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Gain-of-function mutations identify amino acids within transmembrane domains of the yeast vacuolar transporter Zrc1 that determine metal specificity

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Abstract

Cation diffusion facilitator transporters are found in all three Kingdoms of life and are involved in transporting transition metals out of the cytosol. The metals they transport include Zn^{2+} , Co^{2+} , Fe^{2+} , Cd^{2+} , Ni^{2+} and Mn^{2+} ; however, no single transporter transports all metals. Previously we showed that a single amino acid mutation in the yeast vacuolar zinc transporter Zrc1 changed its substrate specificity from Zn^{2+} to Fe^{2+} and Mn^{2+} [Lin, Kumanovics, Nelson, Warner, Ward and Kaplan (2008) J. Biol. Chem. **283**, 33865–33873]. Mutant Zrc1 that gained iron transport activity could protect cells with a deletion in the vacuolar iron transporter (*CCC1*) from high iron toxicity. Utilizing suppression of high iron toxicity and PCR mutagenesis of *ZRC1*, we identified other amino acid substitutions within *ZRC1* that changed its metal specificity. All Zrc1 mutants that transported Fe^{2+} could also transport Mn^{2+} . Some Zrc1 mutants lost the ability to transport Zn^{2+} , but others retained the ability to transport Zn^{2+} . All of the amino acid substitutions that resulted in a gain in Fe^{2+} transport activity were found in transmembrane domains. In addition to alteration of residues adjacent to the putative metal-binding site in two transmembrane domains, alteration of residues distant from the binding site affected substrate specificity. These results suggest that substrate selection involves co-operativity between transmembrane domains.

Keywords

cation diffusion facilitator; iron; manganese; transition metal; vacuole; yeast; zinc

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AUTHOR CONTRIBUTION

Huilan Lin designed the genetic screen and performed most of the experiments. Damali Burton performed the experiments that identified the mutation in *ZRC1* resulting from the transposon screen. David Warner assisted in carrying out most of the experiments. John Phillips was consulted on the structural studies and provided the models showing the relationship between *ZRC1* and YiiP. Diane McVey Ward and Jerry Kaplan were responsible for the overall direction of the project and for writing the manuscript.

INTRODUCTION

Transition metal ions (Cu^{+2+} , Fe^{2+} , Zn^{2+} , Co^{2+} and Mn^{2+}) are essential for all organisms as they serve as cofactors for various proteins. They can also be toxic in excess owing to their participation in redox reactions or by their competing with other metals for protein binding sites. Organisms have evolved mechanisms to regulate uptake, delivery, storage and detoxification of these metals. One mechanism that prevents metal toxicity is metal transport out of the cytosol by CDF (cation diffusion facilitator) transporters. These transporters mediate metal resistance by either exporting metals out of cells or into intracellular organelles, thus reducing cytosolic metal concentration. Some examples include *Escherichia coli* ZitB [1] and mammalian ZnT1 (Slc30a1), which mediate zinc efflux from cells [2] and *Saccharomyces cerevisiae* Zrc1 and Cot1, which transport zinc and cobalt into vacuoles [3,4].

The majority of CDF transporters have six TMDs (transmembrane domains) with the N- and C-termini extending into the cytosol. A recent study classified CDF family members into three major groups (Zn^{2+} , $\text{Fe}^{2+}/\text{Zn}^{2+}$ and Mn^{2+}) based on substrate specificity [5]. Structural studies of the putative Zn^{2+} transporters CzcB from *Thermus thermophilus* [6] and YiiP (also known as FieF) from *E. coli* [7] indicate that the metal-binding sites of the transporter are formed by charged residues in TMDs II and V. The importance of amino acids in determining substrate specificity has been examined by sequence comparisons of different transport groups and by site-specific mutagenesis. Mutations that abrogate metal transport provide little information on substrate selection, because such mutations may affect transport activity independently of substrate selection. For example, loss of function may be due to alteration of residues involved in metal binding, as demonstrated by the study of YiiP (D157A) [7], or due to residues involved in stabilizing the protein structure, as implicated by the study of ZitB (E214A), CzcD (H237R) and CzcD (H280A) [8,9]. In contrast, amino acid mutations that lead to a gain of function by changing the metal transported, by their very existence, indicate the involvement of that amino acid in substrate selection. In this regard, gain-of-function mutations are informative in identifying residues involved in substrate selection.

We identified a mis-sense mutation in the yeast vacuolar Zn^{2+} transporter Zrc1 that resulted in the loss of Zn^{2+} transport and the acquisition of Fe^{2+} and Mn^{2+} transport activity [10]. The finding that one amino acid substitution can change transport specificity provides strong evidence for a role of that residue in substrate selection. Mis-sense mutations that result in a gain, rather than a loss, of function provide a robust approach for examining substrate specificity in CDF transporters. We combined a strong selection, the ability of cells with a deletion in the gene that encodes the vacuolar iron transporter *CCC1* to grow on high iron medium, with random and directed mutagenesis of *ZRC1* to identify residues that alter the transport specificity of Zrc1. We identified gain-of-function mutations that give rise to altered metal specificity. Our studies show that amino acids near the putative metal-binding amino acids in TMD II, as well as amino acids in other TMDs that are distant from the metal-binding residues, are important in substrate selection.

EXPERIMENTAL

Yeast strains and growth media

The following yeast strains (W303 background) were used: wild-type DY150 [*Mat a ade2-1 his3-11 leu2-3,112 trp1-1 ura3-52 can1-100(oc)*]. *ccc1* and *zrc1* strains were generated in the DY150 background by double-fusion PCR, using the *HIS3* gene as a selectable marker as described in [11]. Strains with a *FET3-lacZ* reporter integrated at the *HO* locus were constructed as described in [12]. Wild-type BY4743 (*Mat a/a his3 1/his3 1 leu2 0/leu2 0 lys2 0/+ met15 0/+ ura3 0/ura3 0*) and *pmr1::KanMX* strains in the BY4743 background were obtained from Research Genetics.

Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) or in CM medium (0.67% yeast nitrogen base without amino acids, 2% dextrose and 0.13% amino acid drop-out mix). Plates with high concentrations of metals were made by adding either ferrous ammonium sulfate, zinc sulfate or manganese chloride. Liquid media was supplemented with FeSO_4 to the indicated concentrations, with a final concentration of 1 mM ascorbic acid.

Transposon mutagenesis of *ccc1*

A mTn-lacZ transposon insertion library from Professor Michael Snyder (Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, U.S.A.) was used for mutagenesis. Three separate library pools were digested with NotI and transformed into *ccc1* cells. Cells were replicate plated on to medium containing 3 mM iron, and mutants able to grow on high iron medium were selected for further study. The transposon target site was identified by sequencing as described in [13].

Error-prone PCR and isolation of mutants

Wild-type *ZRC1* with its own promoter and 3' end was generated by PCR from genomic DNA using Pri54 and Pri60 as primers (Table 1). The PCR fragment was digested with BglII and KpnI and inserted into pRS426 (a yeast *URA3*-containing episomal vector) to generate *ZRC1/pRS426*. This plasmid was used as the template for error-prone PCR with PR0064 and Pri56 as primers. Mutations were introduced by the PCR error of Taq DNA polymerase (Fisher Scientific). The PCR conditions were: 95°C for 2 min, 35 cycles of (95°C, 30 s; 55°C, 30 s; 72°C, 2 min) and 72°C for 10 min. Four independent PCR products were pooled from the first round of PCR and used to seed six independent PCRs for the second round of PCR. The PCR products from the second round were pooled and transformed together with a gap plasmid (*ZRC1/pRS426* digested with HindIII and KpnI and the large fragment was purified) into *ccc1* cells. Cells were grown for two days and then replica plated to CM-Ura (complete minimal mix minus uracil) plates containing 5 mM ferrous ammonium sulfate. Cells able to grow on high iron were selected for further study. Plasmids rescued from the high iron-resistant colonies were sequenced using primers pri86, pri87 and pri88, which covered the *ZRC1* coding region.

Site-directed mutagenesis

The generation of *ZRC1*/pYES2, a His₆-tagged version of *ZRC1* under the control of the galactose-inducible promoter *GAL1*, was described previously [10]. Site-directed mutagenesis using *ZRC1*/pYES2 as a DNA template was performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Primers used in the present study are listed in Table 1. All mutations were confirmed by DNA sequencing.

Western blot, β -galactosidase activity and iron content

Cells were disrupted with glass beads in the presence of protease inhibitors (1.0 mM PMSF, 10 μ M pepstatin A and 20 μ M leupeptin) (Sigma). The supernatant from a low speed centrifugation was solubilized using SDS/PAGE sample buffer. Samples (20 μ g protein) were subjected to SDS/4–20% PAGE, transferred to nitrocellulose, and probed with rabbit anti-(His₆ tag) (1:2000, Abcam) or mouse anti-CPY (carboxypeptidase Y) antibody (1:4000, Invitrogen), followed by peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (1:10000, Jackson ImmunoResearch). Membranes were developed using chemiluminescence reagents (PerkinElmer Life Sciences). β -Galactosidase activity was performed in 96-well plates using *o*-nitrophenyl β -galactoside as a substrate [12]. Protein concentration was determined by the bicinchoninic acid method (Pierce) using BSA as standard. Vacuoles were isolated as described previously in [14]. The iron content of isolated vacuoles was determined using a PerkinElmer ICP (inductively coupled plasma)-Optical Emission Spectrometer with a standard curve generated from mixed metal standards.

Immunofluorescence

Immunofluorescence was performed as described in [15]. Cells were incubated with a rabbit anti-(His₆ tag) (1:100, Abcam) overnight at room temperature (20°C) in a humid chamber, and then incubated with an Alexa-594-conjugated goat anti-rabbit antibody (1:750; Invitrogen) for 2 h at room temperature.

RESULTS

Random mutagenesis results in *Zrc1* mutations that have gained iron transport activity

Zrc1 is a yeast vacuolar metal transporter that protects cells from the toxic effect of high concentrations of Zn²⁺ [3,16]. We showed that a single amino acid change (N44I) in *Zrc1* dramatically altered its substrate specificity from Zn²⁺ to Fe²⁺ and Mn²⁺ [10]. As a consequence, expression of *Zrc1* (N44I) was able to rescue the high iron growth defect of *ccc1* cells by transporting and storing iron in the vacuoles. *Zrc1* (N44I) was identified through a UV-mutagenesis screen that selected for *ccc1* mutant cells able to grow on high iron medium. We also utilized a transposon-generated library [13] to identify high iron-resistant mutants of *ccc1* cells. From 15000 colonies, two of the transposon-containing mutants, 22-3 and 25-1, were able to grow on high iron (Figure 1A). These mutants were found to have an insertion of the transposon upstream of *ZRC1* (Figure 1B). The insertion point of the transposon in each mutant was different, demonstrating that they were independent events. The mutants, although iron resistant, were unable to grow on high Zn,

suggesting an alteration in Zrc1 substrate specificity (results not shown). Sequencing of *ZRC1* in each of the mutants revealed base changes resulting in an amino acid substitution, although different in each case. Mutant 22-3 showed an A to T nucleotide change at position 131, resulting in a N44I amino acid substitution in Zrc1. The second mutant, 25-1, showed a G to T nucleotide change at position 99, resulting in a L33F amino acid substitution in Zrc1. Thus two different *ZRC1* mutations lead to a high iron-resistant phenotype. The topology of Zrc1 and the location of the amino acid substitutions within Zrc1 are shown in Figure 2.

