 homologues indicates that the *Chlamydomonas* permease likely has a comparable membrane topology and homologous iron trafficking and permeation motifs, although these features have not been experimentally confirmed (29). If FOX1 and FTR1 function together to support high-affinity iron import in *Chlamydomonas*, this system would prove to be an excellent model to examine, since FOX1 shares more similarity to the mammalian ferroxidases than to the fungal counterparts. Our hypothesis for how FOX1 and FTR1 are assembled in the plasma membrane of *Chlamydomonas* is illustrated in Fig. 1.

Here we provide an analysis of the components of the high-affinity iron uptake system in *Chlamydomonas* and a test of this model. First, iron uptake analysis of *Chlamydomonas* cells under three independent conditions in which FOX1 protein abundance is reduced provides strong evidence that FOX1 is required for high-affinity iron uptake. $^{55}$Fe uptake kinetic data are presented that are consistent with a mechanism of high-affinity iron uptake in *Chlamydomonas* in which FOX1-generated Fe$^{3+}$ is delivered directly to FTR1 for permeation, thus representing another example of coupling between ferroxidation and iron trafficking. Indirect immunofluorescence experiments indicate both FOX1 and FTR1 are trafficked to the plasma membrane, and the topology of FOX1 places the multicopper oxidase domain in the exocyttoplasmic space. The topology and orientation of FTR1 are shown to be identical to those of *S. cerevisiae* Ftr1p, placing potential iron trafficking and permeation motifs in homologous regions. Finally, a soluble (secreted) form of recombinant FOX1 (rFOX1) has been expressed in HEK293E cells and purified from the conditioned medium; this protein exhibits spectral and oxidase characteristics of an MCO and catalyzes the formation of monoferric transferrin from Fe$^{2+}$ and apo-Tf. Based on the homology between FOX1 and the mammalian ferroxidases, these studies provide new insight as to how iron trafficking may occur in higher eukaryotes.

**MATERIALS AND METHODS**

*Chlamydomonas* strains and culture conditions. All *Chlamydomonas* strains used in this work were generously provided by Sabeeka Merchant and were maintained on Tris-acetate-phosphate (TAP) medium plates containing appro-
plasmid DNA and was harvested 5 days posttransfection. For soluble rFOX1-secreting cells, the conditioned medium contained the total culture. Indirect immunofluorescence analysis was carried out 48 h posttransfection. For soluble rFOX1-secreting cells, the conditioned medium was harvested 5 days posttransfection.

**Purification of rFOX1.** To obtain purified rFOX1, conditioned medium from transduced HEK293E cells (10) were used for topology and localization analysis of FOX1 and FTR1 and for secretion of soluble FOX1. The suspension-adapted cells were passed in Erlenmeyer flasks in low-calcium hybridoma serum-free medium (HSFM) (Invitrogen) containing 0.1% Pluronic solution, 10 mM HEPES buffer, and 50 mg/ml Genetecin. The cells were transiently transfected as previously described (4) with FOX1 or FTR1 expression plasmids using-linear polyethylenimine (PEI) (25 kDa) as the transfection vehicle. For transfection, the cells were collected by centrifugation at 500 g, resuspended (1:4,000) in the same medium containing the components listed above, and incubated with shaking for 3 h. The transfection mixture was added dropwise; it contained 1 mg/ml of (total culture volume, not transfection mixture volume) plasmid DNA and 2 mg/ml PEI in a volume of medium representing 5% of the total culture. Indirect immunofluorescence analysis was carried out 48 h posttransfection. For soluble rFOX1-secreting cells, the conditioned medium was harvested 5 days posttransfection.

**Plasmid construction.** Plasmids containing FOX1 and FTR1 cDNA were gifts from Sabeche Merchant and were used to construct plasmids for expression in HEK293E cells. DNA sequence from the FOX1 open reading frame (ORF) representing residues 42 to 1142 (GenBank accession no. AAM45881.1) was PCR amplified using primers that added a NotI site upstream of the M42 (start) location (see Fig. 6 and 7). pTT5SH8Q2 containing a His-tagged hCp ORF was previously described (18). Native hCp was purchased from GenWay Biotech. Tris-borate-EDTA (TBE), and frozen at 20°C. Autooxidation of Fe²⁺ oxidation assays were performed in 96-well microtiter plates. For PPID oxidation, each well contained 5 mM PPID and 0.06% Triton X-100 in 100 mM sodium acetate, pH 5.7. The reaction was initiated by the addition of either 0.5 mM FeCl₃, 0.5 mM native hCp, or 20 mM FOX1 (assuming ~90% purity and containing a mixture of apo and holo forms of the protein). The absorbance at 530 nm was measured every 2 min for 30 min. For Fe²⁺ oxidation, 10 μM ferrous ammonium sulfate in 100 mM sodium acetate, pH 5.7, was incubated with 0.5 μM FeCl₃, 0.5 μM hCp, or 20 μM FOX1. After 30 min, 100 μM ferrozine was added, and the absorbance at 550 nm was measured.

