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Proteomic Analysis of Recombinant *Saccharomyces cerevisiae* upon Iron Deficiency Induced *via* Human H-Ferritin Production

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In our previous study, the expression of active H-ferritins in *Saccharomyces cerevisiae* was found to reduce cell growth and reactive oxygen species (ROS) generation upon exposure to oxidative stress; such expression enhanced that of high-affinity iron transport genes (*FET3* and *FTR1*). The results suggested that the recombinant cells expressing H-ferritins induced cytosolic iron depletion. The present study analyzes metabolic changes under these circumstances *via* proteomic methods. The YGH2 yeast strain expressing H-ferritin, the YGH2-KG (E62K and H65G) mutant strain, and the YGT control strain were used. Comparative proteomic analysis showed that the synthesis of 34 proteins was at least stimulated in YGH2, whereas the other 37 proteins were repressed. Among these, the 31 major protein spots were analyzed *via* nano-LC/MS/MS. The increased proteins included major heat-shock proteins and proteins related to endoplasmic reticulum-associated degradation (ERAD). On the other hand, the proteins involved with folate metabolism, purine and methionine biosynthesis, and translation were reduced. In addition, we analyzed the insoluble protein fractions and identified the fragments of Idh1p and Pgk1p, as well as several ribosomal assembly-related proteins. This suggests that intracellular iron depletion induces imperfect translation of proteins. Although the proteins identified above result from changes in iron metabolism (*i.e.*, iron deficiency), definitive evidence for iron-related proteins remains insufficient. Nevertheless, this study is the first to present a molecular model for iron deficiency, and the results may provide valuable information on the regulatory network of iron metabolism.

Keywords: Two-dimensional gel electrophoresis (2-DE), *Saccharomyces cerevisiae*, H-ferritin, iron deficiency

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Iron is an essential element that is required by all known organisms. It often functions as a cofactor for cellular processes such as oxygen transport, the tricarboxylic acid (TCA) cycle, lipid metabolism, metabolic intermediate synthesis, and the replication and repair of DNA [1]. In excess, iron can become toxic to cells by increasing the formation of reactive oxygen species (ROS). Therefore, iron metabolism, including the absorption, transport, storage, and mobilization of cellular iron, is rigorously regulated in order for organisms to survive [2, 3].

The budding yeast, *Saccharomyces cerevisiae*, is an ideal eukaryotic organism for use in iron mechanism studies [10]. Iron metabolism appears to be well conserved throughout most organisms. Moreover, many of the genes related to iron transport that have been identified in *S. cerevisiae* provide basic information in the search for corresponding genes in mammals [3, 23]. The major iron storage compartment in yeast is the vacuole that can be controlled by either high-affinity iron transporters (Fet5p/Fth1p) or low-affinity iron transporters [21]. Iron is also abundant in mitochondria, mostly in the form of the Fe/S cluster and cytochromes. Recent work demonstrates that mitochondrial iron metabolism plays a central role in cellular iron homeostasis. Excess mitochondrial iron leads to a loss of metabolic and respiratory functions through the generation of free radicals by oxidative stress in mitochondria and the depletion of cytosolic iron.

In the investigation of iron deficiency, the *S. cerevisiae* yeast has demonstrated that cells utilize the iron-responsive transcription factors Aft1p and Aft2p. These proteins regulate many other genes in iron homeostasis, including genes in iron reduction at the plasma membrane, iron uptake, mobilization from intracellular stores, and utilization from heme. Recent studies have shown that mRNA levels of genes involved in biotin synthesis, energy metabolism, and heme assembly are regulated by iron deficiency [24, 27, 36].

Ferritin, an iron storage protein, regulates cellular iron concentrations in order to maintain iron homeostasis [2]. It is found in almost all organisms, with the exception of yeast. In humans, it consists of two main subunits in various tissues, the heavy (H) and light (L) subunits. All ferritins are composed of 24 subunits, which are arranged in a hollow protein shell (apoferritin) that stores up to 4,500 Fe^{3+} atoms [12].

In a previous study, we successfully expressed the human ferritin H-chain gene (*hfH*) in yeast. It was identified that heterologous *hfH* expression altered iron metabolism and induced cytosolic iron deficiency [14, 26].

We have attempted to obtain information on iron metabolism or other types of metabolism that affect iron deficiency at the protein level, *via* two-dimensional gel electrophoresis (2-DE). The results show that iron depletion affects the heat-shock proteins and the proteins of endoplasmic reticulum-associated degradation (ERAD), purine biosynthesis, methionine biosynthesis, folate metabolism, and translation metabolism.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

S. cerevisiae 2805 (*MATα pep4::HIS3 prb1-δ can1 GAL2 his3 ura3-52*) was employed as a host strain for the heterologous expression of the human ferritin H-chain gene (*hfH*). The recombinant yeast strains used in this study were YGT, YGH2 [26], and YGH2-KG (constructed in this work). Cells were grown aerobically at 30°C in uracil-deficient selective medium [ura^- selective; 0.67% (w/v) yeast nitrogen base without amino acids, 0.5% (w/v) casamino acids, 2% (w/v) glucose, 0.03 g/l adenine and tryptophan]. For the expression of the foreign gene, cells were grown in ura^- selective medium supplemented with 2% (w/v) galactose instead of glucose. For the two-dimensional gel electrophoresis (2-DE), the recombinant yeasts were grown in ura^- selective media (containing galactose) for 30 h and harvested by centrifugation. Cells were washed in ice-cold water and stored at −70°C until use.

