THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 23, Issue of June 10, pp. 22181–22190, 2005
© 2005 by The American Society for Biochemistry and Molecular Biology, Inc. *Printed in U.S.A.*

Post-transcriptional Regulation of the Yeast High Affinity Iron Transport System*

Received for publication, December 29, 2004, and in revised form, March 21, 2005
Published, JBC Papers in Press, April 7, 2005, DOI 10.1074/jbc.M414663200

M. Rosa Felice‡§, Ivana De Domenico‡§¶, Liangtao Li¶, Diane McVey Ward¶, Beatrix Bartok¶, Giovanni Musci‡, and Jerry Kaplan¶‖

From the ‡Dipartimento di Scienze Microbiologiche Genetiche e Molecolari, Università di Messina, Salita Sperone 31, I-98166 Villaggio S. Agata, Messina I-98166, Italia and the ¶Division of Immunology and Cell Biology, Department of Pathology, School of Medicine, University of Utah, Salt Lake City, Utah 84132

***Saccharomyces cerevisiae* transcriptionally regulates the expression of the plasma membrane high affinity iron transport system in response to iron need. This transport system is comprised of the products of the *FET3* and *FTR1* genes. We show that Fet3p and Ftr1p are post-translationally regulated by iron. Incubation of cells in high iron leads to the internalization and degradation of both Fet3p and Ftr1p. Yeast strains defective in endocytosis (Δ*end4*) show a reduced iron-induced loss of Fet3p-Ftr1p. In cells with a deletion in the vacuolar protease *PEP4*, high iron medium leads to the accumulation of Fet3p and Ftr1p in the vacuole. Iron-induced degradation of Fet3p-Ftr1p is significantly reduced in strains containing a deletion of a gene, *VTA1*, which is involved in multivesicular body (MVB) sorting in yeast. Sorting through the MVB can involve ubiquitination. We demonstrate that Ftr1p is ubiquitinated, whereas Fet3p is not ubiquitinated. Iron-induced internalization and degradation of Fet3p-Ftr1p occurs in a mutant strain of the E3 ubiquitin ligase *RSP5* (*rsp5-1*), suggesting that Rsp5p is not required. Internalization of Fet3p-Ftr1p is specific for iron and requires both an active Fet3p and Ftr1p, indicating that it is the transport of iron through the iron permease Ftr1p that is responsible for the internalization and degradation of the Fet3p-Ftr1p complex.**

Transition metals are essential for life, yet transition metals in high concentrations can be toxic. Both eukaryotes and prokaryotes tightly regulate the concentration of free intracellular metals by either regulating metal uptake or sequestration. High affinity iron transport in the budding yeast *Saccharomyces cerevisiae* requires the expression of two cell surface proteins, the multicopper oxidase Fet3p and the transmembrane permease Ftr1p (1, 2). Transcription of these genes, as well as genes that encode proteins required for the processing of Fet3p, is regulated by the iron sensing transcription factor Aft1p (3).

In *S. cerevisiae* transporters for the transition metals copper and zinc are regulated post-translationally. High levels of zinc induce the internalization and vacuolar degradation of Zrt1p, the high affinity zinc transporter (4). High levels of copper induce the degradation of Ctr1p, the high affinity copper transporter, whereas Ctr3p, another high affinity copper transporter, is not affected (5). A previous study from our laboratory suggested that regulation of the high affinity iron transport system was predominantly transcriptional (6), although there is evidence that the activity of the iron transport system may be regulated by cAMP (7). Studies in *Schizosaccharomyces pombe*, however, suggested that the multicopper oxidase-based high affinity iron transport system might be regulated post-translationally. Incubation of *S. pombe*, expressing the high affinity transport system, with high concentrations of iron led to a rapid inhibition of iron transport (8). High levels of iron transport activity are seen when *FET3*/*FTR1* are expressed using the iron-independent *GAL10* promoter. There is a 50% reduction in transport activity when such cells are incubated in high iron as opposed to low iron medium (8). Based on these observations, we re-examined whether the Fet3p-Ftr1p transport system is post-translationally regulated. We demonstrate that high levels of iron induce the internalization and degradation of the Fet3p-Ftr1p transport system.

MATERIALS AND METHODS

Strains and Media—The *S. cerevisiae* strains used in this study are listed in Table I. The cells were grown in either medium containing yeast extract-peptone-dextrose (YPD)[1] or yeast nitrogen base synthetic complete medium (CM) with supplements as needed (9). Low iron growth medium was made by adding 40 or 80 μM bathophenanthroline disulfonate (BPS), an iron chelator, to CM or YPD and then adding back varying amounts of $FeSO_4$ (9). Low iron medium used in this work is referred to as BPS (x), in which the media contains BPS and x equals the concentration in micromolar of added $FeSO_4$.

S1 Nuclease Protection Analysis—Total RNA was isolated and analyzed using standard techniques (10). All samples were isolated from mid-log phase cultures grown in either CM or CM BPS (5). The ^{32}P-labeled *FET3* and *CMD1* probes were generated.

Preparation of Antisera against Fet3p—A secreted Fet3p (Fet3p lacking the transmembrane and cytoplasmic domains) was generated as described by Hasset *et al.* (11). Procedures for the isolation and deglycosylation of secreted-Fet3p have been described previously (12). The *N*-glycanase-treated Fet3p was injected into rabbits, and antisera were prepared. The soluble Fet3p was attached to an Amino-link gel using the manufacturer's instructions (Pierce Inc.). The antiserum was applied to the column, and the column was extensively washed with phosphate-buffered saline and eluted with 0.1 M glycine (pH 2.5) and immediately neutralized with 1.0 M Tris-HCl (pH 9.0). The purified antibody was useful for both immunofluorescence and Western analysis.

Immunofluorescence—Cells were prepared for immunofluorescence as described previously (13). For visualization of Fet3p, the rabbit anti-Fet3p antibody was used (1:500) followed by either an Alexa 594- or Alexa 488-conjugated goat anti-rabbit antibody (1:500). All of the

* This work was supported by National Institutes of Health Grant DK30534. Support for DNA oligonucleotides and sequencing was provided by Cancer Center Support Grant CA43014. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.
‖ To whom correspondence should be addressed: Dept. of Pathology, School of Medicine, University of Utah, 50 N. Medical Dr., Salt Lake City, UT 84132. Tel.: 801-581-7427; Fax: 801-581-4517; E-mail: jerry.kaplan@path.utah.edu.

[1] The abbreviations used are: YPD, yeast extract-peptone-dextrose; BPS, bathophenanthroline disulfonate; CM, complete media; GFP, green fluorescent protein; CFP, cyan fluorescent protein.

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

TABLE I
Strains used in this study

Strain	Genotype	Ref.
DY150	*MATa ade2, can1, his3, leu2, trp1, ura3*	(9)
DY1457	*MATα ade6, can1, his3, leu2, trp1, ura3*	(9)
DY150 (*FET3-GFP*)	DY150, *FET3-GFP::KanMX*	This study
DY1457 (*FTR1-CFP*)	DY1457, *FTR1-CFP::KanMX*	This study
Δend4	DY150, *Δend4::LEU2*	(37)
Δftr1 (42C)	*MAT a ade2, his3, leu2, lys2, trp1, Δftr1::TRP1*	This study
Δftr1 42C (*FET3-GFP*)	*Δftr1 (42C) FET3GFP::KanMX*	(38)
Δpep4	*MATa ade2, can1, his3, leu2, trp1, ura3, Δpep4::URA3*	This study
Δpep4Δfet3	*MATa ade2, can1, his3, leu2, trp1, ura3, Δpep4::URA3, Δfet3:KanMX*	This study
Δgef1	*MATα ade6, can1, his3, leu2, trp1, ura3*	(14)
BY4742	*MATα his3 leu2 lys2 ura3*	(21)
Δvta1–5a	*MATα his3 leu2 met 15 ura3, Δvta1::KanMX*	(21)
OCY 354	*MATa ade2, can1, his3, leu2, trp1, ura3, HO::FET3 LacZ*	This study
23344	*MATα, ura3*	(15)
27038 (*rsp5*)	*MATa, ura3, rsp5*	(15)
23344 (*FET3-GFP*)	*MATα, ura3*, FET3-GFP::KanMX	This study
27038 (*rsp5*) (*FET3-GFP*)	*MATa, ura3, rsp5*, FET3-GFP::KanMX	This study
23344 (*FTR1-CFP*)	*MATα, ura3*, FTR1-CFP::KanMX	This study
27038 (*rsp5*) (*FTR1-CFP*)	*MATa, ura3, rsp5*, FTR1-CFP::KanMX	This study
LHY291	*His3, trp1, ade2, ura3, leu2, bar1*	(24)
LHY23	*rsp5–1, ura3, leu2, trp1, bar1 GAL*	(24)