**Indirect immunofluorescence.** For FOX1 topology and localization in *Chlamydomonas*, two 10-ml cultures of cc-425 were grown for 18 h in TAP medium containing either 10 μM Fe-EDTA to reduce FOX1 expression or no iron plus 100 μM ferrozine to maximize FOX1 induction. Cells were collected by centrifugation at 4,000 × g for 10 min and resuspended in phosphate-buffered saline (PBS) to a final concentration of 1 × 10⁷ cells/ml. The cells were added to polystyrene-coated coverslips in 35-mm dishes and allowed to adhere for 1 h. The cells were fixed with 3% formaldehyde for 15 min, washed three times with PBS, and blocked for 1 h in 3% bovine serum albumin (BSA). The unspecifically labeled antibodies were probed for FOX1 using a polyclonal anti-FOX1 antibody at a 1:500 dilution in 3% BSA overnight at room temperature. An additional set of cells was permeabilized with 0.2% Triton X-100 in PBS or left unpermeabilized prior to blocking and probed for ATP synthase (1:500) as a control for membrane integrity. After four washes with PBS, the cells were probed with an antirabbit secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes), diluted 1:200 in 5% BSA for 1 h, and then washed four times before the coverslips were sealed to a microscope slide.

**Iron uptake assays.** *Chlamydomonas* cultures induced for expression of FOX1 and FTR1 were collected by centrifugation at 4,000 × g for 10 min, washed with uptake buffer (100 mM MES, 2 mM potassium acetate, 20 mM sodium citrate, pH 6.0), and resuspended to 10⁶ cells/ml in a minimum of 2.5 ml uptake buffer in a glass scintillation vial. After 15 min of incubation at 30°C with shaking, 20 mM ascorbate was added, followed by a 15-min incubation. A preread 55Fe stock was prepared by diluting the 55FeCl₃ stock to 30 μM in uptake buffer containing 20 mM ascorbate. Uptake reactions were performed in 96-well microtiter plates. For PPD oxidation, each well contained 5 mM PPD and 0.06% Triton X-100 in 100 mM sodium acetate, pH 5.7. The reaction was initiated by the addition of either 0.5 mM FeCl₃, 0.5 mM native hCp, or 20 mM FOX1 (assuming ~90% purity and containing a mixture of apo and holo forms of the protein). The absorbance at 530 nm was measured every 2 min for 30 min. For Fe²⁺ oxidation, 10 μM ferrous ammonium sulfate in 100 mM sodium acetate, pH 5.7, was incubated with 0.5 μM FeCl₃, 0.5 μM hCp, or 20 μM FOX1. After 30 min, 100 μM ferrozine was added, and the absorbance at 550 nm was measured.

**Iron loading and urea-PAGE.** Incorporation of iron into Tf was monitored via a previously described method (16). Each reaction mixture contained 100 μl of 6.25 μM apo-Tf, 200 μM ferrous ammonium sulfate, and 100 μM ascorbate in 100 mM sodium acetate, pH 5.0. The reaction was initiated by the addition of 1 μM total FOX1, and Tf was removed at 35 min by addition of trichloroacetic acid. The samples were immediately added to sample buffer containing 10% glycerol and 0.2% bromophenol blue in 1× Tris-borate-EDTA (TBE), and frozen at −20°C. Autooxidation of Fe²⁺ was monitored in a reaction mixture containing all components except FOX1. Additionally, 10 μl of 10-fold-concentrated conditioned medium from mock-trans-
RESULTS

FOX1 supports high-affinity iron uptake. Initial evidence for FOX1 involvement in high-affinity iron assimilation was the increase in FOX1 transcript and protein abundance under low-iron conditions and the sequence homology that FOX1 has to other ferroxidases (29). Subsequently, Chen et al. produced a fox1 knockdown strain of *Chlamydomonas* that grew poorly in iron-deficient medium, providing more direct evidence of a role for FOX1 in iron metabolism (5). Herbik and colleagues demonstrated that high-affinity uptake was dependent on copper (20); Fe uptake in *S. cerevisiae* exhibits this same dependence, because copper-loaded Fet3p is required for cell ferroxidase activity (7, 58). The copper dependence of iron uptake in *Chlamydomonas* is consistent with involvement of an MCO but does not require that FOX1 (or any MCO) support iron uptake.

In another study, tetrathiomolybdate (TTM) was shown to reduce iron uptake (21). TTM has been shown to inhibit hCp by reducing the T1 copper site, so TTM inhibition of iron uptake in *Chlamydomonas* could be the result of FOX1 inhibition (6). However, TTM is also a known copper chelator; thus, an alternative model is that TTM inhibits iron uptake in *Chlamydomonas* by altering the copper content of the cell, producing an effect similar to growth in a copper-depleted medium (34). To demonstrate that FOX1 contributes to high-affinity iron uptake, 55Fe uptake assays were performed under three separate conditions in which FOX1 protein abundance was reduced.