Construction of the Expression Plasmid

In order to perform site-directed mutagenesis of *hfH*, an oligonucleotide was designed as 5'-CAATCTCATGAGAAGAGGG-AAGGTGCTGAGAAACTGATG-3' (underlined codons indicate the location of the mutation, Glu 62→Lys and His 65→Gly substitution). The PCR amplification and construction of the pYGH2-KG expression plasmid were performed as described previously [26]. Transformation of *S. cerevisiae* Y2805 by pYGH2-KG was performed as described by Ito *et al.* [13], and the resulting cells were denoted as mutant strain YGH2-KG.

Expression of Ferritin and Iron Uptake

Precultures of recombinant yeasts grown in ura^- selective media were used to inoculate 40 ml of ura^- selective medium (containing 2% galactose) supplemented with 5 mM ferric citrate. Cells were harvested and then disrupted using a bead beater (Biospec Products Inc.) in 20 mM Tris-HCl buffer (pH 7.4). The supernatants were examined using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Samples for SDS-PAGE were heated at 100°C for 10 min in SDS gel loading buffer. Subunit assembly and core formation were characterized *via* 7.5% PAGE in nondenaturing gels. Partially purified samples were obtained by heat denaturation at 75°C for 10 min. Gels were immediately stained for protein using 0.2% Coomassie brilliant blue, and for iron using 2% $K_4Fe(CN)_6$ and 2% HCl mixed 1:1, v/v.

Preparation of Protein Extracts

Harvested cells were suspended in 20 mM Tris-HCl buffer (pH 8.0) and disrupted by cell disruption bombs (PARR Instrument Co., Illinois, U.S.A.) at 30,000 psi. The crude extracts were separated by centrifugation (15,000 ×*g*, 30 min, 4°C), and the supernatant (soluble fraction) was collected and dried. Approximately 1 mg of dried samples was solubilized in extraction solution 1 [8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% (w/v) Bio-Lyte 3/10 ampholyte]. The insoluble pellet was washed twice with 20 mM Tris-HCl buffer (pH. 8.0); these washes were discarded. The pellet (insoluble fraction) was used to extract insoluble proteins using the methods of Molly *et al.* [15] with some modifications. The pellet was solubilized using solution 2 [5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 2 mM TBP, 0.2% (w/v) Bio-Lyte 3/10 ampholyte]. Extracted proteins were then used for a separate two-dimensional gel electrophoresis. The protein concentrations were determined using the Bradford method (BIO-RAD, U.S.A.).

Two-Dimensional Gel Electrophoresis (2-DE)

All chemicals used were of electrophoresis grade. 2-DE was performed as reported previously [11]. The samples (approx. 500 μg) were applied on immobilized, pH 3–10 nonlinear gradient IPG-strips (BIO-RAD, USA). Isoelectric focusing (IEF) was carried out at 250 V for 15 min, 10,000 V for 2 h, and then until 30,000 V-h was reached. Prior to the second dimension, the IPG strips were equilibrated for 15min with a solution containing 6 M urea, 0.375 M Tris-HCl (pH 8.8), 2% (w/v) SDS, 2% (w/v) DTT, and 20% (v/v) glycerol, and were then equilibrated for an additional 15 min in the same solution, except that DTT was replaced with 2.5% (w/v) iodoacetamide. After equilibration, the IPG strips were transferred for SDS-PAGE (linear gradient 8–15%). SDS-PAGE was performed at a constant current of 35 mA until the dye front reached the bottom of the gel. The gels were stained with Coomassie brilliant blue (SeePico CBB stain kit; SeePico Co., Republic of Korea). Each experiment was performed in duplicate.

Image Analysis

The gels were scanned using a GS-700 Calibrated Imaging Densitometer (BIO-RAD, U.S.A.). Analysis of the gels was accomplished using PDQuest analysis software (BIO-RAD, U.S.A.). The spot intensities were obtained in pixel units and normalized to the total intensity of the gel. The spot index was calculated as the ratio of spot intensity between YGH2 and standard conditions. Proteins with a spot index higher than two-fold are reported.

In-Gel Digestion with Trypsin

The stained protein spots were excised from the gel and digested with trypsin (sequencing grade modified trypsin; Promega, U.S.A.). After destaining with 50% acetonitrile in 50 mM ammonium bicarbonate (pH 8.0), gel pieces were swollen in 3 μl of digestion

buffer (50 mM ammonium bicarbonate, pH 8.0, 1 μg/spot modified trypsin) and incubated at 30°C for 16 h. The peptides were extracted twice with 0.1% TFA in water for 20 min, and the soluble fraction was pooled and then extracted twice with 50% acetonitrile in 0.1% TFA.

Protein Identification by LC/MS/MS

Deionized water and HPLC-grade acetonitrile were used for the preparation of eluents. Chromatographic separations were performed using a Nano-LC 1D system (Eksigent Technologies, CA, U.S.A.). Samples (6 μl) were injected directly onto a 150 μm×150 mm column (Vydac 218MS5, 1515; Grace Vydac, Hesperia, CA, U.S.A.) and eluted with a linear gradient of 100% acetonitrile (0.1% formic acid) for 120 min. Samples were analyzed by tandem mass spectrometry (MS/MS) using the QqTOF mass spectrometer (QSTAR XL; Applied Biosystems/MDS Sciex, Foster City, CA, U.S.A.). A handmade Nanospray source was used in sample ionization. The spray needle (15 μm PicoTip; New Objective, Inc. MA, U.S.A.) was used for nanospray, and 2,000 volts were applied for ionization. For protein identification, MS/MS spectra were searched using ProID (MDS Sciex, U.S.A.). An NCBI FASTA sequence database file was downloaded to the computer, and an interrogator search database was created in order to find translational modification peptides.