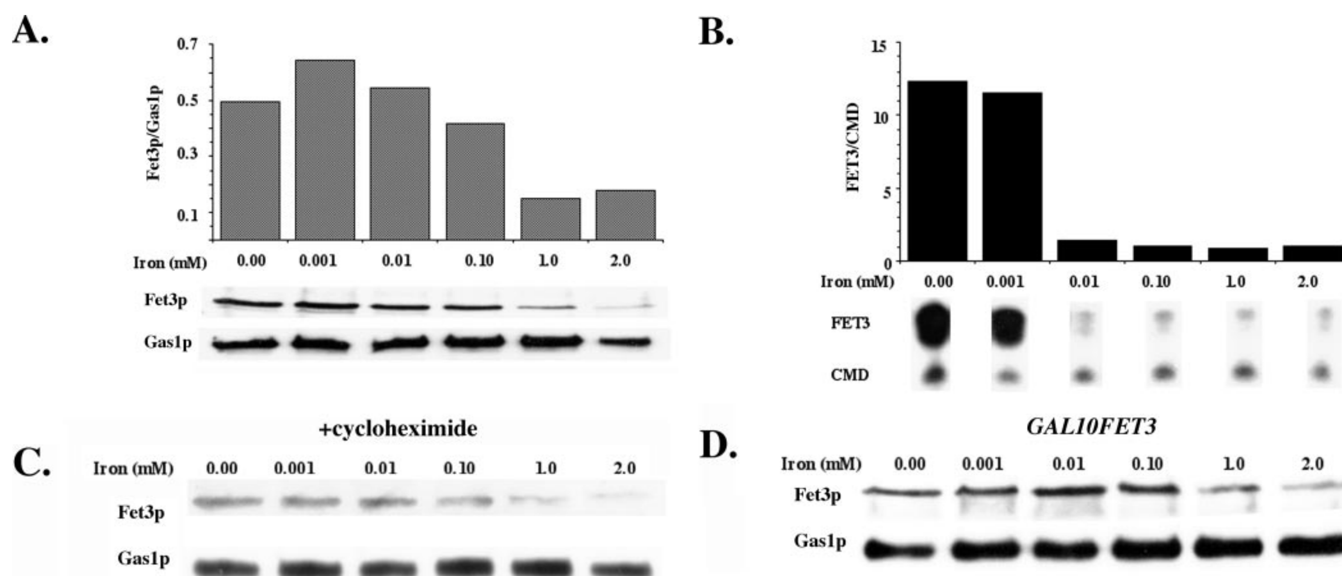


FIG. 1. **Effect of iron on *FET3* protein and mRNA levels.** Cells grown overnight in iron-limited medium BPS (5) were incubated with different concentrations of $FeSO_4$ for 2 h. *A*, samples were taken for Western analysis, and blots were probed with antibodies to Fet3p and to Gas1p. *B*, samples were harvested for S1 nuclease protection analysis using probes for *FET3* and *CMD1*. *C*, cells were treated as in *A* except that 100 μg/ml cycloheximide was added at the same time as cells were incubated in iron-containing medium. *D*, cells (*Δfet3*) were transformed with a plasmid containing a *GAL10*-regulated *FET3*. The cells were incubated in low iron galactose-containing medium overnight and then incubated for 2 h in glucose-containing medium supplemented with different concentrations of iron. The cells were harvested, and extracts were analyzed by Western analysis using antibodies against Fet3p and Gas1p.

fluorescent secondary antibodies were obtained from Molecular Probes.

Western Analysis—Western blot analysis was performed on Fet3p-containing membrane fractions as described previously (14) using our purified rabbit anti-Fet3p (1:1000). The only variation in protocol was that membranes (15 μg) were treated with endoglycosidase Hf per the manufacturer's protocol (New England Biolabs) before being analyzed by SDS-PAGE using 10% gels followed by Western analysis. For Western analysis of Gap1p-GFP or Ftr1p-CFP, membranes were isolated using a procedure described previously (15) and analyzed on 10% SDS-PAGE, and proteins were transferred to nitrocellulose membranes. The membranes were blocked with milk and incubated with either a rabbit anti-Fet3p (1:1,000), rabbit anti-GFP (1:10,000) (Abcam #6556), or rabbit anti-Gas1p (1:30,000, the kind gift of Dr. Howard Riezman University of Basel), followed by peroxidase-conjugated goat anti-rabbit IgG (1:12,500, Jackson ImmunoResearch Laboratories). Proteins were visualized by using a Western Lightning chemiluminescent detection system (PerkinElmer Life Sciences).

Immunoprecipitation—For immunoprecipitation of Fet3p-GFP, Gap1p-GFP, or Ftr1p-CFP, proteins were extracted as described for Western analysis. Following extractions, samples were incubated with rabbit anti-GFP antibody (1:10,000, Abcam #6556) and 40 μl of Sepharose-conjugated Protein A/G beads (Santa Cruz Biotechnology) overnight at 4 °C. Protein A/G beads were pelleted and washed ten times in extraction/lysis buffer, and samples were eluted in 2× SDS-PAGE sample buffer without β-mercaptoethanol. Immunoprecipitated samples were examined by SDS-PAGE followed by Western analysis using either rabbit anti-Fet3p, mouse anti-GFP (1:10,000, Covance), or mouse anti-ubiquitin (1:1,000, Covance) as the primary antibody and peroxidase-conjugated goat anti-mouse or rabbit IgG as the secondary antibody (1:10,000, Jackson ImmunoResearch Laboratories, Inc.).

Atomic Absorption Assay—Cells were grown to log phase in low iron medium and then transferred to medium containing a range of $FeSO_4$ for 2 h. Log phase cells were collected and washed by centrifugation with 50 mM Tris-HCl, pH 6.5, 10 mM EDTA. Cell pellets were digested in 200 μl of 5:2 nitric acid:perchloric acid at 80 °C for 1 h. After digestion, the samples were diluted to 1.0 ml with deionized water and then flamed in a PerkinElmer Life Sciences inductively coupled plasma atomic absorption spectrometer. All samples were measured in duplicate, and the experiment was performed at least twice.