Iron uptake analyses were first performed on the fox1 knockdown strain, kd11, and the parental strain, NE3. Initial velocities of 55Fe uptake were quantified as a function of [Fe2+] and fit to the standard Michaelis-Menten equation (Fig. 2A). The apparent values for Fe2+ were as follows: NE3 (wild type), 4.1 ± 0.9 μM; kd11 (knockdown), 4.2 ± 0.6 μM. The identical Michaelis constant for ferrous iron is strong evidence that the same iron import complex is being quantified in each strain. The *V* max value found for NE3 was 4.0 ± 0.4 pmol 55Fe/107 cells/20 min; for the fox1 knockdown strain, kd11, this value was 1.1 ± 0.1 pmol 55Fe/107 cells/20 min. This 75% decrease in apparent *V* max correlated well with the decrease in the FOX1 protein as determined by immunoblot analysis of extracts from the cells used in these uptake experiments (Fig. 2A). The loading control for this analysis was ATP synthase, which is unaffected by cellular iron status (29). Iron uptake and FOX1 protein abundance was also analyzed in the copper-deprived wild-type strain NE3 (Fig. 2B). Cells were grown for 5 days in medium containing either 6 μM Cu (the concentration in TAP) or 100 μM bathocuproine disulfonate (BCS) to deplete the cells of copper before overnight induction of FOX1 using medium containing no iron. To determine if uptake could be restored, 6 μM Cu was added to the copper-depleted cells 24 h prior to initiating the 55Fe uptake experiments. The immunoblot in Fig. 2B indicates that FOX1 protein abundance is reduced in copper-depleted cells. Correlated with this reduction, copper-depleted cells exhibited ~40% high-affinity iron uptake in comparison to copper-replete ones, confirming the results obtained by Herbik et al. (20). Iron uptake is almost completely restored in cells re-supplemented with copper (Fig. 2B); the FOX1 protein recovers as well (data not shown).
Iron uptake analysis also was performed on a *Chlamydomonas* strain with a mutation at the CRD2 locus, an allele conferring copper-conditional iron deficiency (11). The immunoblot in Fig. 2C indicates that the crd2 strain produces less FOX1 protein than to the wild-type strain 34+ when cells are iron deprived but grown under normal copper conditions. A decrease in iron uptake correlates with the decrease in protein abundance in this strain as well. Thus, the three conditions evaluated show that a decrease in iron uptake correlates with a decrease in FOX1 protein abundance, providing consistent evidence in support of the conclusion that FOX1 is required for high-affinity Fe uptake in this alga.

**Kinetic mechanism of high-affinity iron uptake.** Our model for high-affinity iron uptake in *Chlamydomonas* is that Fe$^{3+}$ produced by FOX1 is supplied to FTR1 for permeation of the cell. The channeling mechanism proposed for Fe uptake in *S. cerevisiae*, in which the product of Fet3p is handed off directly to residues on Ftr1p, was demonstrated through the use of Fe$^{3+}$-specific chelators in kinetic iron uptake analyses (27). If FOX1 and FTR1 function as do their yeast counterparts, a possible mechanism for iron uptake is one in which Fe$^{3+}$ produced by FOX1 is channeled directly to residues on FTR1. In order to test this possibility, the effect of ferric iron chelators as inhibitors of iron uptake in *Chlamydomonas* was examined.

A chelator can act as an inhibitor of iron uptake in either of two ways. If the Fe$^{3+}$ product of FOX1 equilibrates into bulk solvent kinetically much faster than FTR1 uses it as a substrate for permeation, as would be the case for a dissociative, nonchanneling mechanism, a Fe$^{3+}$ chelator would bind the FTR1 substrate free in solution with an efficacy that reflected the stability constant of the corresponding Fe(III)-chelate complex. In the case where iron uptake occurs via a strict channeling mechanism, the Fe$^{3+}$ produced by FOX1 dissociates into solution much more slowly than it traffics to FTR1 for Fe$^{3+}$ permeation. In this kinetic mechanism, a ferric iron chelator could inhibit uptake only through association with the iron uptake complex. A quantitative analysis of the relationship between chelator strength (its stability constant, $K_I$) and the uptake inhibition constant ($K_I$) for each chelator allows for distinguishing between the two mechanisms. In a standard rate, equilibrium linear free energy plot, where the log $K_I$ value is plotted versus the log $K_I$ value, the relationship between chelator strength and inhibition is represented by the slope of the line, $\beta$. This relationship is given by equation 1,

$$\log K_I = -\beta \log K_I + C \quad (1)$$

where the intercept $C$ reflects the hypothetical value of log $K_I$ when the Fe(III)-chelate complex has zero stability. Note that the slope of the correlation is negative since $K_I$ is an association constant while $K_I$ is a dissociation constant. If the mechanism is a dissociative, nonchanneling one, $\beta$ equals 1, since there would be a 1:1 relationship between chelator strength and the chelator's ability to inhibit uptake by scavenging Fe$^{3+}$ that equilibrates with bulk solvent. A fractional $\beta$ value indicates this 1:1 relationship does not hold and is consistent with an associative, iron channeling mechanism (24, 33).

To examine the kinetic mechanism of high-affinity iron uptake in *Chlamydomonas*, reductase-independent $^{55}$Fe uptake assays were performed on the cell wall mutant strain cc-425 using ferric iron chelators of various strengths. Cells were grown in TAP medium containing 5 µM iron to a cell density of $3 \times 10^6$ cells/ml and then washed, resuspended, and allowed to shake in TAP containing no iron for 18 h to allow FOX1 and FTR1 induction before an experiment. To ensure the experiment was reductase independent, a $^{55}$FeCl$_3$ stock was prepared in 20 mM ascorbate. The apparent $K_m$ for Fe$^{2+}$ in this strain was 1.4 ± 0.2 µM, while the $V_{max}$ was 3.7 ± 0.3 pmol $^{55}$Fe/10$^7$ cells/20 min. To quantify the kinetics of Fe$^{3+}$ chelator inhibition of iron uptake, assays were performed using 1 µM $^{55}$Fe at various chelator concentrations. The inhibition constants, $K_I$s, for five Fe$^{3+}$-specific chelators with log stability constants ranging from 10 to 19, were determined (Table 1). The relationship between chelator strength and the inhibition constant was examined by plotting the log $K_I$ value versus the log $K_I$ value according to equation 1 (Fig. 3). This analysis gave a $\beta$ value equal to $-0.39 \pm 0.07$; this value states that a 1,000-fold increase in chelator strength supports only a 15-fold decrease in $K_I$ (increase in inhibition), far from the 1:1 relationship ($\beta = 1$) expected if the chelator were binding FOX1-produced Fe$^{3+}$, which was released to bulk solvent. This result eliminates the dissociative mechanism as describing the kinetic mechanism of Fe uptake through the FOX1, FTR1 system. This value of $\beta$ compares favorably with the value derived from a similar study in yeast, $-0.46$, suggesting that iron uptake in *Chlamydomonas* occurs via a mechanism of iron channeling in a manner similar to that for yeast.