Results and Discussion

Construction of the Expression Vector

We attempted to gain information on the cellular response to iron deficiency in *S. cerevisiae* using a proteomic method. The recombinant YGH2 strain was used as a model organism for iron deficiency [14]. The YGT strain was used as a control, and the mutant YGH2-KG strain producing H-ferritin variants was used to eliminate the influence of the expression of foreign genes in yeast.

To produce the YGH2-KG strain, the *hfH* gene was modified by the substitutions Glu 62→Lys and His 65→Gly. According to a previous report, the two sites are responsible for ferroxidase activity in this protein [4]. The *hfH* variant was cloned into the pYGT yeast expression vector, and the cloned vector was used to transform *S. cerevisiae* 2805. This recombinant yeast was denoted as YGH2-KG.

Expression of the Recombinant H-Ferritin Variants

Recombinant yeast cells were cultured for 2 days in ura$^-$ selective medium containing 5 mM ferric citrate. The crude cell extracts were then analyzed by 10% SDS-PAGE and 7.5% PAGE in nondenaturing gels. In the SDS-PAGE gel (Fig. 1A), all of the extracts from the induced YGH2 and YGH2-KG cells showed the band for the H-subunit (21 kDa). The amount of H-subunit variant produced in YGH2-KG was relatively similar to that of the native H-subunit produced in YGH2. Heat-denatured, partially purified samples were used to examine protein assembly.

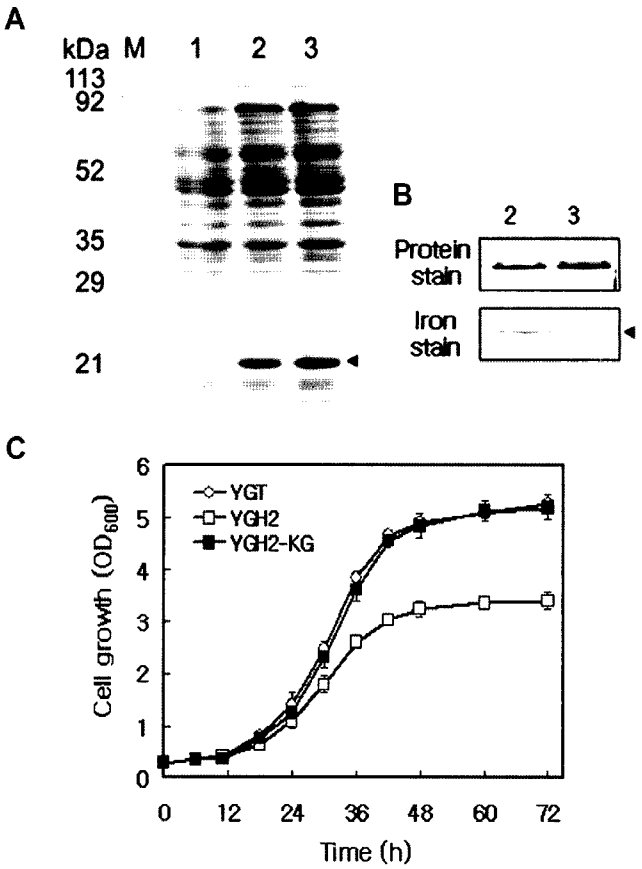


Fig. 1. Expression of H-ferritin variant and cell growth of the recombinant yeasts.

The yeast cells were cultured in uracil-deficient selective (ura$^-$ selective) medium containing 2% galactose and 5 mM ferric citrate for 2 days. **A.** 10% SDS-PAGE analysis of the cell extract of YGH2 and YGH2-KG. **B.** 7.5% nondenaturing gel of H-ferritin expressed in YGH2 and its variant expressed in YGH2-KG. For the 7.5% nondenaturing gel, heat-labile proteins were removed *via* 10 min of heat treatment at 75°C. The samples were stained for protein with Coomassie brilliant blue and stained for iron with Prussian blue. The lanes are M, molecular mass size marker; 1, cell extract of YGT; 2, cell extract of YGH2; 3, cell extract of YGH2-KG. **C.** Cell growth of the YGH2, YGH2-KG, and YGT. The yeast cells were cultured in ura$^-$ selective medium containing 2% galactose.

The expressed H-subunit and its variants were spontaneously assembled into holoproteins *in vivo*, and these holoproteins were observed on nondenaturing gel (Fig. 1B). The produced ferritin variants migrated in a similar manner to H-ferritins. This band negatively stained for iron, suggesting that the expressed ferritin variants were not able to uptake iron into the core (Fig. 1B). This finding implies that the ferroxidase activity of produced H-ferritin variant is lost, which is consistent with the previous result where the *E. coli*-derived H-ferritin variants lost ferroxidase activity through substitution of Glu 62 and His 65 with Lys and Gly, respectively [4].

Upon examining the growth of the recombinant strains, significant differences were found between YGH2 and YGH2-KG, which were grown in ura$^-$ selective medium supplemented with 2% galactose (Fig. 1C). The recombinant

YGH2 cells exhibited reduced growth compared with YGT. However, the cell growth of the recombinant YGH2-KG was similar to that of YGT. This suggests that the difference of cell growth in YGH2 and YGH2-KG may be associated with the ferroxidase activity of H-ferritin.

Comparative 2-DE Analysis of the Soluble Proteins Upon Iron Deficiency

We performed comparative proteomic analysis of soluble fractions *via* two-dimensional gel electrophoresis (2-DE) and nano-LC/MS/MS. The YGT, YGH2, and YGH2-KG strains were cultured for 30 h in ura$^-$ selective medium, and were subsequently analyzed.