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

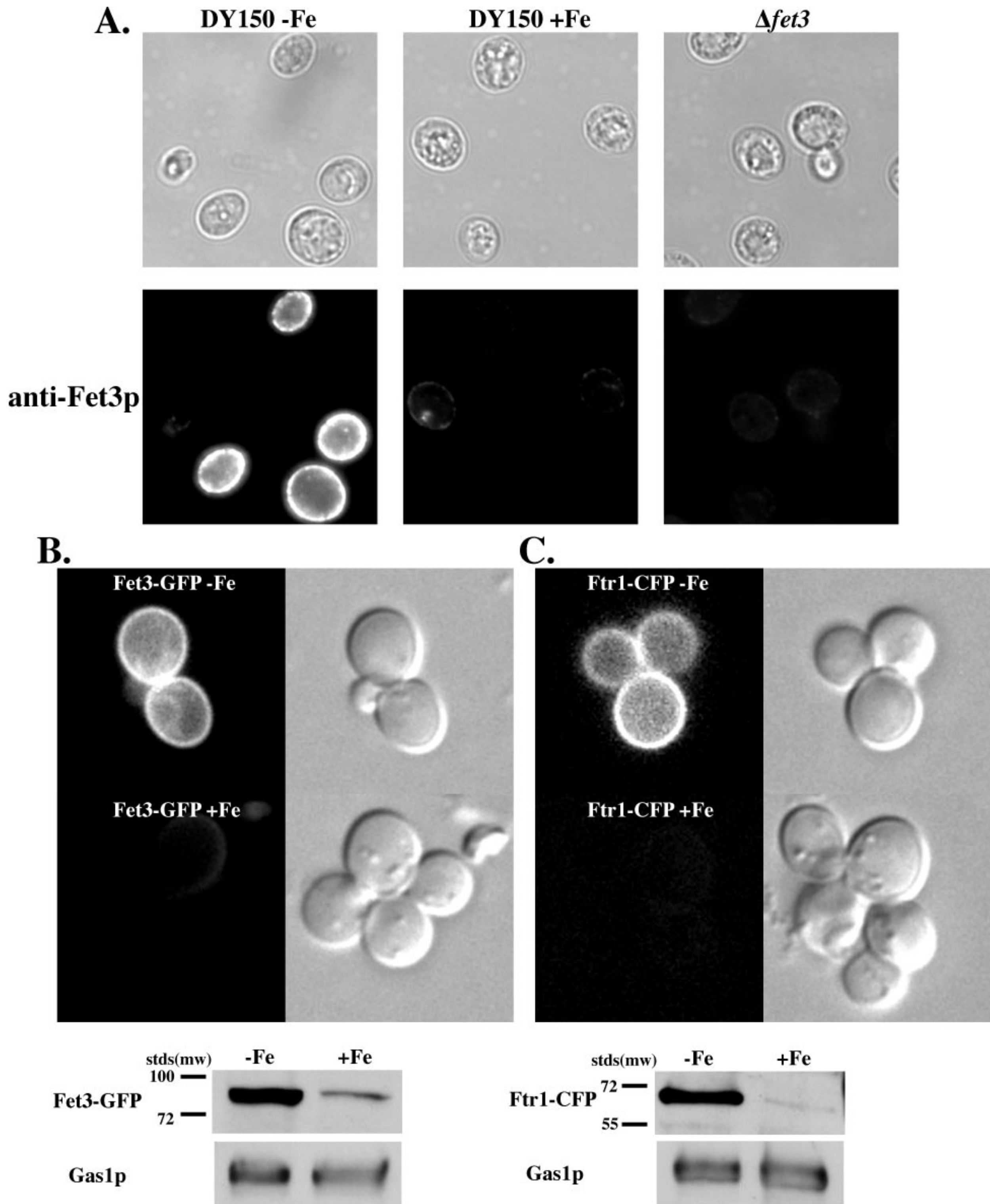


FIG. 2. **Iron induces the loss of cell surface Fet3p and Ftr1p.** *A*, wild type or Δ*fet3* cells were grown overnight in iron-limited medium BPS (5). The cells were washed and then incubated in fresh iron-limited medium or in iron-limited medium supplemented with 1.0 mM $FeSO_4$ for 2 h. Cells were examined for the presence of Fet3p by immunofluorescence. The lack of fluorescence on Δ*fet3* cells that had been incubated in low iron medium is added as a control for the specificity of the antibody. *B*, cells containing a chromosomal copy of *FET3* with a GFP terminal epitope (*C*) or a chromosomal copy of *FTR1* with a CFP terminal epitope were incubated as described in *A*. Cells were harvested, and extracts were analyzed by Western analysis using antibodies to GFP, which also detects CFP.

RESULTS

To determine if the Fet3p-Ftr1p transport system is post-transcriptionally regulated by iron, we exposed wild type cells expressing the transport system to high iron medium and then examined Fet3p levels by Western analysis. When cells were exposed to high iron medium there was a concentration-dependent decrease in Fet3p. Relative to Gas1p, employed as a loading control, exposure of cells to 1 mM $FeSO_4$ resulted in the disappearance of 50% of Fet3p within 1 h (data not shown) and 80% within 2 h (Fig. 1*A*). It may be possible that the disappearance of surface Fet3p is the result of the steady-state turnover of Fet3p, as *FET3* transcription is iron-sensitive (9).

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

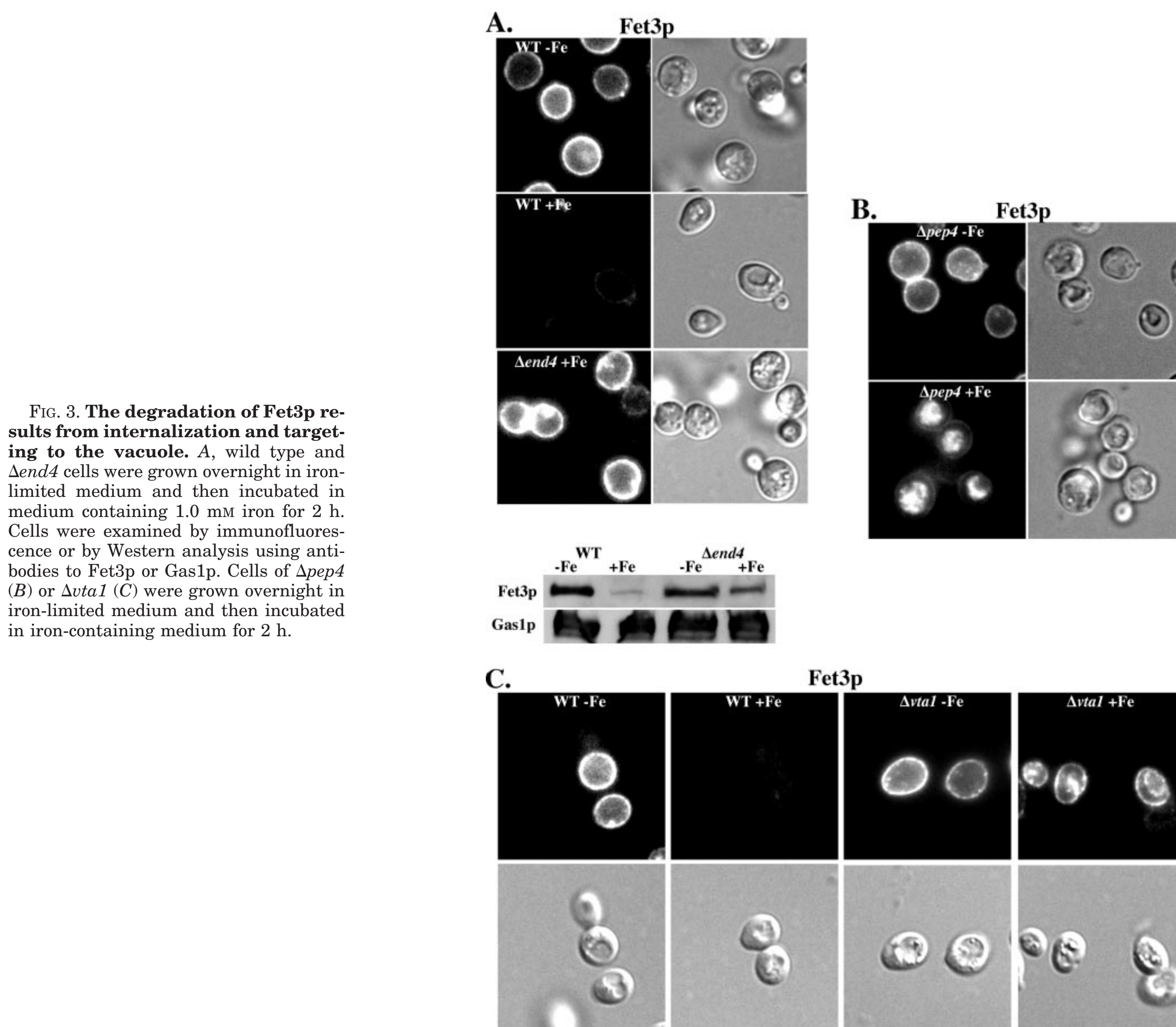


FIG. 3. **The degradation of Fet3p results from internalization and targeting to the vacuole.** *A*, wild type and Δ*end4* cells were grown overnight in iron-limited medium and then incubated in medium containing 1.0 mM iron for 2 h. Cells were examined by immunofluorescence or by Western analysis using antibodies to Fet3p or Gas1p. Cells of Δ*pep4* (*B*) or Δ*vta1* (*C*) were grown overnight in iron-limited medium and then incubated in iron-containing medium for 2 h.

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

We observed that *FET3* mRNA levels were dramatically decreased when cells were incubated with as little as 10 μM iron (Fig. 1*B*). There was little further change in transcript level with increased medium iron. Examination of Fet3p levels revealed little decrement in Fet3p when cells were incubated with 10 μM iron for 2 h. Decreased protein levels were only seen at higher concentrations of iron (Fig. 1*A*).

These results suggest that Fet3p levels may be regulated independently of *FET3* mRNA. We confirmed this result using two different approaches. First, we measured Fet3 protein in cells treated with the protein synthesis inhibitor cycloheximide. Cells were grown in low iron medium, and cycloheximide was added at the same time as high iron. In the presence of cycloheximide, there was an iron-dependent decrease in Fet3p (Fig. 1*C*). Second, we examined changes in Fet3p levels in Δ*fet3* cells transformed with a plasmid containing *GAL10FET3* (8). High affinity iron transport occurs under low iron conditions in the presence of galactose but not in the presence of glucose. Cells were exposed to galactose in low iron medium to induce the high affinity iron transport system. The cells were then incubated in glucose media to prevent transcription of *FET3* mRNA. Addition of glucose leads to inhibition of transcription of galactose-regulated genes within 4 min (16). Addition of iron to glucose media resulted in a concentration-dependent loss of Fet3p (Fig. 1*D*). These experiments demonstrate that iron has an effect on Fet3p independent of *FET3* transcription.

The post-translational regulation of the high affinity iron transport system was confirmed using immunofluorescence. Cells incubated in iron-depleted medium showed fluorescent staining of the cell surface, whereas Δ*fet3* cells stained with the same anti-Fet3p antibody showed no fluorescence (Fig. 2*A*). Addition of iron for 2 h resulted in the disappearance of Fet3p fluorescence. Expression of *FET3* regulated by the *GAL10* promoter leads to abundant Fet3p on the cell surface, and there was little change in the surface expression of Fet3p when galactose-grown cells were incubated in glucose-containing medium. Upon addition of iron, there was a dramatic decrease in fluorescence (data not shown). We generated strains containing an integrated *FET3-GFP*. As shown previously, addition of an epitope to either Fet3p or Ftr1p does not alter their ability to transport iron (17). Addition of iron also resulted in the loss of surface fluorescence in cells that had a chromosomal copy of *FET3* with a carboxyl-terminal GFP (Fig. 2*B*). The loss of Fet3p was confirmed by Western analysis.