Other mutations in Zrc1 that resulted in a change in metal substrate specificity were discovered through a second genetic screen. Previously, we identified *MRS3* and *MRS4* as high-copy suppressors of iron toxicity in a *ccc1* strain [17]. To identify other high-copy suppressors of poor growth of *ccc1* on high iron and circumvent the identification of *MRS3* and *MRS4*, we generated a high-copy genomic library from *mrs3 mrs4* cells. The genomic library was transformed into *ccc1* cells, and cells capable of growth on high iron medium were selected. As expected, we identified plasmids containing *CCC1* (identified by colony PCR) as suppressors that conferred high iron resistance (results not shown). We also identified two high copy suppressors that contained *ZRC1* with T to C nucleotide mutations at positions 119 and 120, resulting in the same mis-sense mutation F40S in Zrc1.

Directed evolution of Zrc1 reveals that different mutations lead to acquisition of iron transport activity

To identify other Zrc1 mutants that were able to transport iron, the *ZRC1* coding sequence was randomly mutagenized through error-prone PCR. The PCR products were transformed together with a gap plasmid into *ccc1* cells. We performed PCR mutagenesis on two independent preparations of *ZRC1*. Transformed colonies were replica plated on to medium containing 5 mM ferrous ammonium sulfate. Colonies (50000) were screened and resistant colonies (250) were identified. Plasmids from 62 resistant colonies were rescued, their ability to support the growth of *ccc1* cells reconfirmed and rescued plasmids were sequenced. Many mutant *ZRC1* plasmids showed more than one base change, but often the second base change was silent, as it did not result in a change in amino acid sequence (Table 2). We identified a number of mutants that had the same amino acid substitution (e.g. F40S). We know that those mutants were independently generated, as some mutants had a silent base change in addition to the mis-sense mutation (compare pZm165 with pZm225). We also identified the same amino acid substitution occurring by it self and in conjunction with other amino acids (compare pZm165 with pR3 and pR26). We also identified different substitutions of the same amino acid (L33S/L33F and I275N/I275F). These observations suggest that the mutagenesis was extensive; however, as shown below, we do not think it was saturated.

We first focused on plasmids in which there was only one amino acid substitution. All the *ZRC1* mutants were able to protect *ccc1* cells from high iron toxicity (Figure 3A). For comparison purposes we included *ZRC1(D45A)*, which we had generated previously [10]. Aspartic acid in the corresponding position in the *E. coli* YiiP is a critical zinc-binding residue [7]. Zrc1 (D45A) does not have Zn^{2+} or Fe^{2+} transport activity. To further demonstrate that the mutant proteins altered cellular iron metabolism, they were

overexpressed in wild-type cells containing an iron-sensitive reporter *FET3-lacZ*. Overexpression of *Ccc1*, by transporting iron into the vacuoles, lowers cytosolic iron, resulting in induction of *FET3-lacZ* [14]. Overexpression of wild-type *Zrc1* did not change the expression level of this reporter; however, all *Zrc1* mutants were able to induce *FET3-lacZ*, indicating their ability to lower cytosolic iron, although less efficiently than *Ccc1* (Figure 3B). Consistent with loss of transport activity, *Zrc1* (D45A) had no effect on expression of *FET3-lacZ*. Previously, we demonstrated that *Zrc1* (N44I) protects *ccc1* from high iron toxicity by transporting iron into vacuoles [10]. Overexpression of selected mutant *Zrc1* proteins increased vacuolar iron content in *ccc1* cells relative to cells transformed with a vector control or with wild-type *ZRC1* (Figure 3C).

Two amino acid substitutions in *Zrc1* are less efficient in transporting iron than a single amino acid substitution

To test whether combining amino acid substitutions might result in an additive effect generating a more efficient Fe^{2+} transporter, three independently isolated mutants in TMD II (L33F, F40S and N44I) were chosen to generate double mutants. *Zrc1* with two amino acid substitutions was not more efficient in Fe^{2+} transport activity than *Zrc1* with a single substitution, as assayed by growth on high iron medium (Figure 3A) and induction of *FET3-lacZ* (Figure 4A). We note that *Zrc1* (L33F, F40S) showed a low induction of *FET3-lacZ*, yet conferred high iron resistance when assayed on plates (Figure 3A). This difference may be ascribed to a difference in affinity, as the induction of *FET3-lacZ* was performed in moderate iron medium, whereas the plate assay utilized high iron medium. *Zrc1* (L33F, N44I) conferred less protection from high Fe^{2+} toxicity than the single substitution mutants, and its ability to induce the low iron sensor *FET3-lacZ* was also reduced. It is possible that some of the double amino acid substitutions give rise to an unstable *Zrc1*. To test this, wild-type *Zrc1*, single mutants and double mutants were epitope tagged at the C-terminus with His₆ and expressed using the *GALI* promoter. The expression levels of single mutants and double mutants were similar to that of the wild-type *Zrc1* (Figure 4B). We also tested the possibility that the double-mutant proteins were mistargeted and did not accumulate in the vacuole. Immunofluorescence showed that all *Zrc1* mutants were targeted to the vacuole (Figure 4C). These data suggest that combining some amino acid substitutions in *Zrc1* did not have an additive effect on iron transport.

Gain of iron transport activity in *Zrc1* mutants does not necessarily mean the loss of intrinsic zinc transport activity

As described previously, *Zrc1* (N44I) gained Fe^{2+} transport activity but lost its inherent Zn^{2+} transport activity [10]. Loss of Zn^{2+} transport is not, however, sufficient to protect *ccc1* from high concentrations of iron. *Zrc1* (D45A) does not have Zn^{2+} transport activity and does not protect *ccc1* cells from high iron [10]. In our previous study [10], we used a low copy plasmid to test Zn^{2+} transport activity of mutant *Zrc1*. In the present study we used a high copy plasmid to determine if overexpressed mutant *Zrc1* retained any Zn^{2+} transport activity, as assayed by protection of *zrc1* cells from high zinc toxicity. Some mutants (L33F and L87H) clearly lost Zn^{2+} transport activity, however, others (F40S, A52T, G79S, F86S, R101G and I275F) did not show significant differences from the wild-type *Zrc1* in protecting *zrc1* cells from zinc toxicity (Figure 5). Other mutants of *Zrc1* (L33S, N44I,

S272P and I275N) showed decreased Zn^{2+} transport activity compared with wild-type Zrc1. It is interesting that the double mutant Zrc1 (F40S, N44I) lost Zn^{2+} transport activity, whereas individually each of the single mutants had Zn^{2+} transport activity.