Changes in the spot intensity of the 2-DE of YGT, YGH2, and YGH2-KG cells were quantified *via* densitometry and PDQuest analysis software. Approximately 350 protein spots were detected as the result of the 2-DE of the soluble fraction of YGH2 using a pH 3–10 IPG strip. The 2-DE comparison of YGH2 with YGT and YGH2-KG showed that 34 proteins were notably induced and the levels of 37 other proteins were repressed in YGH2 cells. These differentially expressed protein spots and recombinant ferritins are indicated with circles and squares, respectively, in Fig. 2. Among those 71 proteins, 31 proteins showed a difference of at least 2.0-fold mean intensity in the 2-DE gels. The protein spots were excised from the Coomassie brilliant blue staining gels and digested by trypsin. For protein identification, the digested peptide fragments were analyzed by nano-LC/MS/MS. Some of the identified proteins including ferritin were identified in more than one spot in

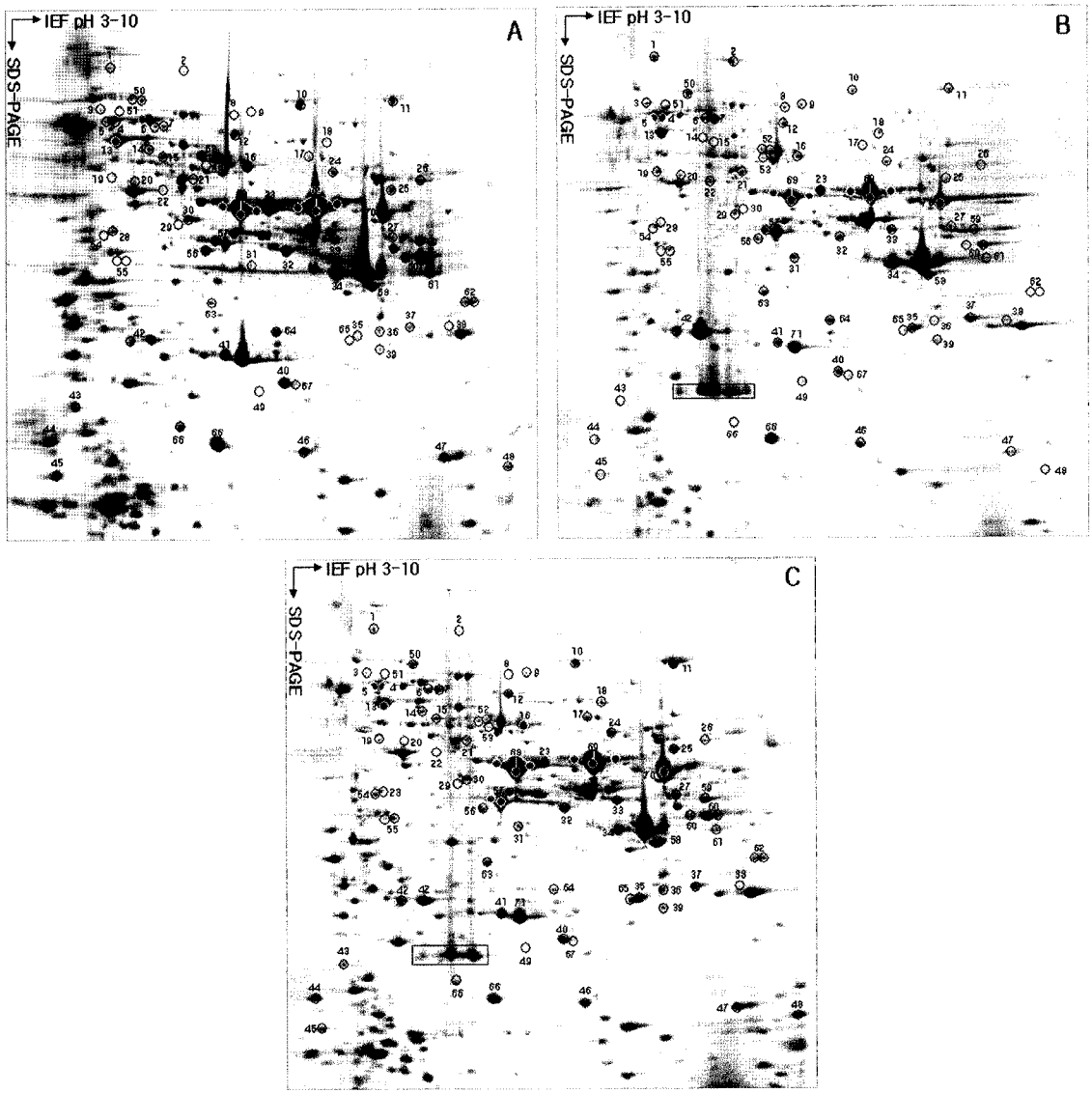


Fig. 2. Comparative two-dimensional gel electrophoresis (2-DE) of the soluble fractions of YGT (**A**), YGH2 (**B**), and YGH2-KG (**C**). The protein extracts were separated on pH 3–10 nonlinear immobilized pH-gradient strips, followed by a 8–15% SDS-polyacrylamide gel. The gels were stained with Coomassie brilliant blue. Analysis of the gels was accomplished using PDQuest analysis software. These differentially expressed protein spots and recombinant ferritin are indicated with circles and squares, respectively.

Table 1. Searching results of differentially expressed proteins in *Saccharomyces cerevisiae via* heterologous expression of ferritin.