Both components of the high affinity iron transport system, Fet3p and Ftr1p, have to be synthesized simultaneously for appropriate cell surface targeting (2). In the absence of Fet3p, Ftr1p does not localize to the surface and is degraded, as is

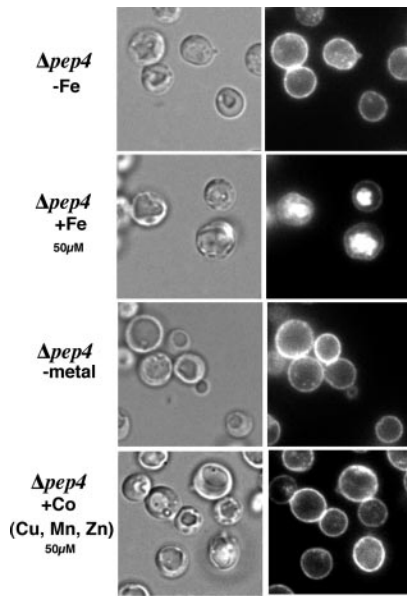


FIG. 4. **Internalization of Fet3p is specific for iron.** Cells (Δ*pep4*) were grown overnight in iron-limited medium and then incubated in medium containing 50 μM of the specified metals for 2 h. The cells were then processed for immunofluorescence using an antibody to Fet3p.

Fet3p in the absence of Ftr1p. These results suggest that Fet3p and Ftr1p form a complex. Based on the observation that iron induced the loss of Fet3p, we asked whether iron also induced the loss of Ftr1p. We generated strains containing an integrated *FTR1-CFP*, because published studies show the utility of this fusion protein (17). Expression of Frt1p-CFP permitted those cells to grow on low iron media, indicating that the protein was functional. When Ftr1p-CFP-expressing cells were incubated with high iron there was a loss of cell surface Ftr1p-CFP fluorescence (Fig. 2*C*). The predicted molecular mass of Ftr1p is 45.7 kDa, and addition of CFP would add 27 kDa. Our data show Ftr1p-CFP migrating on SDS-PAGE with a predicted molecular mass of 70 kDa, which is close to the predicted size of the fusion protein.

We took advantage of mutant cell lines to show that the loss of surface Fet3p was due to internalization and vacuolar degradation. A deletion of *END4* attenuates but does not completely inhibit endocytosis, as shown by decreased uptake of the fluorescent dye FM4–64 (18). In Δ*end4* cells, the iron-induced loss of surface Fet3p was reduced (Fig. 3*A*). In cells that lack the vacuolar protease Pep4p, the iron-induced loss of cell surface fluorescence correlated with the appearance of fluorescence in the vacuole (Fig. 3*B*). The targeting of many cell surface proteins to the vacuoles requires their sorting in the

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

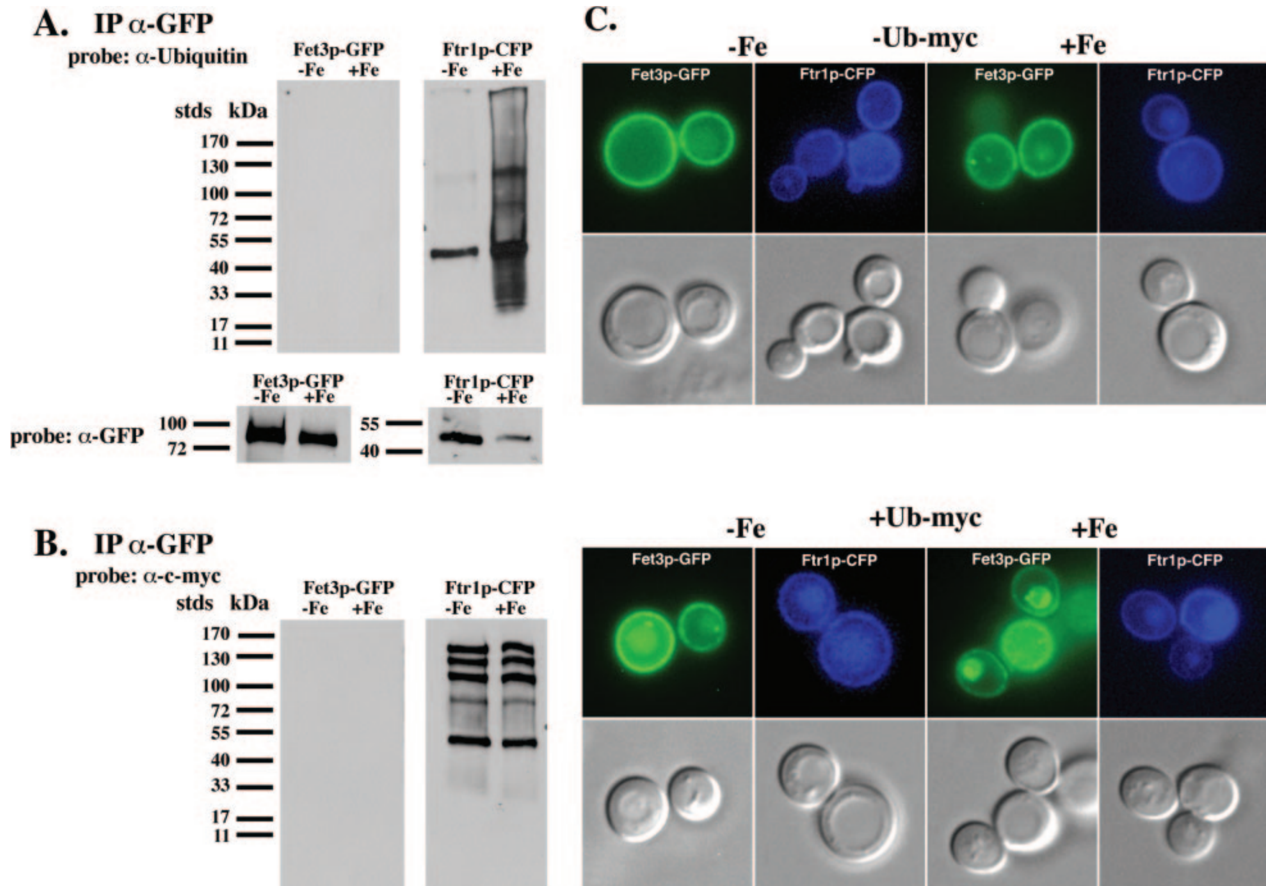


FIG. 5. **Ftr1p is ubiquitinated.** *A*, wild type cells containing an integrated copy of *FET3-GFP* or *FTR1-CFP* were grown in low iron medium overnight and then incubated in either low iron or high iron containing medium for 2 h. Cells were harvested; membranes were detergent-extracted and immunoprecipitated with antibodies against GFP/CFP. The immunoprecipitates were analyzed by Western blot using antibodies to either ubiquitin or GFP/CFP. *B*, cells with a chromosomal copy of *FET3-GFP* or *FTR1-CFP* were transformed with either a control plasmid or a plasmid containing a *CUP1* regulated ubiquitin c-*myc* construct. The cells were incubated in low iron medium overnight and then transferred to copper containing low or high iron containing medium for 2 h. Cells were harvested; membranes were detergent-extracted and immunoprecipitated with antibodies against GFP/CFP. The immunoprecipitates were analyzed by Western blots using antibodies to c-*myc*. *C*, cells treated as in *B* were incubated in low iron medium overnight then incubated in either low iron or high iron medium in the presence or absence of copper for 45 min and then examined for fluorescence. Note that, in the absence of ubiquitin expression, fluorescence was seen in vacuoles only in cells incubated with iron. In contrast, when ubiquitin was expressed both Fet3p-GFP and Ftr1p-CFP were found in the vacuole even in low iron medium.