Zrc1 mutants that transport iron also transport manganese

The Golgi membrane P-type ATPase Pmr1 is responsible for transporting Ca^{2+} and Mn^{2+} into the Golgi [16]. *pmr1* cells accumulate Mn^{2+} in the cytosol and show poor growth on high concentrations of Mn^{2+} [17]. Overexpression of Ccc1, by transporting Mn^{2+} into the vacuoles, protects *pmr1* cells from toxic amounts of manganese in the environment. To test whether the Zrc1 mutants were able to transport Mn^{2+} into vacuoles and detoxify it, *pmr1* cells were transformed with these mutant constructs and grown on plates with 3 mM Mn^{2+} . All the mutants showed protection of *pmr1* cells compared with wild-type Zrc1 (Figure 6), although some mutants were less efficient than others. Surprisingly, although the D45A substitution lost both Fe^{2+} and Zn^{2+} transport activity, it was able to partially rescue the growth defect of *pmr1*.

Effect of amino acid substitutions in TMD II on substrate selection

The sequence adjacent to the putative metal-binding residues in TMD II is different in different CDF family members. These amino acids were hypothesized to determine the substrate specificity of CDF families. We identified two residues, Phe⁴⁰ and Asn⁴⁴, adjacent to the putative metal-binding residues His⁴¹ and Asp⁴⁵ in TMD II, which, when mutated, resulted in iron transport activity (F40S and N44I). The F40S mutation occurred quite often and no other mutations of Phe⁴⁰ were found. One interpretation of this result is that the hydroxy group of the introduced serine residue is required for Fe^{2+} transport. To test this possibility, we determined whether other substitutions in this amino acid would affect substrate selection. We generated Zrc1 (F40T) and Zrc1 (F40A) and examined their effect on metal resistance. Zrc1 (F40S) and Zrc1 (F40T) were able to protect *ccc1* cells from high iron toxicity (Figure 7A). Complementation by F40A was less efficient than for F40S or F40T, but was still noticeable (note vector alone). This result indicates that the hydroxy group at position 40 enhances, but is not absolutely required for, Fe^{2+} transport. All the Phe⁴⁰ mutants retained Zn^{2+} transport activity, with Zrc1 (F40S) having slightly decreased activity (Figure 7B). Zrc1 (F40S) protected *pmr1* cells from Mn^{2+} toxicity to a much greater extent than F40T (Figure 7C). The fact that F40T did not lead to Mn^{2+} transport shows that it is not simply the presence of a hydroxy group that permits transport.

A hydrophobic amino acid substitution in Zrc1 at position 44 (N44I) resulted in Fe^{2+} transport activity. This residue is adjacent to the putative metal-binding residue Asp⁴⁵ and may alter the structure of the metal-binding site. To examine whether the size of the side chain at this position affects substrate selection, we generated N44I, N44A and N44V mutations. All Asn⁴⁴ substitutions were able to protect *ccc1* cells from iron toxicity (Figure 7A). Zrc1 (N44A) retained Zn^{2+} transport activity, whereas Zrc1 (N44I) and Zrc1 (N44V) did not (Figure 7B). All three mutants were able to transport Mn^{2+} , as shown by their ability to protect *pmr1* cells from Mn^{2+} toxicity (Figure 7C). A summary of phenotypes for all mutant Zrc1 isoforms is detailed in Table 3.

DISCUSSION

Residues that impart metal specificity to CDF transporters have been identified through site-specific mutagenesis, sequence comparisons and structural studies [5]. Sequence comparisons of CDF transporters implicated residues in TMDs II and V as critical determinates of metal specificity. The crystal structure of the only CDF member resolved, YiiP, shows a substrate-binding site formed by residues from TMDs II and V [7]. Recombinant YiiP has Zn^{2+} transport activity *in vitro* and the crystal structure shows bound zinc [7]. Confusing the issue is that YiiP appears to protect cells from iron toxicity [7], but no iron was found bound to the protein. Mutation of aspartic acid residues in TMD II (Asp⁴⁹) and TMD V (Asp¹⁵⁷) affects Zn^{2+} transport activity and metal binding [20]. These data suggest that these aspartic acid residues are important in metal binding, but it is not clear what their role is in metal selectivity.

Gain-of-function mutations in *ZRC1* that alter substrate selection provide insight into the role of specific amino acids in metal specificity. Gain-of-function *ZRC1* mutations were identified through their ability to suppress the phenotype of poor growth of *ccc1* cells on high iron medium. Previously, we demonstrated that suppression of the high iron toxicity phenotype resulted from the transport of iron into the vacuole [10]. We confirmed that result with surrogate assays for cytosolic iron, including the expression of iron-sensitive reporter constructs *FET3-lacZ*. We have not been able to successfully measure the transport of ⁵⁹Fe into vacuoles. To date there are only two published papers that measured transition metal uptake into vacuoles *in vitro*: one on cadmium [21] and one on zinc [4]. There are no published studies of direct measurements of iron transport into isolated organelles, such as mitochondria or vacuoles. Measurement of direct iron transport into organelles is problematic, as iron is 'sticky' and non-specific binding of iron to organelles is so high that specific transport cannot be assessed. Published studies measuring iron transport in to mitochondria have utilized surrogate assays such as iron incorporation into haem or the activity of iron-sulfur-containing enzymes for just that reason (for example [22–24]). The measurement of iron-dependent toxicity is an indirect measure of iron transport activity. It is of interest that the only high copy suppressors of iron-dependent growth toxicity we identified are organelle iron transporters: the vacuolar iron transporter Ccc1 [14], the yeast mitochondrial transporters Mrs3 and Mrs4 [17], Rim2 (L. Li, unpublished work) and mutant vacuolar zinc transporters [10]. No other genes have been found to suppress high iron toxicity, giving us confidence that our toxicity assays are indeed measuring the activity of iron transporters.