**FOX1 and FTR1 localization and topology.** In our model for high-affinity iron uptake in *Chlamydomonas*, the import of Fe$^{3+}$ is coupled directly to the ferroxidase activity of FOX1. This model requires not only FOX1 expression at the plasma membrane, but also protein stability in this strain as well. Thus, the three conditions evaluated show that a decrease in iron uptake correlates with the decrease in FOX1 protein abundance, providing consistent evidence in support of the conclusion that FOX1 is required for high-affinity Fe uptake in this alga.

| Chelator | Stability constant ($\log K_I$) | Inhibition constant, $K_I$ (mM) |
|----------|--------------------------------|---------------------------------|
| IDA      | 10                             | 13.7 ± 2.8                      |
| EDDP     | 11                             | 10.9 ± 2.5                      |
| HIDA     | 13                             | 8.7 ± 1.3                       |
| NTA      | 15                             | 0.64 ± 0.22                     |
| HEDTA    | 19                             | 0.005 ± 0.001                   |

**FIG. 3.** The linear relationship between the iron uptake inhibition constant of a Fe$^{3+}$ chelator and its stability constant represented as a standard rate, equilibrium linear free energy plot (see equation 1). The fitted $K_I$ values determined for each chelator (Table 1) were plotted against the corresponding log stability constants. A $\beta$ value of $-0.39$, given by the slope of the line, indicates inhibition of uptake by a chelator is 1.5% as probable as chelation of aqueous Fe$^{3+}$. The correlation coefficient for this fit was 0.916.
membrane but an exocyttoplasmic orientation of the protein’s MCO domain. Topology analyses predict that FOX1 contains two possible transmembrane domains: an amino-terminal plasma membrane targeting sequence that also serves as a membrane anchor and a carboxy-terminal membrane-spanning region (29). A structural homology model of FOX1 places the latter carboxy-terminal portion in the last of the protein’s six cupredoxin domains, indicating that it is unlikely to be a membrane-spanning region (51). As depicted in Fig. 1, we predict that FOX1 is anchored in the plasma membrane by a single amino-terminal membrane-spanning helix (a type Ib membrane protein lacking a cleavable signal sequence) with all six cupredoxin domains in the extracellular space.

To demonstrate this localization and orientation of FOX1, indirect immunofluorescence was carried out with the Chlamydomonas cell wall mutant strain, cc-425, using an antibody directed against FOX1 residues 394 to 646, which include the second, third, and fourth cupredoxin domains (29). Cultures were grown overnight in medium containing either 10 μM iron to suppress FOX1 expression or 100 μM ferrozine to induce it. Cells were plated and left unpermeabilized prior to treatment with anti-FOX1 and Alexa fluor 488 conjugate antibodies. As a control for membrane integrity, permeabilized (C) and unpermeabilized cells (D) were probed for ATP synthase which is localized to the chloroplast. Dim fluorescence is visualized in the unpermeabilized cells (D), which is likely due to background autofluorescence. The bottom panels represent phase-contrast images of the same cells under each condition.

**FIG. 4.** FOX1 localizes to the plasma membrane in iron-deprived Chlamydomonas cells and is oriented with the MCO domain outside the cell. The cell wall mutant strain, cc-425, was grown in TAP medium containing either 10 μM iron for FOX1 repression (A) or no iron plus 100 μM ferrozine for FOX1 induction (B). Cells were plated and left unpermeabilized prior to treatment with anti-FOX1 and Alexa fluor 488 conjugate antibodies. As a control for membrane integrity, permeabilized (C) and unpermeabilized cells (D) were probed for ATP synthase which is localized to the chloroplast. Dim fluorescence is visualized in the unpermeabilized cells (D), which is likely due to background autofluorescence. The bottom panels represent phase-contrast images of the same cells under each condition.
Based on sequence homology to the *S. cerevisiae* ferric permease Ftr1p, we proposed that FTR1 has a similar topology (Fig. 1). Ftr1p is a 7-transmembrane protein in which the two REXXE motifs involved in iron permeation are located in the first and fourth transmembrane domains (45). The DASE motif located in extracellular loop 6 is involved in the hand-off of iron from Fet3p to Ftr1p (27, 45). Three of six topology prediction programs (HMMTOP, TMHMM, and TMPRED) predicted FTR1 contains seven transmembrane domains (22–23, 43, 52–54). The ClustalW alignment of FTR1 with Ftr1p, shown in Fig. 6, reveals that the seven transmembrane domains of FTR1 predicted by these programs align well with the structurally homologous domains of Ftr1p (30). Furthermore, this alignment shows that the two REXXXE motifs in FTR1 align with their yeast counterparts, placing them in transmembrane domains 1 and 4. There is no DASE motif in FTR1; however, we propose that the EPTD motif within loop 6 of FTR1 is homologous to the DASE motif in *S. cerevisiae* Ftr1p (ScFtr1p) and may play a role in iron trafficking between FOX1 and FTR1 (Fig. 1).