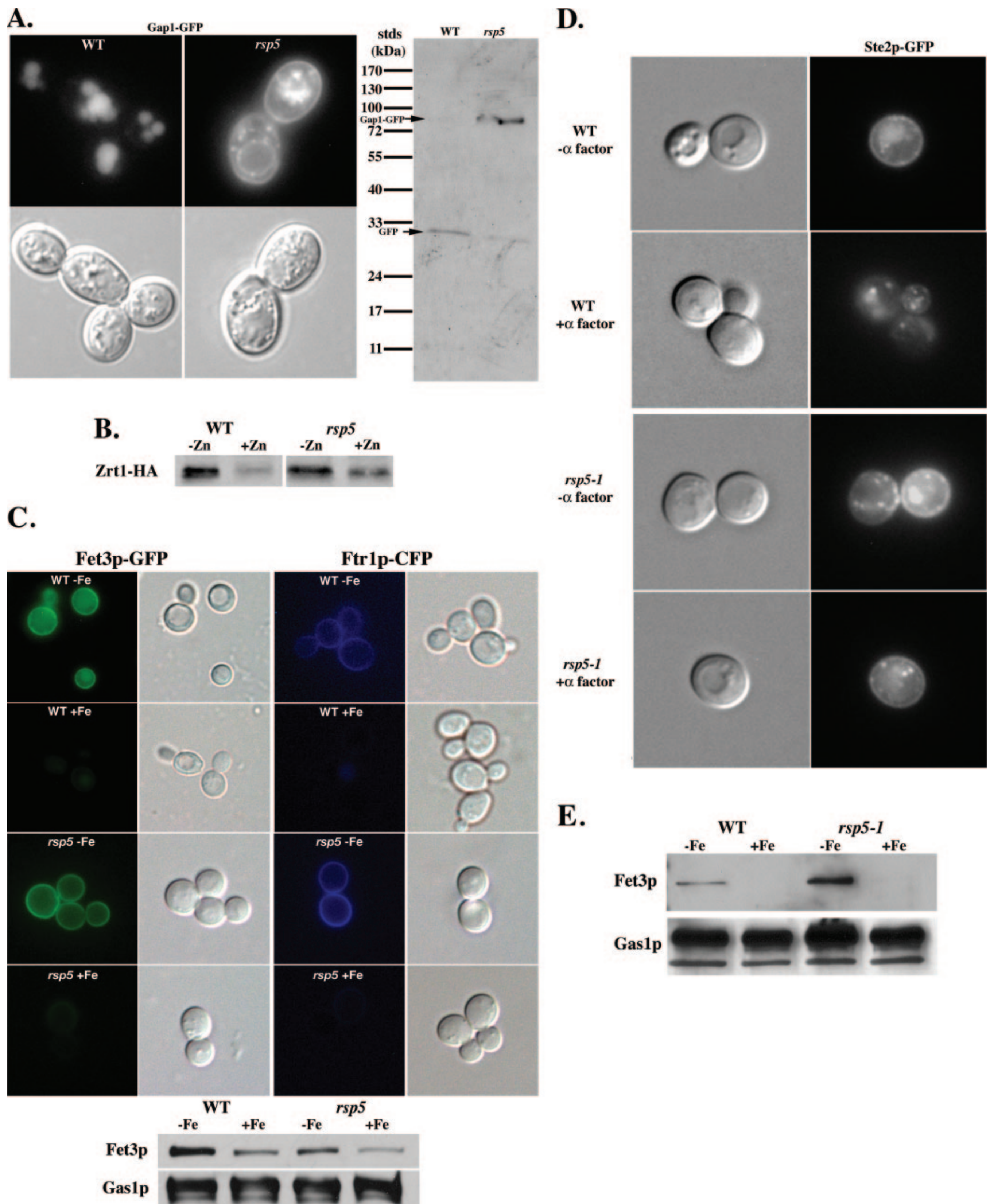


FIG. 6. **The iron-induced degradation of Fet3p is independent of Rsp5p.** *A*, internalization and degradation of the ammonia transporter Gap1p is reduced in *rsp5* cells. Wild type and *rsp5* cells, transformed with a *GAP1-GFP*-containing plasmid, were grown in ammonia-containing medium overnight. The cells were examined for Gap1-GFP by both fluorescence and Western analysis in which an anti-GFP antibody was used to probe Western blots. *B*, wild type and *rsp5* cells were transformed with a plasmid containing *ZRT1* with a hemagglutinin epitope. The cells were grown overnight in low Zn^{2+} medium and then incubated with Zn^{2+} for 1 h. The cells were then examined by Western analysis, and the blots were probed with an antibody directed against hemagglutinin. *C*, wild type and *rsp5* cells, containing an integrated copy of *FET3-GFP* or *FTR1-CFP*, were incubated overnight in low iron medium. The cultures were divided into aliquots, and iron was added to one of the aliquots for 2 h. Samples were taken for fluorescence or for Western analysis in which the blots were probed with either Gas1p or an antibody to GFP. *D*, wild type cells and *rsp5-1* cells were transformed with an STE2-GFP plasmid. Cells were incubated at the restrictive temperature for 15 min, and then α-factor was

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

multivesicular body (19). Vta1p is a class E protein involved in multivesicular body sorting (20, 21). In iron-exposed Δ*vta1* cells, Fet3p was not degraded and was found at the plasma membrane as well as in a class E prevacuolar compartment (Fig. 3*C*).

The Fet3p-Ftr1p transport system is highly specific for iron and will not transport other transition metals. Cells with a deletion of *PEP4* (Δ*pep4*) were incubated overnight in low iron medium and then exposed to different transition metals. At a concentration of 50 μM, only iron led to the vacuolar accumulation of Fet3p. Incubation of cells in metals such as Cu^{+}, Mn^{2+}, and Zn^{2+} in concentrations as high as 50 μM did not lead to internalization of Fet3p (Fig. 4). These results show that internalization of surface Fet3p is specific for iron.

Entry of most plasma membrane proteins into the vacuole through the multivesicular body is a consequence of their being ubiquitinated. We therefore examined whether either Fet3p or Ftr1p was ubiquitinated. Cells expressing either Fet3p-GFP or Ftr1p-CFP were incubated in the presence of iron for 2 h and harvested, and detergent extracts were immunoprecipitated with anti-GFP antibodies. Western blots of the immunoprecipitates were probed with an anti-ubiquitin antibody. No ubiquitin was seen in the immunoprecipitate from Fet3p-GFP-expressing cells, but ubiquitin was found in extracts from Ftr1p-CFP-expressing cells (Fig. 5*A*). Ubiquitin could be seen on Ftr1p-CFP from cells grown in low iron medium; however, the addition of iron resulted in a significant increase in ubiquitin levels. Immunoprecipitated Ftr1p (detected by Western analysis) had an apparent molecular mass of 45 kDa, much lower than that of Ftr1p-CFP seen in extracts of cells probed by Western analysis. We think that it is likely that the lower molecular mass results from proteolytic cleavage occurring during immunoprecipitation, because we did not observe this change in molecular mass prior to immunoprecipitation (compare Figs. 2*B* and 5*A*).

We confirmed the presence of ubiquitin on Ftr1p by taking advantage of cells transformed with a plasmid containing a copper regulated (*CUP1*) ubiquitin with a carboxyl-terminal c-*myc* epitope. Cells were grown in high copper-containing medium to induce the expression of ubiquitin-c-*myc*, and detergent extracts were immunoprecipitated using antibodies to GFP. Again, no ubiquitin was seen in immunoprecipitates from Fet3p-GFP cells, but ubiquitin was seen in immunoprecipitates from Ftr1p-CFP cells (Fig. 5*B*). We observed that Ftr1p was ubiquitinated in both low and high iron medium with multiple ubiquitin-containing bands. Other plasma membrane proteins have been found to be hyper-ubiquitinated in cells overexpressing ubiquitin (15). If ubiquitination of Ftr1p was responsible for the internalization of the Fet3p-Ftr1p complex, then we might expect increased internalization of hyper-ubiquitinated Ftr1p-CFP in low iron medium. In Fet3p-GFP- or Ftr1p-CFP-expressing cells incubated in low iron medium, fluorescence was found predominately on the cell surface. Under the same conditions, in cells expressing ubiquitin, fluorescence was now found in the vacuole. Addition of iron to cells overexpressing ubiquitin resulted in an increase in the rate of vacuolar accumulation of Fet3p-Ftr1p. These results suggest that ubiquitination of Ftr1p leads to internalization of the Fet3p-Ftr1p complex.