The strength of this phenotype permitted facile identification of rare mutations. For example, we identified mutations that were generated through use of a transposon mutagenesis screen. The transposon mutagenesis occurs by activating transposon movement in bacteria carrying a yeast genomic library [11]. The library is then transformed into yeast and the transposon-carrying chromosomal fragments are selected to replace the endogenous piece of chromosome by homologous recombination. Using this procedure, we identified two independent *ZRC1* missense mutations, even though the transposon was inserted 5' to the coding region. Generation of a genomic library from yeast with a wild-type *ZRC1* also resulted in gain-of-function *ZRC1* mutants. Both results suggest that transformation into

bacteria or yeast is mutagenic, or that some level of expression of *ZRC1* in bacteria is toxic and leads to the selection of mutations.

The finding of different mutations in *ZRC1* that led to a gain of function was the basis for performing random mutagenesis of *ZRC1*. The mutagenesis was extensive, as the same substitution (for example F40S) was identified independently more than once. As shown by site-directed mutagenesis of Asn⁴⁴ (Figure 7), there were substitutable amino acids we did not find in the screen, leading us to conclude that, although the mutagenesis was extensive, it was not saturating. All amino acid substitutions occurred in, or immediately adjacent to, the TMDs (Figure 2). No mutations in the cytosolic side of Zrc1 were identified. We did not find mutations distributed throughout the protein, suggesting that the cytosolic domain is not involved in substrate selection. This portion of CDF transporters is thought to be involved in sensing metals or in the docking of metal chaperones [6]. The fact that a single mutation can change the substrate specificity calls into question the nature or existence of a chaperone. If chaperone binding is critical to metal transport activity, how can a single amino acid change affect chaperone selectivity and transport activity? Would a zinc chaperone be promiscuous and permit iron binding?

Structural studies on YiiP demonstrated that four residues from TMD II and TMD V form a zinc-binding site, and Zn²⁺ may cross the membrane through this site [7]. The corresponding aspartate residue in Zrc1, when mutated to an alanine, results in a loss of Zn²⁺ transport activity, suggesting a conservation of function of the corresponding residues in Zrc1. Consistent with this finding, we found that mutations in four critical residues in TMD II (Leu³³, Phe⁴⁰, Asn⁴⁴ and Ala⁵²) affected metal specificity. It is of interest that we did not identify mutations in TMD V, as this domain is expected to comprise the metal-binding domain. We did, however, identify mutations in TMD III (G79S, L86S, L87H, E97G and R101G) and TMD VI (S272P, I275F and I275N) that affected metal specificity.

Sequence alignment of YiiP and Zrc1 shows significant homology within the TMDs (Figure 8). The crystal structure of YiiP has been solved to 3.8 Å (1 Å = 0.1 nm) resolution [7]. The protein functions as a homodimer. Using the structure of YiiP (PDB entry 2QFI) as a model for Zrc1, we generated a homologous model of the Zrc1 dimer. There may be differences in the number of amino acids in the TMDs between YiiP and Zrc1, as the TMDs are predicted and have not been experimentally verified. The model for Zrc1, based on YiiP, provides a tool to discuss structure–function relations. In this model, many of the Zrc1 residues shown to affect metal transport specificity were distant from the predicted tetraco-ordinate metal-binding residues (site identified in YiiP) and by homology analysis in other CDF members [5]. By realigning the monomer subunits to approximate the assumed biological dimer, we generated a model that shows that many of the residues that alter metal specificity of Zrc1 are located at the dimer interface (Figure 9). This model suggests that interaction between subunits is as important in determining metal specificity as amino acids found near the essential metal-binding site. We believe that these mutations make the active site flexible so that it can accommodate metal ions of different co-ordination geometries. Mutation of Asn⁴⁴ to a smaller hydrophobic group, N44A, led to both Zn²⁺ and Fe²⁺ transport activity, suggesting that the size of the amino acid side chain affects the geometry of the active site. There are mutations that affect substrate specificity that are not adjacent to the active site or

in the dimer interface. This result suggests that the metal-binding site is altered through allosteric interactions.

Zrc1 mutations that conferred Fe^{2+} transport activity also conferred Mn^{2+} transport activity, as demonstrated by complementation of the growth defect of *pmr1* cells under high concentration of Mn^{2+} . Many transporters that transport Fe^{2+} also transport Mn^{2+} , such as Nramp1 (natural-resistance-associated macrophage protein 1) [25], Smf1 (suppressor of mitochondrial import function 1) [26], Ccc1 [14] and IRT1 (iron-regulated transporter 1) [27]. Mutations in the plant transition-metal transporter IRT1 affect substrate specificity, although the mutations do not result in a gain of new transport capabilities, but rather a selective loss of transport for some metals and increased activity for others [28]. It was noted that the ability to transport Fe^{2+} tracks with that of Mn^{2+} , but not with that of Zn^{2+} , as loss of Fe^{2+} transport was concomitant with loss of Mn^{2+} transport. There are, however, transporters that are specific for Mn^{2+} . Pmr1 is an ATP cassette transporter that transports Mn^{2+} in to the Golgi. Overexpression of *PMR1* in *ccc1* cells did not confer iron resistance. There are CDF members that are specific to Mn^{2+} [5]. Our selection system focused on mutations that led to resistance to high iron toxicity. It might be of interest to repeat the selection process but focusing on resistance to Mn^{2+} toxicity to determine if there are mutations that can discriminate between Fe^{2+} and Mn^{2+} .