This FTR1 model was tested using a series of (HA)2-tagged species as shown in Fig. 1 and 6. An (HA)2-encoding sequence was cloned into FTR1 cDNA to be located after residue 131, 171, or 390, corresponding to predicted loop 3, 4, or 6, respectively (noted in Fig. 6). Indirect immunofluorescence was performed on HEK293E cells expressing each of the tagged FTR1 proteins using an anti-HA antibody. To determine the orientation of the carboxy terminus, indirect immunofluorescence was performed on the GFP-tagged version of FTR1 probed with a GFP-specific antibody. For each set of transfections, cells were plated on coverslips, fixed, and either permeabilized with Triton X-100 or left untreated prior to probing with primary and secondary antibodies. Figure 7 reveals that the 131(HA)2-tagged and GFP-tagged versions of FTR1 were accessible to antibody only if the cells were permeabilized. Note that one cell in the 131(HA)2-tagged unpermeabilized sample was fluorescent, which was possibly due to slight permeabilization in the membrane of that cell. In contrast, the 171 and 390(HA)2-tagged versions were accessible regardless of the state of the membrane, indicating these epitopes are located extracellularly. We conclude that loop 3 and the carboxy-terminal domain are oriented toward the inside of the cell while loops 4 and 6 are oriented exocytoplasmically. Since loop 6 contains the potential iron trafficking motif EPTD, the topology determined here is consistent with our model that iron transfer from FOX1 to FTR1 is a key aspect of the mechanism of iron import in this alga.

**FOX1 is a ferroxidase that catalyzes holotransferrin formation.** Based on the similarity between FOX1 and hCp, we predicted that a soluble form of FOX1 could be produced in HEK293E cells and secreted into the medium, allowing purification of rFOX1. The coding sequence of FOX1 corresponding to residues 86 to 1142 was cloned into the pTTSSH8Q2 vector downstream of the hCp coding sequence corresponding to residues 1 to 25, which include the cleavable, hCp signal peptide, residues 1 to 19. A 100-ml culture of HEK293E cells was transfected with this hCp-FOX1 fusion construct, and after 5 days, the conditioned medium was harvested for purification over Mono-Q equilibrated in 50 mM MES, pH 6.5. The column was eluted with a gradient from 0 to 1 M NaCl.

The resulting fractions from the Mono-Q column were analyzed by SDS-PAGE and immunoblotting for rFOX1. Previous data indicate that copper-loaded hCp migrates at ~70 kDa when the sample is left unheated prior to electrophoresis (44). Apo-hCp (heated or unheated) and heated hCp (heated and thus Cu depleted) migrate according to the protein’s molecular mass, 125 kDa. We considered that rFOX1 produced from HEK293E cells might be a mixture of metalated forms from fully apo to fully copper loaded and would exhibit similar electrophoretic behavior; thus, the fractions eluted from the mono-Q resin were left unheated prior to electrophoretic analysis. The rFOX1 protein migrating at the expected mass of 125 kDa was detectable in several fractions from the Mono-Q column, as shown in the immunoblot in Fig. 8A, indicating that much of the protein secreted was in the apo form. However, fractions 4 and 5 contained a species recognized by anti-FOX1 that migrated as a lower-molecular-mass protein, indicating that it could be Cu replete. The slower-migrating species was more abundant, however, and as a result, the longer film ex-

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**FIG. 5. FOX1 and FTR1 localization in HEK293E cells.** (A) FOX1 localization was analyzed by indirect immunofluorescence in cells cotransfected with FOX1 and FTR1-GFP using the anti-FOX1 antibody. The cells were unpermeabilized prior to antibody treatment, indicating that the MCO domain resides outside the cell. (B) GFP fluorescence reveals localization of FTR1-GFP at the plasma membrane. (C) The fluorescence overlay indicates the colocalization of FOX1 and FTR1-GFP at the plasma membrane. (D) Differential interference contrast image of cells shown in panels A to C.
posure required to detect the lower band resulted in the extreme overexposure of this putative apo-FOX1 species.

To determine the purity of the fractions containing the highermobility species, 2 μg of the fourth fraction (F4) was analyzed by SDS-PAGE and Coomassie staining (Fig. 8B). Sample buffer was added with or without SDS, dithiothreitol (DTT), and boiling in order to detect both apo and holo forms of the protein. As a control, 2 μg of hCp was also analyzed. The predominant band in both F4 samples migrates at a molecular mass of 110 kDa, confirming that the fraction contains relatively pure rFOX1; however, several less-distinct bands are apparent in the undenatured sample both above and below the predominant 130-kDa band, making it difficult to determine the ratio of holo-rFOX1 to apo-rFOX1 according to this analysis.

Fraction F4 also exhibited a slight blue hue. All MCO proteins have a characteristic absorbance at 608 nm due to the Cu²⁺ at the T1 site(s). This fraction was concentrated 7.5-fold, confirming that the fraction contains relatively pure rFOX1; however, several less-distinct bands are apparent in the undenatured sample both above and below the predominant 130-kDa band, making it difficult to determine the ratio of holo-rFOX1 to apo-rFOX1 according to this analysis.