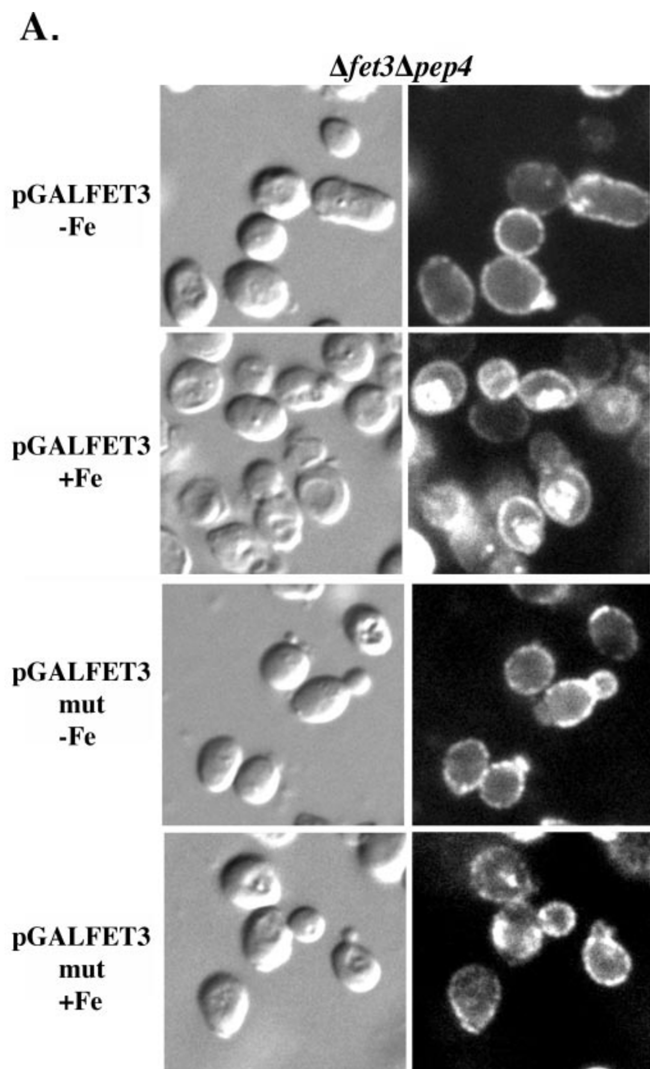
Most plasma membrane transporters are ubiquitinated by the ubiquitin ligase Rsp5p (for reviews see Refs. 19 and 22). To determine if Rsp5p is required for the iron-induced internalization of Fet3p-Ftr1p transport system, we utilized a yeast strain with a mutation in *RSP5*. We first confirmed that *rsp5* cells showed a defect in ubiquitination by following the degradation of the high affinity amino acid transporter Gap1p. Wild type and *rsp5* cells were transformed with a *GAP1-GFP* plasmid. In cells grown in ammonia-free (nitrogen-poor) media Gap1p is localized to the plasma membrane (15, 23). Addition of ammonia results in the internalization of Gap1p-GFP and its localization in the vacuole, as seen by fluorescence or by the presence of cleaved GFP on Western blots. In the *rsp5* cells, Gap1p-GFP is found at the plasma membrane and in a prevacuolar compartment (Fig. 6*A*). Western analysis of *rsp5* extracts showed full-length Gap1p-GFP, whereas in wild type cells only GFP is seen, indicating that Gap1-GFP is degraded. Gitan and Eide (4) reported a decrease in the Zn^{2+}-mediated internalization and degradation of the high affinity zinc transporter Zrt1p in *rsp5* cells. We confirmed that result, as shown in Fig. 6*B*. To determine if Rsp5 was necessary for the iron-induced degradation of Fet3p-Ftr1p, we generated an *rsp5* strain with an integrated *FET3-GFP* or *FTR1-CFP*. We were surprised to find no reduction in iron-induced internalization or degradation of Fet3p or Ftr1p in *rsp5* cells (Fig. 6*C*). It is possible that the rsp5 mutant retains sufficient activity to ubiquitinate Ftr1p. We therefore took advantage of a temperature-sensitive allele of *RSP5* (*rsp5-1*), which shows a severe defect in ubiquitination (24). We confirmed that *rsp5-1* has a temperature-sensitive defect in ubiquitination by showing a severe alteration in α-factor-mediated internalization of Ste2p-GFP (Fig. 6*D*). Fet3p-GFP showed no of iron-dependent loss in *rsp5-1* cells at the restrictive temperature (Fig. 6*E*). These results suggest that Rsp5p is not required for the iron-induced loss of the high affinity iron transport system.

We considered three mechanisms to explain how iron signals the internalization and degradation of Fet3p-Ftr1p: a signal generated by iron at the cell surface, a signal generated by iron inside the cell, or a signal generated as a consequence of movement of iron through the transport system. To test these possibilities, we transformed a Δ*fet3*Δ*pep4* strain with a *GAL10*-regulated allele of *FET3* that was unable to transport iron due to a mutation in one of the amino acids that ligate the Type 1 copper (25). Cells were grown in galactose to induce the expression of the mutant Fet3p. Although the inactive Fet3p is still translocated to the cell surface, incubation of cells with high levels of iron did not result in the degradation of the mutant Fet3p, as assessed by either Western blot analysis (data not shown) or by immunofluorescence (Fig. 7*A*). We then examined the effect of mutations in Ftr1p by expressing a form of Ftr1p that had defective iron transport. A mutation in the putative iron binding REXLE domain of Ftr1p does not prevent Ftr1p and Fet3p from being localized to the cell surface but reduces iron transport by ~80% (17). When exposed to high iron, the degradation of the transport system was reduced compared with wild type cells (Fig. 7*B*). The concentration of media iron in these experiments was high, because it was sufficient to support the growth of Δ*fet3* cells but was also sufficient to inhibit transcription of a *FET3lacZ* reporter construct in Δ*fet3* cells (data not shown).

To further show that a Fet3-Ftr1p transport system is required for iron-induced internalization, we took advantage of cells with a deletion in the *GEF1* gene. Gef1p is a voltage-regulated chloride channel present in the post-Golgi compart-

added to cells at both the permissive and restrictive temperatures. Cells were examined for fluorescence after 60 min. *E*, cells, which had been grown in low iron medium, were incubated with high iron at the restrictive and permissive temperatures. The loss of Fet3p was assessed by Western analysis using a polyclonal antibody to Fet3p as described under "Materials and Methods." The results show that *rsp5-1* cells show a defect in internalization of Ste2p but no defect in the degradation of Fet3p.

Downloaded from http://www.jbc.org/ by guest on July 24, 2018



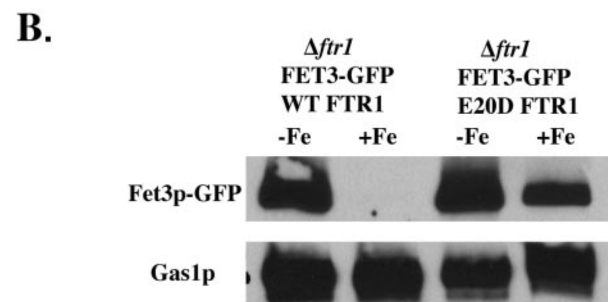


FIG. 7. **Iron does not induce the degradation of an inactive iron transport system.** *A*, cells (Δ*fet3*Δ*pep4*) were transformed with either a plasmid containing a *GAL10*-regulated *FET3* or a *FET3* in which one of the amino acids that ligate the Type I copper had been mutated resulting in an enzymatically inactive molecule. Cells were grown in low iron galactose medium overnight and then incubated for 2 h in glucose- and iron-containing medium with cycloheximide. The cells were processed for immunofluorescence using an antibody against Fet3p. *B*, cells (Δ*ftr1* containing an integrated *FET3-GFP*) were transformed with a plasmid containing a wild type *FTR1* or an *FTR1* with a mutation in the REXLE domain. Cells were incubated overnight in low iron medium and then for 2 h in iron-containing medium with cycloheximide. Cells were examined by Western analysis using an antibody to GFP.

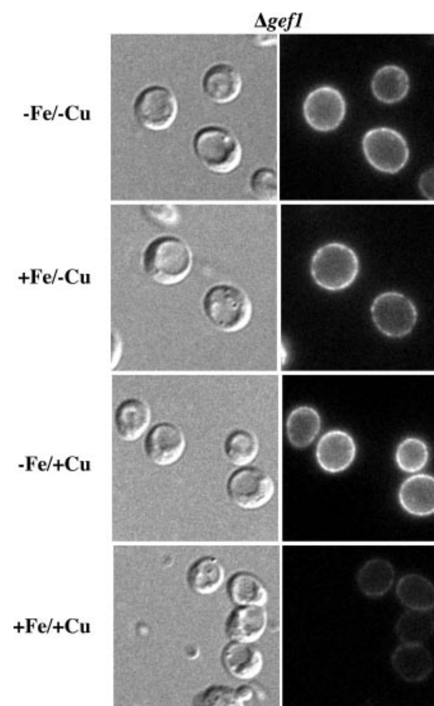


FIG. 8. **Degradation of Fet3p requires iron be transported through the iron channel.** Wild type and Δ*gef1* cells were incubated in low iron medium overnight. Cells were then incubated at 0 °C for 30 min in the absence (*−Cu*) or presence (*+Cu*) of Cu^+, Cl^-, and ascorbate. The cells were resuspended in fresh medium and incubated in either low iron medium (*−Fe*) or medium containing 100 μM iron (*+Fe*) for 2 h. Cells were processed for immunofluorescence using an antibody to Fet3p. Δ*gef1* cells are defective in copper loading of apo-Fet3p, leading to an apo-Fet3p on the cell surface. The presence of iron did not result in the internalization and degradation of the apo-Fet3p. Cells with an apo-Fet3p could be made functional by copper loading at 0 °C. A now functional Fet3p could be degraded in the presence of high iron.

ment in which apo-Fet3p is copper-loaded (14, 26). In the absence of Gef1p, apo-Fet3p is not copper-loaded but is still targeted to the cell surface. Cell surface apo-Fet3p, lacks multicopper oxidase activity and is unable to transport iron. Incubation of Δ*gef1* cells with iron did not lead to the degradation of apo-Fet3p (Fig. 8). Apo-Fet3p on the cell surface can be copper-loaded by incubation of cells at 0 °C in the presence of Cl^-, Cu^+, and reduced pH (14). Copper loading of apo-Fet3p resulted in increased iron transport activity and increased multicopper oxidase activity. Once copper-loaded, addition of iron leads to the internalization and degradation of Fet3p. These results demonstrate that an active iron transport system is required for iron to induce the internalization of Fet3p-Ftr1p.