Spot No.	Gene name	Accession No.[a]	Description	Folding	
				H2/T	H2/KG
Heat-shock protein					
2	HSP104	P31539	Heat-shock protein 104	9.9	5.9
4	SSA1	P10591	Heat-shock protein 70 family	6.1	2.1
5	SSA2	P10592	Heat-shock protein 70 family	3.8	2.7
7	SSB2	P40150	Heat-shock protein 70 family	11.8	2.3
9	HSP78	P33416	Heat-shock protein 78	4.4	2.1
42	HSP26	P15992	Heat-shock protein 26	6.7	4.2
Endoplasmic reticulum-associated degradation					
1	CDC48	P25694	Cell division control protein 48	16	4
3	KAR2	P16474	78 kDa glucose-regulated protein homolog	4.8	3.8
19	RPT5	P33297	26S protease regulatory subunit 6A	15.4	3.5
Methionine/folate/purine biosynthesis					
10	MET6	P05694	N5-methyltetrahydrofolate homocysteine methyltransferase	0.26	0.27
26	SHM1	P37292	Mitochondrial serine hydroxymethyltransferase	0.04	0.05
41	ADK1	P07170	Adenylate kinase	0.22	0.39
47	YNK1	P36010	Nucleoside diphosphate kinase	0.29	0.52
Transcription/translation metabolism					
30	SBP1	P10080	Single-stranded nucleic acid binding protein	0.12	0.13
45	RPP2B	P02400	Ribosomal protein P2-beta	0.18	0.34
51	GCD3	Q2GCD3	Negative regulator gene in general amino acid biosynthesis	3.75	2.9
Other metabolism enzymes					
8	TKL2	P33315	Transketolase 2	12.2	7.2
16	ALD4	P46367	K^+-activated aldehyde dehydrogenase	0.35	0.34
20	PDX1	P16451	Pyruvate dehydrogenase protein X component	3.62	2.55
27	GAL7	P08431	Galactose-1-phosphate uridyl transferase	2.16	0.40
29	LEU2	P04173	3-Isopropylmalate dehydrogenase	32.9	4.85
56	ARA1	P38115	Large subunit of arabinose dehydrogenase	0.26	0.34
Unclassified proteins					
31	YDL124W	Q07551	NADPH-dependent alpha-keto amide reductase	4.3	2.9
37	POR1	P04840	Outer mitochondrial membrane protein porin 1	9.9	2.0
40	SOD2	P00447	Superoxide dismutase [Mn]	0.50	0.54
43	YDL110C	Q12513	Unknown	0.15	0.33
44	QCR6	P00127	Ubiquinol-cytochrome *c* reductase complex 17 kDa protein	0.15	0.38
48	IGO2	Q9P305	Unknown	nd[b]	nd
49	YER067W	P40043	Unknown	48.7	3.9
62	ATP3	P38077	ATP synthase gamma chain	0.27	0.26

[a]Accession numbers are entries in the SWISS-PROT.
[b]nd , not determined in YGH2.
Proteins were identified in this work by mass spectrometry.

the 2-DE gel (spot Nos. 6, 42, 50, 55, 57, 60, 62, 66, 68, and 69). This can probably be attributed to the presence of various isoforms or to co- and post-translational processing. A similar phenomenon appeared in the 2-DE of rat intestine under iron deficiency [28]. Among the identified proteins, the results of a search of differentially expressed proteins in *S. cerevisiae via* heterologous expression of ferritin are summarized in Table 1. The increased proteins under iron deficiency were sorted into four different functional classes (Table 1). (i) Heat-shock proteins (HSPs): Hsp104p, Ssa1p, Ssa2p, Ssb2p, Hsp78p, and Hsp26p. These proteins showed a 2- to 6-fold increase in YGH2 compared with YGH2-KG, and a 4- to 12-fold increase compared with YGT. (ii) Proteins of endoplasmic reticulum-associated degradation (ERAD): cell division control protein Cdc48p, glucose-regulated protein homolog Kar2p, and 26S protease regulatory protein Rpt5p. These proteins are enhanced 3.5-fold in YGH2 compared with YGH2-KG, and 5- to 16-fold compared with YGT. (iii) Other metabolism enzymes: transketolase 2 Tkl2p, pyruvate dehydrogenase Pdx1p, galactose-1-phosphate uridyl transferase Gal7p, 3-isopropylmalate dehydrogenase Leu2p, and negative

regulator in amino acid biosynthetic pathway Gcd3p. This class shows different expression indices, ranging from 0.4 to 32.9, depending on the protein. (iv) Unclassified proteins: NADPH-dependent alpha-keto amide reductase YDL124W, mitochondria porin Por1p, and protein of unknown function YER067W. Considering the overall changes under iron deficiency, the majority of significantly increased proteins were HSPs. It is suggested that the iron deficiency produced many unfolded or misfolded proteins in the cytosol, and cells then produced HSPs to get rid of these abnormal proteins. It is well known that expression of numerous HSPs allows them to refold or remove these abnormal proteins, which have the potential to be cytotoxic [9]. In addition, proteins of Cdc48p, Kar2p, and Rpt5p that are related to ERAD were induced in the iron-deficient strain, YGH2. ERAD is a quality control process that eliminates the misfolded and improperly processed secreted proteins, or the unassembled oligomeric proteins, during the ER process [25]. ERAD proteins are closely connected to HSP function. If protein repair by HSPs is unsuccessful, abnormal proteins are cleared by the ERAD process. During ERAD, abnormal proteins are translocated back to the cytosol and degraded by the ubiquitin-proteasome system [18, 25]. Thus, increased expression of HSPs and ERAD proteins suggests that many iron-proteins might undergo a process of misfolding in the absence of sufficient iron supply. Consequently, the accumulation of misfolded proteins might induce the expression of HSPs and ERAD proteins. In addition, Leu2p related to leucine amino acid biosynthesis was found to be increased in YGH2. Shakoury-Elizeh *et al.* [27] reported that the genes involved in amino acid biosynthesis were increased in iron-deficient yeast.