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Abbreviations used

CDF	cation diffusion facilitator
CM-Ura	complete minimal mix minus uracil
CPY	carboxypeptidase Y
ICP	inductively coupled plasma
TMD	transmembrane domain

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Figure 1. Identification of transposon-generated genomic mutants that protect *ccc1* cells from high iron toxicity

(A) Wild-type (WT), *ccc1* and *ccc1* mutants 22-3 and 25-1 were grown on CM-Ura plates with 3 mM ferrous ammonium sulfate. Plates were incubated at 30 °C for 2 days. (B) The transposon insertion sites were identified by sequencing the rescued plasmids. The transposon was inserted upstream of *ZRC1* in both mutants, as denoted by the arrow.

Figure 2. Model for the topology of Zrc1

A model for the topology of Zrc1 is shown with predicted TMDs in the centre (I to VI), cytoplasmic loops below and vacuolar loops above. Black circles are residues in TMDs II and V that co-ordinate zinc based on the homologous bacterial CDF transporter YiiP. The numbered grey circles are residues identified in the present study that, when mutagenized, result in iron transport activity. The histidine-rich and cytosolic domains are also indicated. Transmembrane domains were predicted using the TMHMM program.

Figure 3. Zrc1 mutants protect *ccc1* cells from high iron toxicity

(A) DY150 (WT) and *ccc1* cells, transformed with the indicated plasmids, were grown in CM-Ura medium to exponential phase and serially spotted on to CM-Ura plates with or without 5 mM ferrous ammonium sulfate. The plates were incubated at 30 °C for 2 days. (B) WT cells with an integrated *FET3-lacZ* reporter construct were transformed with pRS426 (empty vector), *ZRC1*, *ZRC1* mutants and *CCC1* regulated by their endogenous promoters. Cells were grown to exponential phase in CM medium and β -galactosidase activity was determined. (C) *ccc1* cells transformed with pRS426, *ZRC1* or mutant *ZRC1* under the control of the *ZRC1* promoter and were grown in CM-Ura medium with 100 μ M FeSO₄ overnight. Vacuoles were isolated and the iron content was determined by ICP optical emission spectrometry. All data were normalized for protein concentration and error bars represent S.D. from three experiments.

Figure 4. Zrc1 double mutants are not more efficient than single mutants in iron transport activity

(A) Wild-type cells with an integrated *FET3-lacZ* were transformed with pRS426, *ZRC1*, *ZRC1* single mutants and double mutants. Cells were grown to exponential phase in CM medium and β -galactosidase activity was determined. (B) *ccc1* cells were transformed with pYES2/CT, *ZRC1-His₆* and *ZRC1-His₆* single or double mutants under the control of the *GAL1* promoter. Cells grown in CM-Ura medium with 2 % galactose were harvested, disrupted by glass beads and protein levels were measured by Western blot using a rabbit anti-His₆ antibody or a mouse anti-CPY antibody followed by peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. The vacuolar protein CPY was used as a loading control. The lanes were: 1, pYES2/CT; 2, *ZRC1*; 3, L33F; 4, F40S; 5, N44I; 6, L33F, F40S; 7, L33F, N44I; 8, F40S, N44I. (C) *ccc1* cells were transformed as in (B) and grown in galactose medium. Cells were stained with a rabbit anti-His₆ antibody followed by an Alexa Fluor[®]-594-conjugated goat anti-rabbit antibody. Nomarski interference contrast and the corresponding immunofluorescence images are shown. All Zrc1 single and double mutants localized to the vacuole. Representative images are shown.

Figure 5. Analysis of zinc transport activity in Zrc1 mutants

Wild-type and $\Delta zrc1$ cells, transformed with pRS426, *ZRC1* and *ZRC1* mutants, were spotted on CM-Ura plates with or without 6 mM ZnSO₄. Plates were incubated at 30 °C for 2 days.

Figure 6. All gain-of-function *ZRC1* mutants confer resistance to high manganese toxicity Wild-type cells (BY4743) or *pmr1* cells were transformed with pRS426, *CCCI*, *ZRC1* or *ZRC1* mutants under their native promoters. Cells were spotted on to CM-Ura plates with or without 3 mM $MnCl_2$. Plates were incubated at 30 °C for 2 days.

Figure 7. The effect of amino acid substitutions at position 40 and 44 of Zrc1 on metal transport activity

(A) *ccc1* cells transformed with the indicated plasmids were spotted on to CM-Ura plates with or without 5 mM ferrous ammonium sulfate. (B) *zrc1* cells transformed with indicated plasmids were spotted on CM-Ura plates with or without 6 mM ZnSO₄. (C) *pmr1* cells were transformed with indicated plasmids and spotted on to CM-Ura plates with or without 3 mM MnCl₂. All plates were incubated at 30 °C for 2 days.