Extracellular iron is not the signal for the internalization and degradation of the high affinity iron transport system. *S. cerevisiae* can acquire iron through the low affinity iron transport system, Fet4p, as well as through siderophore-iron transporters. There are two separate routes by which *S. cerevisiae* can acquire iron provided by iron-siderophore complexes (27, 28). The first route involves reduction of siderophore-iron complexes at the cell surface followed by the uptake of iron by the Fet3p-Ftr1p transport system. The impermeable Fe(II) chelator BPS can inhibit uptake of iron by this route. The second route of uptake of siderophore iron involves transport of the siderophore iron complex through a siderophore transporter. This route of iron acquisition cannot be inhibited by BPS. In the presence of high concentrations of BPS, yeast can grow on siderophore-iron complexes (28, 29). Addition of high concentrations of ferrioxamine-iron to cells grown in BPS did not lead to the internalization of Fet3p-Ftr1p (Fig. 9*A*). The same concentration of ferrioxamine-iron, however, can provide enough iron to support the growth of cells and to prevent the expression of a *FET3lacZ* reporter construct (Fig. 9*B*). It may be possible that the amount of siderophore-iron accumulated

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

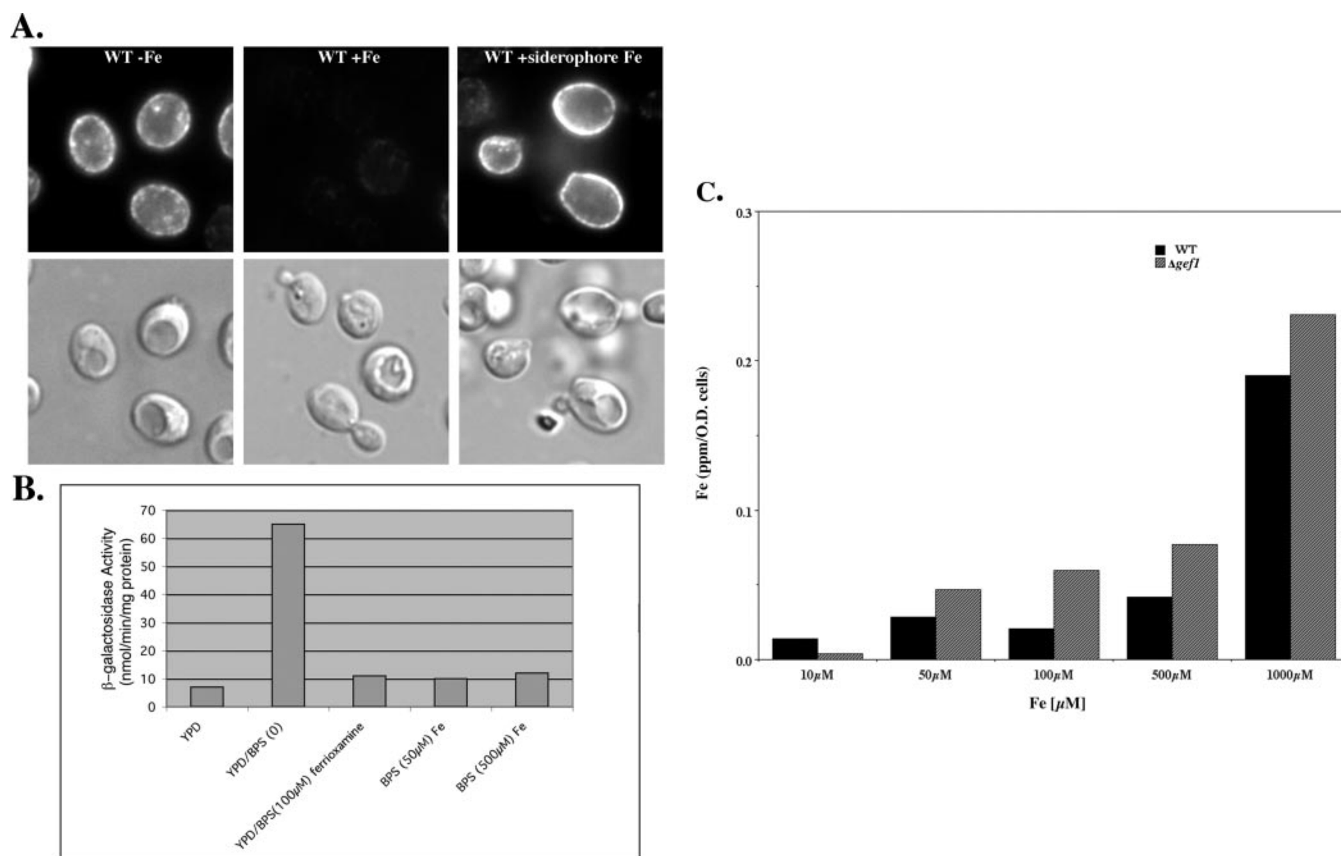


FIG. 9. **Accumulation of iron by siderophore iron complexes or by the low affinity iron transport system does not lead to the internalization and degradation of Fet3p.** Cells containing an integrated reporter construct *FET3lacZ* were grown overnight in iron-limited medium. The cells were then incubated in medium with no available iron resulting from the addition of 80 μM BPS. The cells were incubated with 80 μM BPS, 80 μM BPS, and 1.0 mM ferrioxamine-iron, or 1 mM Fe SO_4 for either 2 or 6 h. *A*, at 2 h cells were processed for immunofluorescence using an antibody to Fet3p. *B*, at 6 h cells were harvested and homogenized, and β-galactosidase activity and cell protein were determined. *C*, wild type and Δ*gef1* cells were grown in low iron media and then incubated with media containing the specified amounts of Fe SO_4 for 2 h. The cells were harvested and washed, and iron content was determined by inductively coupled plasma atomic absorption spectroscopy.

within cells may be enough to suppress the transcription of the iron-regulon but is insufficient to induce the internalization of Fet3p-Ftr1p. To examine the effect of intracellular iron on the degradation of Fet3p-Ftr1p, we took advantage of the observation that, in the absence of high affinity, iron transport system cells increase the expression of the low affinity transition metal transporter Fet4p (30). As shown above, Δ*gef1* cells do not internalize Fet3p-Ftr1p. At low concentrations of media iron wild type cells accumulated more iron than Δ*gef1* cells (Fig. 9*C*). This is expected, because Δ*gef1* cells do not have a functional high affinity iron transport system. As media iron increases, Δ*gef1* cells showed a greater accumulation of iron than wild type cells. Increased iron accumulation reflects the increased expression of the Fet4p low affinity iron transporter on Δ*gef1* cells and low levels of Fet4p on wild type cells. Even in the face of greater than wild type levels of cellular iron, no degradation of Fet3p-Ftr1p was observed (see Fig. 8). These results suggest that intracellular iron does not provide the signal for the internalization of Fet3p-Ftr1p.

DISCUSSION

Transcriptional regulation of the high affinity iron transport system, comprising the products of the *FET3* and *FTR1* genes, by the transcription factor Aft1p has been well described (3, 31–33). We now demonstrate that Fet3p and Ftr1p are regulated post-translationally, as iron induces the internalization and degradation of both Fet3p and Ftr1p. The simultaneous synthesis of Fet3p and Ftr1p is required for their appropriate targeting to the cell surface suggesting that these molecules are in a complex (2, 8). The observation that iron induces the simultaneous internalization of both Fet3p and Ftr1p provides further support for the view that these two proteins exist in an obligate complex. Iron-induced internalization of the iron transport system is consistent with studies showing post-translational regulation of copper (5), zinc (4, 34), and manganese (35) transport systems. For both iron and zinc transport systems, transcriptional regulation is more sensitive than post-translational regulation. Iron, at concentrations as low as 10 μM, can reduce transcription of *FET3* by >90%, whereas the same concentration only had minimal effects on protein levels.