Fig. 6. ClustalW alignment of Chlamydomonas FTR1 (CrFtr1) with Saccharomyces cerevisiae Ftr1p (ScFtr1) (30). Stars and dots indicate identical and similar residues, respectively. The 7 predicted transmembrane domains (boxed in blue) align well with the yeast transmembrane domains. The two REXXE motifs (boxed in yellow) align with their yeast counterparts, placing them in transmembrane domains 1 and 4, while the possible iron-channeling EPTD motif in Loop 6 corresponds to the essential DASE motif in ScFtr1p (boxed in yellow). (HA)₂ tags were cloned in CrFtr1 in loops 3, 4, and 6 between the residues boxed in purple, and GFP was fused to the carboxy terminus.

MCO proteins catalyze the oxidation of p-phenylenediamine (PPD) regardless of their substrate specificity (2, 48); thus, the PPD oxidase activity of F4 was measured in comparison to those of Fet3p and hCp (Fig. 9A). Conditioned medium from mock-transfected HEK293E cells was harvested 5 days post-treatment, concentrated 10-fold, and used as a negative control; various amounts of the latter sample were assayed to determine if HEK293E cells themselves contributed to oxidase activity. The amount of F4 used in the assay corresponded to approximately 1 μM holo-rFOX1 (20 μM total rFOX1) based on the 608-nm absorbance and extinction coefficient as outlined above. The rate of oxidation of PPD by this amount of F4 was comparable to that with 0.5 μM Fet3p and hCp, or about 50% of the activity of these two ferroxidases used as positive controls. The concentrated control medium exhibited no oxidase activity, indicating that the secreted rFOX1 contributed all the oxidase activity observed in F4.

A ferrozine-based assay was also used to determine the reactivity of this rFOX1 protein toward ferrous iron. Ferrozine forms a stable complex with Fe²⁺ that absorbs at 550 nm. In this endpoint assay, Fe²⁺ is incubated with ferroxidase for 30
min prior to addition of ferrozine. After 10 min of incubation with ferrozine, the absorbance at 550 nm is measured. In the absence of a ferroxidase, a strong absorbance is observed, whereas samples containing ferroxidase have an $A_{550}$ that is reduced due to the conversion of Fe$^{2+}$/H$^{+}$ to Fe$^{3+}$/H$^{+}$. As shown in Fig. 9B, the amount of remaining Fe$^{2+}$ after 30 min was smaller in the F4 sample than in the samples containing no ferroxidase and concentrated conditioned medium, suggesting rFOX1 exhibits Fe$^{2+}$/H$^{+}$ oxidase activity in addition to activity toward PPD.

In mammals, the ferroxidase activities of Cp and Hp are proposed to contribute to the Fe$^{3+}$ loading of apotransferrin (Tf) for systemic iron delivery. In a recent study, Griffiths et al. used urea-PAGE to show that purified recombinant hHp catalyzed the formation of diferric Tf from Fe$^{2+}$/H$^{+}$ and apo-Tf (16). We used this assay to examine the ability of rFOX1 (F4) to catalyze Fe$^{3+}$/Tf formation. In each sample, apo-Tf was incubated with Fe$^{2+}$/H$^{+}$ in the presence of 0.1 mM ascorbate and the reaction was initiated by the addition of 0.4 μM ferroxidase; yeast Fet3p was used as a positive control. As a function of incubation time, samples were removed and electrophoretically fractionated on a 6% urea polyacrylamide gel (Fig. 9C). Lanes 1 and 2 show the migration differences between apo- and diferric Tf, respectively. When apo-Tf was incubated with 0.2 mM Fe$^{2+}$/H$^{+}$ in the presence of ascorbate, very little mono- or diferric Tf was formed after 3 h (lane 3). As indicated in lanes 4 to 6, an increase in monoferric Tf was observed in the F4 sample as a function of incubation time. In the negative control containing concentrated conditioned medium from mock-transfected HEK293E cells (lane 7), very little mono- or diferric Tf was observed. We conclude that the monoferric Tf formed in the F4 sample can be attributed to rFOX1-dependent ferroxidase activity.

**DISCUSSION**

Our model for high-affinity iron uptake in *Chlamydomonas* is one in which the extracellular ferroxidase, FOX1, hands off its ferric iron product to the ferric importer, FTR1. In support of this model, we show that FOX1 directly supports high-affinity iron uptake in *Chlamydomonas* utilizing a mechanism consistent with channeling between a ferroxidase and permease. Additionally, the data presented here confirm the predicted localization, topology, and orientation of FOX1 and FTR1. Finally, the spectral and catalytic characteristics of purified recombinant FOX1 clearly indicate it is a ferroxidase.
FIG. 8. Purified rFOX1 exhibits electrophoretic and spectral properties characteristic of an MCO. rFOX1 was purified from conditioned medium of HEK293E cells transfected with a plasmid containing the MCO coding sequence of FOX1 fused to the ER targeting signal coding sequence of hCp. (A) Fractions eluted from Mono-Q resin were analyzed by immunoblotting for rFOX1. The samples were left unheated prior to electrophoresis in order to detect both apo and holo forms of FOX1. The prominent band in all fractions migrates according to the molecular mass of rFOX1, ~120 kDa; fractions 4 and 5 exhibit a species migrating around 60 kDa as well, which could correspond to holo-rFOX1. (B) Fraction 4 from the Mono-Q column was analyzed for purity by a Coomassie-stained SDS polyacrylamide gel. Protein (3 μg) was incubated in sample buffer with or without SDS, DTT, and boiling prior to electrophoresis (2nd and 3rd lanes, respectively). Although the fraction contains one prominent band corresponding to the size of rFOX1, a discrete band corresponding to the higher-mobility band in the immunoblot was not visualized in the undenatured sample, indicating the majority of the protein is apo-rFOX1. The 4th and 5th lanes show the mobility of 2.5 μg denatured and undenatured native hCp, respectively. (C) A UV-visible spectrum of fraction 4 concentrated 7.5-fold was obtained using a BMB Labtech FLUOstar Omega microplate reader. The spectrum exhibits an absorbance peak at 608 nm, which is characteristic of all MCO proteins.