ScZrc1 --MITGKELR--IISLLTLDTVFFLEITIGYMSHSLALIADSFHMLNDIISLLVALWAV 56
EcYiiP MNQSYGRIVSRRAIIATAMASLLLLIKIFAWWYTGVSILAALVDSLVDIGASLTNLVV 60
*: I: *: : : : : : : : *: : : : : : * II: * * * * : * . * *

ScZrc1 DVAKNRGPDAKYTYGWKRAEILGINAVFLIALCFSIMIEALQRIEIQNPRVLVY 116
EcYiiP RYSLQP-ADDNHSFGHGKAESLAALQSMFISGSALFLFLTGIQHLISPTPMTDPGVGVI 119
: : . * : : : * III: * * * * : : : . : : : : * : * : *

ScZrc1 VGVAGLISNVVGLFLFDHGHGSDSLHSHSGSVESGNNDLIESNATHSHSHASLPNDLA 176
EcYiiP VTIVALICTITILVSFOR----- 136
* : IV: * * . : : : : :

ScZrc1 IDEDAISSPGPSGGIGEVLPQSVVNRLSNESQPLLNHDDHDHSHESKKPGHRSLNMHGVF 236
EcYiiP -----WVVR-----TQSAVRADM 151
* * * V: * * : : :

ScZrc1 LHLVDGALGNIGVIAAALFIWKTEYSWRYSDPVLSLIITIIIFSSALPLSRRASRILLQ 296
EcYiiP LHYQSDVMMNGAILLALGLSW--YGWHR-ADALFALGIGIYILYLSALRMGYEAVQSLLD 207
* * . * : * : * * * VI: * . : : * * * : * * : . * * : * :

ScZrc1 ATPSTISADQIQREILAVPGVIAVDFHVNLTESIYIASIHVQIDCAPDKFMSSAKLIR 356
EcYiiP RALPDEERQEIIDVTSWPGVSGAHLRLTRQSGPTRFIQ-IHLEMEDS----- 254
: . : : : : : * * * . : : : : : : : : * * * : : :

ScZrc1 KIFHQHGIHSATVQPEFVSGDVNEDIRRFSIIAGGSPSSQEAFDShGNTHEGRKKRSP 416
EcYiiP -----LPLVQAHMVADQVEQAILRRF-----PGSDVIIHQDPCSVVPREGKRSM 298
* * . : * : : : * * * * * . * . . . * * *

ScZrc1 TAYGATTASSNCIVDDAVNCNTSNCL 442
EcYiiP LS----- 300

Figure 8. Amino acid sequence alignment of *S. cerevisiae* Zrc1 and *E. coli* YiiP
Amino acid identity is denoted as *, similarity as : and .; TMDs of YiiP are underlined and designated I–VI [7] and arrows denote amino acid substitution sites identified in the screen.

Figure 9. Model of the TMDs of Zrc1

A model of the Zrc1 dimer TMDs is shown with one monomer in blue and the other monomer in cyan. The model was generated by homology modelling using YiiP as template. The mutant residues that give rise to iron transport activity are shown in green in the blue monomer and magenta in the cyan monomer. The metal co-ordinating residues identified in YiiP are shown in orange. (A) Side view. (B) Top view.

Table 1

Primers used in the present study

Primers	Sequence	Purpose
Pri54	GATATGAAAGTAGTTGCATT	Clone <i>ZRC1</i>
Pri60	TTGGTACAGGAGGGAACAAG	Clone <i>ZRC1</i>
Pri86	CCTTCTCAGATGGCCTTGA	Sequence <i>ZRC1</i>
Pri87	CCCAAGGTTGGTTTTATACG	Sequence <i>ZRC1</i>
Pri88	TGCCCTTATCACGTAGAGCT	Sequence <i>ZRC1</i>
PR0599	AATATACCTCTATACTTTAACGTC	Sequence <i>ZRC1</i>
Pri56	ATGATCACCGGTAAAGAATT	Error-prone PCR
PR0064	CGCCAGGGTTTTCCAGTCACGAC	Error-prone PCR
Pri115	CCATAGGTTATATGTACATTTCATTGCTTGATTGCCGATTC	<i>ZRC1</i> (L33F)
Pri116	GAATCGGCAATCAAGGCaAATGAATGTGACATATAACCTATGG	<i>ZRC1</i> (L33F)
Pri94	GGCCTTGATTGCCGATTCATcCACATGTTGAATGATATC	<i>ZRC1</i> (F40S)
Pri95	GATATCATTC AACATGTGggATGAATCGGCAATCAAGGCC	<i>ZRC1</i> (F40S)
Pri64	GGCCTTGATTGCCGATTCATTTACATGTTGATTGATATCATCTCTCTTTTAGTGGC	<i>ZRC1</i> (N44I)
Pri65	GCCACTAAAAGAGAGATGATATCAATCAACATGTGAAATGAATCGGCAATCAAGGCC	<i>ZRC1</i> (N44I)
Pri102	CACATGTTGAATGCTATCATCTCTCTTTTAGTGGCAC	<i>ZRC1</i> (D45A)
Pri103	GTGCCACTAAAAGAGAGATGATAGCATTCAACATGTG	<i>ZRC1</i> (D45A)
Pri117	GCCTTGATTGCCGATTCATcTCACATGTTGAfTGATATCATCTCTC	<i>ZRC1</i> (F40S, N44I)
Pri118	GAGAGATGATATCAaTCAACATGTGAgATGAATCGGCAATCAAGGC	<i>ZRC1</i> (F40S, N44I)
Pri127	GCCGATTCATTTACATGTTGgTGATATCATCTCTCTTTTAGTGGC	<i>ZRC1</i> (N44V)
Pri128	GCCACTAAAAGAGAGATGATATCAacCAACATGTGAAATGAATCGGC	<i>ZRC1</i> (N44V)
Pri129	GCCGATTCATTTACATGTTGgcTGATATCATCTCTCTTTTAGTGGC	<i>ZRC1</i> (N44A)
Pri130	GCCACTAAAAGAGAGATGATATCAgcCAACATGTGAAATGAATCGGC	<i>ZRC1</i> (N44A)
Pri149	CATTGGCCTTGATTGCCGATTCAacTCACATGTTGAATGATATC	<i>ZRC1</i> (F40T)
Pri150	GATATCATTC AACATGTGAgfTGAATCGGCAATCAAGGCCAATG	<i>ZRC1</i> (F40T)
Pri151	CATTGGCCTTGATTGCCGATTCAgcTCACATGTTGAATGATATC	<i>ZRC1</i> (F40A)
Pri152	GATATCATTC AACATGTGAgcTGAATCGGCAATCAAGGCCAATG	<i>ZRC1</i> (F40A)

Mutations are shown in lower case.