Ubiquitination is the signal that targets most membrane proteins for degradation (19, 22). Our data indicate that Ftr1p can be ubiquitinated and that ubiquitination is required for degradation, because deletions of genes required for sorting into the multivesicular body pathway prevent the vacuolar localization of Fet3p-Ftr1p. Most plasma membrane proteins are ubiquitinated by Rsp5p (22). Rsp5p, in combination with Bsd2p, is responsible for ubiquitination of the Mn^{2+} transporter Smf1p in the biosynthetic pathway (36). The rate of degradation of the amino acid transporter Gap1p and the zinc transporter Zrt1p was severely reduced in *rsp5* cells. It was surprising to find that the iron-induced degradation of Fet3p-Ftr1p was not affected in *rsp5* cells. Furthermore, no iron-induced change in surface Fet3p was seen at the restrictive temperature in cells that had temperature-sensitive allele of *RSP5*, although effects were seen on the internalization of Ste2p. These results suggest that a ubiquitin ligase other than

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

Rsp5p is required for the ubiquitination of Ftr1p.

Given that metals can induce the internalization of surface transporters via ubiquitination leading to transporter degradation, what is the signal that leads to ubiquitination? The metal-induced event that leads to Rsp5p-mediated ubiquitin addition in Smf1p may be a conformational change in the transporter resulting from transport of the metal (36). Mutations that abolish transport activity for Smf1p abolish metal-induced internalization. These studies suggest that Rsp5p (in combination with Bsd2p) recognizes alterations in the hydrophobic domain of membrane proteins. Transport of substrate might be expected to lead to perturbation in the lipid bilayer resulting from movements in the transporter as substrate is passed through the bilayer. This is an attractive model for post-translational regulation of the iron transport system, because it would correlate transport activity with both cellular metal requirement and transcriptional regulation of the high affinity iron transporter. In conditions of iron sufficiency, iron transport will lead to degradation of the transporter. In the face of iron insufficiency, even though transport of iron increases the rate of degradation of the transporter, the increased degradation rate will be offset by increased transcription of the transporter. Linking transport activity to degradation rate provides a simple feedback mechanism that ensures tight control of cytosolic metal levels, as well as assuring the specificity of membrane targeting to the specific transporter.

The demonstration that the Fet3p-Ftr1p transport system must be active to effect iron-induced internalization indicates that cell surface iron is not the signal for internalization/ubiquitination. There are two issues that must be resolved before accepting a model for iron-induced internalization in which the movement of iron through the channel is responsible for ubiquitination. First, why are high concentrations of iron required for post-translational regulation? The K_m for the Fet3p-Ftr1p transport system is in the sub-micromolar (0.15–0.2 μM) range (9), yet much higher concentrations of iron (>50 μM) are required for substantial rates of transporter degradation. Second, what is the ubiquitin ligase responsible for "marking" Ftr1p? We have ruled out Rsp5p, although it is formally possible that, even in the *rsp5* mutant strain or the temperature-sensitive *rsp5-1* strain, residual enzyme activity is sufficient to ubiquitinate Ftr1p. The best way to show that Rsp5p is not required is to identify the ubiquitin ligase that is required. Those studies are in progress.

Acknowledgments—We thank Drs. David Eide (University of Wisconsin), Chris Kaiser (Massachusetts Institute of Technology), Linda Hicke (Northwestern), and Howard Riezman (University of Basel) for their generous gifts of plasmids, cells, and antibodies.

REFERENCES

1. Askwith, C. C., de Silva, D., and Kaplan, J. (1996) *Mol. Microbiol.* **20,** 27–34
2. Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D., and Dancis, A. (1996) *Science* **271,** 1552–1557
3. Yamaguchi-Iwai, Y., Dancis, A., and Klausner, R. D. (1995) *EMBO J.* **14,** 1231–1239
4. Gitan, R. S., Luo, H., Rodgers, J., Broderius, M., and Eide, D. (1998) *J. Biol. Chem.* **273,** 28617–28624
5. Ooi, C. E., Rabinovich, E., Dancis, A., Bonifacino, J. S., and Klausner, R. D. (1996) *EMBO J.* **15,** 3515–3523
6. Eide, D., Davis-Kaplan, S., Jordan, I., Sipe, D., and Kaplan, J. (1992) *J. Biol. Chem.* **267,** 20774–20781
7. Lesuisse, E., Horion, B., Labbe, P., and Hilger, F. (1991) *Biochem. J.* **280,** 545–548
8. Askwith, C., and Kaplan, J. (1997) *J. Biol. Chem.* **272,** 401–405
9. Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., and Kaplan, J. (1994) *Cell* **76,** 403–410
10. Chen, O. S., Hemenway, S., and Kaplan, J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 16922–16927
11. Hassett, R. F., Yuan, D. S., and Kosman, D. J. (1998) *J. Biol. Chem.* **273,** 23274–23282
12. Harris, Z. L., Davis-Kaplan, S. R., Gitlin, J. D., and Kaplan, J. (2004) *Blood* **103,** 4672–4673
13. Davis-Kaplan, S. R., Ward, D. M., Shiflett, S. L., and Kaplan, J. (2004) *J. Biol. Chem.* **279,** 4322–4329
14. Davis-Kaplan, S. R., Askwith, C. C., Bengtzen, A. C., Radisky, D., and Kaplan, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13641–13645
15. Springael, J. Y., and Andre, B. (1998) *Mol. Biol. Cell* **9,** 1253–1263
16. Mason, P. B., and Struhl, K. (2003) *Mol. Cell. Biol.* **23,** 8323–8333
17. Severance, S., Chakraborty, S., and Kosman, D. J. (2004) *Biochem. J.* **380,** 487–496
18. Raths, S., Rohrer, J., Crausaz, F., and Riezman, H. (1993) *J. Cell Biol.* **120,** 55–65
19. Katzmann, D. J., Odorizzi, G., and Emr, S. D. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 893–905
20. Yeo, S. C., Xu, L., Ren, J., Boulton, V. J., Wagle, M. D., Liu, C., Ren, G., Wong, P., Zahn, R., Sasajala, P., Yang, H., Piper, R. C., and Munn, A. L. (2003) *J. Cell Sci.* **116,** 3957–3970
21. Shiflett, S. L., Ward, D. M., Huynh, D., Vaughn, M. B., Simmons, J. C., and Kaplan, J. (2004) *J. Biol. Chem.* **279,** 10982–10990
22. Hicke, L., and Dunn, R. (2003) *Annu. Rev. Cell Dev. Biol.* **19,** 141–172
23. Soetens, O., De Craene, J. O., and Andre, B. (2001) *J. Biol. Chem.* **276,** 43949–43957
24. Dunn, R., and Hicke, L. (2001) *Mol. Biol. Cell* **12,** 421–435
25. Askwith, C. C., and Kaplan, J. (1998) *J. Biol. Chem.* **273,** 22415–22419
26. Gaxiola, R. A., Yuan, D. S., Klausner, R. D., and Fink, G. R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4046–4050
27. Lesuisse, E., Simon-Casteras, M., and Labbe, P. (1998) *Microbiology* **144,** 3455–3462
28. Yun, C. W., Ferea, T., Rashford, J., Ardon, O., Brown, P. O., Botstein, D., Kaplan, J., and Philpott, C. C. (2000) *J. Biol. Chem.* **275,** 10709–10715
29. Kosman, D. J. (2003) *Mol. Microbiol.* **47,** 1185–1197
30. Li, L., and Kaplan, J. (1998) *J. Biol. Chem.* **273,** 22181–22187
31. Casas, C., Aldea, M., Espinet, C., Gallego, C., Gil, R., and Herrero, E. (1997) *Yeast* **13,** 621–637
32. Rutherford, J. C., Jaron, S., and Winge, D. R. (2003) *J. Biol. Chem.* **278,** 27636–27643
33. Yamaguchi-Iwai, Y., Stearman, R., Dancis, A., and Klausner, R. D. (1996) *EMBO J.* **15,** 3377–3384
34. Gitan, R. S., and Eide, D. J. (2000) *Biochem. J.* **346,** 329–336
35. Liu, X. F., and Culotta, V. C. (1999) *J. Biol. Chem.* **274,** 4863–4868
36. Hettema, E. H., Valdez-Taubas, J., and Pelham, H. R. (2004) *EMBO J.* **23,** 1279–1288
37. Li, L., Chen, O. S., McVey Ward, D., and Kaplan, J. (2001) *J. Biol. Chem.* **276,** 29515–29519
38. Spizzo, T., Byersdorfer, C., Duesterhoeft, S., and Eide, D. (1997) *Mol. Gen. Genet.* **256,** 547–556

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

Post-transcriptional Regulation of the Yeast High Affinity Iron Transport System
M. Rosa Felice, Ivana De Domenico, Liangtao Li, Diane McVey Ward, Beatrix Bartok, Giovanni Musci and Jerry Kaplan

J. Biol. Chem. 2005, 280:22181-22190.
doi: 10.1074/jbc.M414663200 originally published online April 7, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M414663200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 26 of which can be accessed free at
http://www.jbc.org/content/280/23/22181.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on July 24, 2018