The repressed proteins were also sorted into four functional classes (Table 1). (i) The enzymes involved with folate metabolism, purine and methionine biosynthesis: serine hydroxymethyl-transferase Shm1p, adenylate kinase Adk1p, diphosphate kinase Ynk1p, and methionine synthase Met6p. Shm1p was expressed very little in YGH2 compared with controls. Met6p was reduced to about a quarter in YGH2 compared with YGH2-KG and YGT. Other proteins were repressed to about half in YGH2 compared with YGH2-KG, or to about a quarter compared with YGT. (ii) The enzymes related to translation: single-stranded nucleic acid binding protein Sbp1p and ribosomal protein P2-beta Rpp2bp. Sbp1p was repressed to about one-eighth (1/8) in YGH2 compared with two controls. Rpp2bp was reduced to one-third (1/3) in YGH2 compared with YGH2-KG, and to one-sixth (1/6) in comparison with YGT. (iii) Other metabolism enzymes: aldehyde dehydrogenase Ald4p and the large subunit of $NADP^+$-dependent arabinose dehydrogenase Ara1p. These proteins were found to be reduced to one-third in YGH2 as compared with the controls. (iv) Unclassified proteins: superoxide dismutase Sod2p, cytochrome *c* reductase Qcr6p, ATP synthase Atp3p, and unknown function proteins (YDL110C and Igo2p). Interestingly, an exception was noted where the presence of the negative regulator in the general amino acid biosynthetic pathway, Gcd3p, was approximately tripled in YGH2 compared with controls, although it should be noted that it belongs to the aforementioned repressed protein class (ii). Shm1p is a folate metabolic enzyme and a major provider for one-carbon units of cells. One-carbon units are essential to purine, thymidine, and methionine biosynthesis [8]. Our data are in line with previous reports that expression of the folate metabolic enzyme at the transcriptional level was inhibited under iron depletion. Genes related to purine biosynthesis were also reduced by iron deficiency [19, 27]. In this study, Sbp1p and Rpp2bp were reduced and Gcd3 was only expressed in cells under iron deficiency. These results suggest that cellular iron depletion suppressed amino acid biosynthesis by regulating proteins that are involved in translation. Sbp1p is a single-stranded RNA binding protein that is located in the nucleus. This protein is an integral part of a nucleolar snRNP that is involved in one of the steps of rRNA maturation or ribosome assembly [6]. Rpp2bp is a key component of the ribosomal stalk, and is involved in the interaction between translational elongation factors and the ribosome [20]. Gcd3p plays a central role in the depression of amino acid biosynthetic genes, as indicated by the finding that the deletion strain of *GCD3* elevates transcription levels in amino acid biosynthesis [16]. However, Leu2p, which is related to leucine biosynthesis, showed different results from the above evidence found using our data. In our investigation, the expression level of Leu2p was observed to increase rather than decrease, and further investigation is required to resolve this discrepancy. It has been reported that *S. cerevisiae* similarly increased the transcription of genes involved in amino acid biosynthesis under iron deprivation [27].

Comparative 2-DE Analysis of the Insoluble Fractions Upon Iron Deficiency

For the proteomic analysis of the soluble fraction in YGH2 cells, the insoluble proteins were extracted according to the method described previously [15] prior to the performance of 2-DE gel analysis.

Approximately 300 protein spots were detected in the insoluble fraction of YGH2 (Fig. 3). Comparative image analysis revealed that 10 protein spots had exhibited profoundly increased levels in YGH2, but were nearly absent in the YGH2-KG. The protein spots were excised from the staining gels, digested by trypsin, and analyzed *via* MS/MS. The identified proteins of the insoluble fraction under iron deficiency are summarized in Table 2. Some of these were identified as metabolic enzymes, including phosphoglycerate kinase Pgk1p, pyruvate dehydrogenase Lat1p, isocitrate dehydrogenase Idh1p, and malate dehydrogenase Mdh1p, which functioned in both