The iron uptake analyses indicate FOX1 directly supports high-affinity iron uptake. Iron uptake was analyzed under three independent conditions in which FOX1 protein abundance was reduced, and in each case a decrease in uptake activity correlated with the decrease in FOX1 protein abundance. For example, the $V_{\text{max}}$ for Fe uptake in the fox1 knockdown strain kd11 was reduced by about 70% relative to that of the wild type; perhaps more significant was that the Fe$^{2+}$ $K_m$ values were the same, indicating that the same kinetic process was responsible for Fe uptake in both strains. This result indicates that the residual uptake in the knockdown strain was not due to a secondary, kinetically distinguishable Fe uptake system. Consistent with previous results, we found that Cu-depleted cells have reduced FOX1 protein levels and diminished Fe uptake (20, 29). Finally, the crd2 strain also exhibited decreased iron uptake that correlated with decreased FOX1 abundance. In addition to the reduction in the FOX1 protein, Eriksson et al. previously indicated that mutations at this locus result in copper-conditional iron deficiency, suggesting CRD2 may function in copper transport (11). It should be noted, however, that the crd2 strain is not Cu depleted when grown under Cu-replete conditions; thus, the reduction of FOX1 protein abundance (and Fe uptake) does not appear to reflect the cellular Cu status solely.

In order to probe the mechanism of iron uptake, we used Fe$^{3+}$-specific chelators of various strengths as inhibitors of the iron uptake reaction. We found that the inhibitory effects quantitatively represented only 1.5% of the strength of the chelator. The $\beta$ value (−0.39) associated with this correlation suggests that a direct transfer of Fe$^{3+}$ from FOX1 to FTR1 is approximately 70-fold more likely than dissociation of Fe$^{3+}$ from FOX1 into bulk solvent. By analogy to the fungal system, a model is proposed in which the ferric iron product of FOX1 is handed off to an extracellular loop in FTR1 and passed into the cytosol via the RExxE motifs residing in the first and fourth membrane-spanning helices of the permease (cf. Fig. 1). This channeling mechanism implies that there are specific residues on each protein responsible for the “hand-off” from FOX1 to FTR1. Our topology analysis places the EPTD motif, similar to the DASE motif in Ftr1p, outside the cell; the acidic residues...
in this motif could potentially serve as “hands” in Fe$^{3+}$ channeling. Certainly, mutational analysis of FTR1 is required to assess the role of the EPTD motif in uptake. Furthermore, an iron-channeling mechanism implies that the two proteins are in a complex or at least close enough to one another for such a “hand-off” to occur. Evidence of any interaction between FOX1 and FTR1 has yet to be obtained, however.

Iron uptake supported by FOX1 and FTR1 requires their expression at the plasma membrane. FOX1 was previously detected in the plasma membrane fraction from *Chlamydomonas* by immunoblotting (21); we used the FOX1 antibody for analysis of the orientation of FOX1 in intact cells. The indirect immunofluorescence data confirmed that FOX1 localizes at the plasma membrane while demonstrating that the MCO domain is exocytosplasmic. Identical results were obtained with recombinant FOX1 expressed in HEK293E cells. FTR1 localization, topology, and orientation were also determined using a series of (HA)$_2$- and GFP-tagged versions of FTR1 heterologously expressed in these cells. The direct fluorescence from FTR1::GFP and the indirect immunofluorescence results demonstrate FTR1 plasma membrane localization. Furthermore, the topology of FTR1 illustrated in our model (Fig. 1) is consistent with the indirect immunofluorescence patterns observed in unpermeabilized versus permeabilized cells, patterns which matched those reported for Ftr1p (45); these patterns place loops 4 and 6 outside and loop 3 and the carboxy terminus of both proteins in the cytoplasm. Although the Fe-trafficking DASE motif in loop 6 of Ftr1p is not strictly conserved in loop 6 of FTR1, we predict a comparable Fe$^{3+}$-trafficking role for the EPTD motif in this loop in the algal permease. Overall, the topologic analyses for FOX1 and FTR1 provide a picture of these two proteins in the algal plasma membrane that is consistent with the hypothesis that they function as a ferroxidase and a permease in Fe uptake in this organism.