Table 2

Nucleotide and amino acid changes in mutant Zrc1

Mutants	Amino acid change	Nucleotide change
pR3	F40S, G410D	t119c, g1229a
pR4	L33F	g99t
pR8	F40S, silent	t119c, a1245g
pR9	N44I, P371L	a131t, c1112t
pR10	A52V, I94T, V239I, L273F,	c155t, t281c, g715a, a819t
pR13	N44I, I94T	a131t, t281c
pR15	A37G, silent, silent	c110g, t726c, t1284c
pR16	I275F	a823t
pR17	L33F, silent	g99t, t628c
pR18	Silent, I275N	t783c, t824a
pR19	F40S	t119c
pR20	Silent, I177F, I275F, I387T	g321a, a529t, a823t, t1163c
pR22	F40S	t119c
pR26	F40S, I94T	t119c, t281c
pR28	I275N	t824a
pR31	F40S, S403G	t119c, a1207c
pR32	Silent, I94T, G248C, I274V	a90t, t281c, g742t, a820g
pR124	N44I, I270T	a131t, t809c
pR127	Silent, F40S, D342E, silent, silent	t54a, t119c, c1026g, t1248c, a1275t
pR129	F40S, silent, I94T, silent	t119c, t237c, t281c, t1281c
pR130	F86S, I94T, D173G, silent, silent, I337T	t257c, t281c, a518g, t804c, a903g, a990g, t1010c
pR132	N44T, silent, T259A	a131c, t342c, a775g
pR133	K224R, I275N, silent	a671g, t824a, c948a
pR134	L33F, V58A	g99t, t173c,
pR137	F40S, I94T, I177N, I388F	t119c, t281c, t530a, a1162t
pR139	I275N	t824a
pR145	L33F	g99t
pZm152	F19V, F40S, S222P	t55g, t119c, t664c
pZm155	I275N	t824a
pZm160	F40S	t119c
pZm163	E97G, silent	a290g, t795c
pZm165	F40S	t119c
pZm167	L33S, S166P, Y419C	t98c, t496c, a1256g
pZm169	L87H	t260a
pZm171	L87H, S387P	t260a, t1159c
pZm175	E104G, N231S	a311g, a692g
pZm176	E97G, silent, silent	a290g, t831c, t856c
pZm177	L33S, silent	t98c, a717g
pZm184	A52T, silent	g154a, t1191c

Mutants	Amino acid change	Nucleotide change
pZm185	Low quality but has A52T	g154a
pZm187	L87H	t260a
pZm188	E97G	a290g
pZm192	L33S, silent	t98c, t583c
pZm194	E97G	a290g
pZm195	F20L, silent, P105L	t58c, t81c, c314t
pZm207	L33S	t98c
pZm208	Silent, S272P	c516t, t814c
pZm210	E97G, E107D	a290g, a321t
pZm211	R101G, silent	a301g, a939g
pZm213	R101G, silent, F401L	a301g, a903g, t1203a
pZm214	M232T, S272P	t695c, t814c,
pZm217	E97G, silent, E179G	a290g, t417c, a536g
pZm218	L33F	g99t
pZm219	I36N, silent, L203S, E399A	t107a, t171c, t608c, a1196c
pZm223	G79S	g235a
pZm224	G79S	g235a
pZm225	F40S, silent	t119c, t309c
pZm226	S272P	t814c
pZm229	Silent, S272P	a564c, t814c
pZm230	F86S	t257c
pZm235	P269L, S272P	c806t, t814c
pZm236	F40S, T276S	t119c, c827g

Table 3

Summary of the Zrc1 mutants in the present study

Amino acid ^a	Base ^b	Number ^c	Location ^d (TMD)	Fe ^e	Zn ^f	Mn ^g	Induction of FET3-lacZ ^h
ZRC1				-	+	-	No
Single mutations identified from error-prone PCR and subsequent selection							
L33F	g99t	4	II	+	-	+	Yes
L33S	t98c	3	II	+	+	+	Yes
F40S	t119c	3	II	+	+	+	Yes
N44I	at131t	2	II	+	±	+	Yes
A52T	g154a	1	II	+	+	±	Yes
G79S	g235a	2	III	+	+	+	Yes
F86S	t357c	1	III	+	+	±	Yes
L87H	t260a	2	III	+	-	+	Yes
E97G	a290g	4	III	+	+	+	Yes
R101G	a301g	1	III	+	+	+	Yes
S272P	t814c	3	VI	+	±	+	Yes
I275F	a823t	1	VI	+	+	+	Yes
I275N	t824a	3	VI	+	+	+	Yes
Mutations made by site-directed mutagenesis							
D45A			II	-	-	±	No
L33F, F40S			II	±	-	+	Yes
L33F, N44I			II	±	-	+	Yes
F40S, N44I			II	+	-	+	Yes
N44V			II	+	-	+	N.T.
N44A			II	+	+	+	N.T.
F40A			II	±	+	-	N.T.
F40T			II	+	+	-	N.T.

^a Amino acid substitutions in Zrc1 protein.
^b Nucleotide changes found in ZRC1 coding region corresponding to the amino acid change.
^c Number of independent plasmids identified.

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^dThe position of the amino acid substitution relative to the transmembrane domains.

^eThe ability to transport iron was assayed by the growth of *ccc1* cells on 5 mM of iron, +, growth; -, no growth; ±, partial growth.

^fThe ability to transport Zn^{2+} was assayed by the growth of *zrc1* cells on 6 mM Zn^{2+} .

^gThe ability to transport Mn^{2+} was assayed by the growth of *pmr1* cells on 3 mM Mn^{2+} .

^hThe ability to induce the *FET3-lacZ* reporter was assayed as a result of overexpression of the mutant allele.

N.T., not tested.