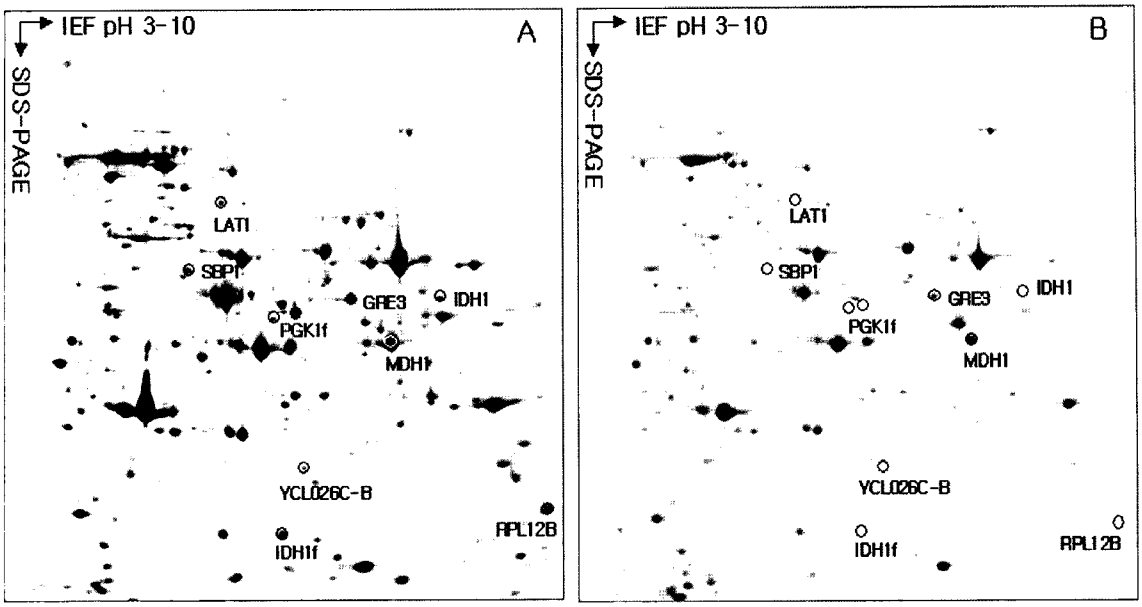


Fig. 3. Two-dimensional gel electrophoresis of proteins from the insoluble fractions of the YGH2 (**A**) and YGH2-KG (**B**).
The proteins were extracted and separated on pH 3–10 nonlinear immobilized pH-gradient strips, followed by a 8–15% SDS-polyacrylamide gel. The gels were stained with Coomassie brilliant blue.

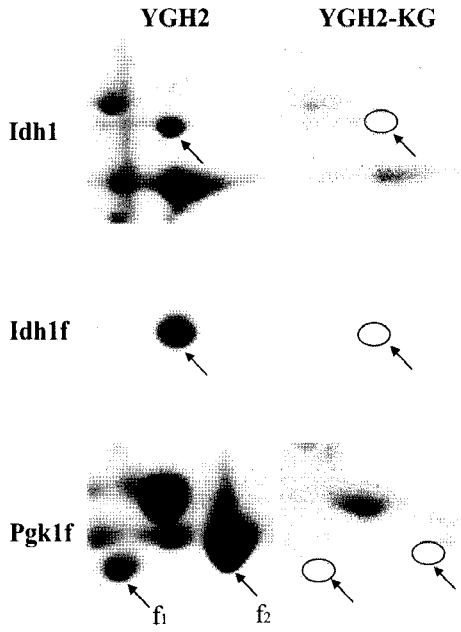
glycolysis and the tricarboxylic acid (TCA) cycle. These proteins appear to be a prerequisite for the generation of ATP utilized in the cytosolic and ER chaperones. The energy source may be employed for the refolding or proteolysis of misfolded proteins. Proteins expressed in the insoluble fraction may be of inactivated forms, due to protein unfolding or misfolding. In other investigations, it has been suggested that enzymes related to TCA, such as citrate synthase, aconitase, isocitrate dehydrogenase, and succinate dehydrogenase, were reduced by iron depletion [17, 22].

Interestingly, Idh1p and Pgk1p are found at other positions in addition to the original position corresponding to its molecular mass (Fig. 4). These were identified as the fragments of Idh1p and Pgk1p, referred to as Idh1f, Pgk1f$_1$, and Pgk1f$_2$, respectively. The molecular mass of Idh1f was 17.0 kDa, whereas the native form of Idh1p was 39.3 kDa. The molecular masses of Pgk1f$_1$ and Pgk1f$_2$ were 37 kDa and 38 kDa, respectively, whereas that of Pgk1p was 44.6 kDa. Finding this type of fragments was unexpected. These protein spots did not appear in the soluble fraction of YGH2. The observation was further supported by the fact that the ribosomal assembly-related protein, Rpl12bp, and a nucleolar single-stranded nucleic acid binding protein, Sbp1p, were increased in parallel in this fraction. The direct relevance remains unclear, but it has been theorized that unprocessed rRNA may affect the process of translational elongation [5]. It is currently believed that iron deficiency may indirectly influence nuclear pre-rRNA processing and rRNA modification, thereby leading to an increase in unfolded protein levels. In general, this modification can

Table 2. Identification of proteins expressed differentially in insoluble fraction *via* heterologous expression of ferritin.

Accession No.[a]	Gene name	Description	Theoretical M_r (kDa)/pI	Protein expression
P00560	PGK1*	Phosphoglycerate kinase	44.6/7.09	Up
P12695	LAT1	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	51.8/7.60	Up
P28834	IDH1*	Isocitrate dehydrogenase [NAD] subunit 1	39.3/8.99	Up
P17505	MDH1	Malate dehydrogenase	35.6/8.46	Up
P10080	SBP1	Single-stranded nucleic acid binding protein	33.0/5.48	Up
P17079	RPL12B	60S ribosomal protein L12	17.8/9.43	Up
P38715	GRE3	NADPH-dependent aldose reductase GRE3	37.1/6.60	Up
Q96VH4	YCL026C-B	Hypothetical protein	21.0/6.43	Up

[a]Accession numbers are entries in the SWISS-PROT.
*This protein had N-terminal truncated protein fragments.
Proteins were identified in this work by mass spectrometry.