The *in vitro* spectral and kinetic analyses of rFOX1 clearly define this protein as an MCO with specificity toward Fe$^{2+}$. The PPD and ferroxidase activities of FOX1 as determined here are comparable to those exhibited by Fet3p and hCp. The indirect immunofluorescence data confirmed that FOX1 localizes at the plasma membrane while demonstrating that the MCO domain is exocytosplasmic. Identical results were obtained with recombinant FOX1 expressed in HEK293E cells. FTR1 localization, topology, and orientation were also determined using a series of (HA)$_2$- and GFP-tagged versions of FTR1 heterologously expressed in these cells. The direct fluorescence from FTR1::GFP and the indirect immunofluorescence results demonstrate FTR1 plasma membrane localization. Furthermore, the topology of FTR1 illustrated in our model (Fig. 1) is consistent with the indirect immunofluorescence patterns observed in unpermeabilized versus permeabilized cells, patterns which matched those reported for Ftr1p (45); these patterns place loops 4 and 6 outside and loop 3 and the carboxy terminus of both proteins in the cytoplasm. Although the Fe-trafficking DASE motif in loop 6 of Ftr1p is not strictly conserved in loop 6 of FTR1, we predict a comparable Fe$^{3+}$-trafficking role for the EPTD motif in this loop in the algal permease. Overall, the topologic analyses for FOX1 and FTR1 provide a picture of these two proteins in the algal plasma membrane that is consistent with the hypothesis that they function as a ferroxidase and a permease in Fe uptake in this organism.

The *in vitro* spectral and kinetic analyses of rFOX1 clearly define this protein as an MCO with specificity toward Fe$^{2+}$. The PPD and ferroxidase activities of FOX1 as determined here are comparable to those exhibited by Fet3p and hCp. The kinetic data were limited, however, by the fact that <10% of the purified rFOX1 was isolated in a fully Cu-replete state. This limited Cu activation could be due to an imbalance between the efficiency of rFOX1 protein production and of rFOX1 Cu-loading in HEK293E cells; these cells do not normally express hCp or hHp. Nonetheless, the data here clearly show that rFOX1 catalyzes Fe$^{3+}$ loading of Tf. After 3 h, FOX1 catalyzed the complete formation of monoferric Tf from apo-Tf. Diferric Tf was not formed as might have been expected; however, the assay was limited to the amount of rFOX1 that could be used before interfering with the migration of Tf on the urea gel, and thus, an increased concentration of holo-rFOX1 may have given different results.

While the data presented here indicate the similarity between the components of iron uptake in *Chlamydomonas* and *S. cerevisiae*, structural differences between FOX1 and Fet3p indicate that the iron-trafficking contributions of each ferroxidase to iron uptake may be different. A homology-based structural model of FOX1 suggests that, like hCp, FOX1 is comprised of six cupredoxin domains containing T1 Cu sites in domains 2, 4, and 6 and a trinuclear copper cluster between domains 1 and 6 (51). In all ferroxidases, at least one acidic residue within hydrogen bonding distance of a His ligand at the T1 Cu site is required for the efficient outer-sphere electron transfer that occurs from Fe$^{2+}$ to the T1 Cu(II) atom; these acidic residues adjacent to T1 Cu sites confer Fe$^{2+}$ specificity to a ferroxidase (41–42, 47). In FOX1, E349, E727, and D1077 reside close to the T1 Cu sites in domains 2, 4, and 6, respectively, suggesting that Fe$^{2+}$ binding and oxidation occur independently at each site (51). In contrast, Fet3p has a single substrate oxidation site where E185 not only supports Fe$^{2+}$ specificity and electron transfer to the T1 Cu atom but also contributes to Fe$^{3+}$ channeling to the DASE motif in Ftr1p (27, 45, 47). In the crystal structure of hCp, Lindley et al. observed that acidic residues close to each of the T1 Cu sites collectively contribute to a negative charge distribution that is formed at the top surface of the molecule; these were suggested to be involved in Fe$^{2+}$ binding and oxidation (31). This negative charge distribution is conserved in the FOX1 homology model and may provide a holding site for Fe$^{3+}$ that awaits dissociation into solution or trafficking to FTR1 (46). Thus, the three negatively charged cation binding sites apparent in the FOX1 model may cooperatively form a pool of Fe$^{3+}$ to which FTR1 has access, rather than the permease obtaining Fe$^{3+}$ from each site independently. Further analysis of FOX1 and FTR1 is certainly required to determine the precise mechanism of high-affinity iron uptake in this alga.

Nonetheless, the similarities between FOX1 and hCp make *Chlamydomonas* an excellent model for understanding iron-trafficking mechanisms that are applicable to higher eukaryotes. If the charge distribution on FOX1 described above provides an iron binding platform accessible to FTR1, it is appealing to infer from this how proteins in other organisms might interact with a ferroxidase in order to traffic iron. For instance, in humans, both Fpn and Tf may extend motifs into the negatively charged surface of Cp, thus coupling this enzyme’s ferroxidase activity to the release of Fe$^{2+}$ from Fpn to the binding of Fe$^{3+}$ by Tf. The halotolerant alga *D. salina* could serve as a particularly useful iron-trafficking model since it expresses an iron uptake system involving a ferroxidase (D-Fox) and a transferrin (Tf). Tf is known to bind Fe$^{3+}$ and is found in complex with D-Fox, but there has been no demonstration that D-Fox is essential to Fe$^{3+}$ loading of Tf (13, 37). Clearly, mechanistic analyses of high-affinity Fe uptake in both algal systems will undoubtedly benefit our understanding of iron-trafficking mechanisms in higher eukaryotes.

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