Idh1p, 39.3 kDa

```
  1 MLNRTIAKRT LATAAQAERT LPKKYGGRFT VTLIPGDGVG KEITDSVRTI FEAENIPIDW  60
 61 ETINIKQTDH KEGVYEAVES LKRNKIGLKG LWHTPADQTG HGSLNVALRK QLDIYANVAL 120
121 FKSLKGVKTR IPDIDLIVIR ENTEGEFSGL EHESVPGVVE SLKVMTRPKT ERIARFAFDF 180
181 AKKYNRKSVT AVHKANIMKL GDGLFRNIIT EIGQKEYPDI DVSSIIVDNA SMQAVAKPHQ 240
241 FDVLVTPSMY GTILGNIGAA LIGGPGLVAG ANFGRDYAVF EPGSRHVGLD IKGQNVANPT 300
301 AMILSSTLML NHLGLNEYAT RISKAVHETI AEGKHTTRDI GGSSSTTDFT NEIINKLSTM 360
```

Idh1f, 17.0 kDa

```
  1 MLNRTIAKRT LATAAQAERT LPKKYGGRFT VTLIPGDGVG KEITDSVRTI FEAENIPIDW  60
 61 ETINIKQTDH KEGVYEAVES LKRNKIGLKG LWHTPADQTG HGSLNVALRK QLDIYANVAL 120
121 FKSLKGVKTR IPDIDLIVIR ENTEGEFSGL EHESVPGVVE SLKVMTRPKT ERIARFAFDF 180
181 AKKYNRKSVT AVHKANIMKL GDGLFRNIIT EIGQKEYPDI DVSSIIVDNA SMQAVAKPHQ 240
241 FDVLVTPSMY GTILGNIGAA LIGGPGLVAG ANFGRDYAVF EPGSRHVGLD IKGQNVANPT 300
301 AMILSSTLML NHLGLNEYAT RISKAVHETI AEGKHTTRDI GGSSSTTDFT NEIINKLSTM 360
```

Pgk1p, 44.6 kDa; Pgkf$_1$, 37 kDa

```
  1 MSLSSKLSVQ DLDLKDKRVF IRVDFNVPLD GKKITSNQRI VAALPTIKYV LEHHPRYVVL  60
 61 ASHLGRPNGE RNEKYSLAPV AKELQSLLGK DVTFLNDCVG PEVEAAVKAS APGSVILLEN 120
121 LRYHIEEEGS RKVDGQKVKA SKEDVQKFRH ELSSLADVYI NDAFGTAHRA HSSMVGFDLP 180
181 QRAAGFLLEK ELKYFGKALE NPTRPFLAIL GGAKVADKIQ LIDNLLDKVD SIIIGGGMAF 240
241 TFKKVLENTE IGDSIFDKAG AEIVPKLMEK AKAKGVEVVL PVDFIIADAF SADANTKTVT 300
301 DKEGIPAGWQ GLDNGPESRK LFAATVAKAK TIVWNGPPGV FEFEKFAAGT KALLDEVVKS 360
361 SAAGNTVIIG GGDTATVAKK YGVTDKISHV STGGGASLEL LEGKELPGVA FLSEKK     416
```

Pgkf$_2$, 38 kDa

```
  1 MSLSSKLSVQ DLDLKDKRVF IRVDFNVPLD GKKITSNQRI VAALPTIKYV LEHHPRYVVL  60
 61 ASHLGRPNGE RNEKYSLAPV AKELQSLLGK DVTFLNDCVG PEVEAAVKAS APGSVILLEN 120
121 LRYHIEEEGS RKVDGQKVKA SKEDVQKFRH ELSSLADVYI NDAFGTAHRA HSSMVGFDLP 180
181 QRAAGFLLEK ELKYFGKALE NPTRPFLAIL GGAKVADKIQ LIDNLLDKVD SIIIGGGMAF 240
241 TFKKVLENTE IGDSIFDKAG AEIVPKLMEK AKAKGVEVVL PVDFIIADAF SADANTKTVT 300
301 DKEGIPAGWQ GLDNGPESRK LFAATVAKAK TIVWNGPPGV FEFEKFAAGT KALLDEVVKS 360
361 SAAGNTVIIG GGDTATVAKK YGVTDKISHV STGGGASLEL LEGKELPGVA FLSEKK     416
```

Fig. 4. Identification of the fragments of Idh1p and Pgk1p.
The proteins were obtained from the insoluble fractions of YGH2 and YGH2-KG. Idh1f and Pgk1f were the fragments of Idh1p and Pgk1p, respectively. Underlined letters represent the protein sequence covered by the measured peptide masses. Total sequence coverage by QqTOF analysis were reached as following; Idh1p, 42.8%; Idh1f, 7.8%; Pgk1f$_1$, 47.4%; and Pgk1f$_2$, 41.8%.

contribute to the stabilization of functional rRNA structures, and to the control of the binding of tRNA and mRNA, thus regulating the translational activity of the ribosomes [7]. In addition, NADPH-dependent aldose reductase Gre3p and proteins of unknown function, YCL026C-B, were identified in the insoluble fraction.

In conclusion, iron deficiency seems to increase the production of HSPs and ERAD-associated proteins to protect the cell through degradation or refolding of abnormal proteins produced under the deficiency conditions. Under the circumstances, on the other hand, the reduced proteins include proteins related to energy metabolism, biosynthesis of purine and methionine, folate metabolism, and translation. In the present study, an iron regulon that is directly involved in iron deficiency, including *FET3* and *FTR1*, has not been identified. This may be due to the relatively low expression of these proteins compared with other proteins, or to weak staining of the proteins by Coomassie brilliant blue. The changes in cellular metabolism described above are likely to be general metabolic responses of the cells to the deficiency, and will provide further information to improve our broad understanding of iron metabolism in mammals and plants. In addition, the study presents a model organism that can be utilized in investigating the cellular response to iron.

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References

1. Aisen, P., C. Enns, and M. Wessling-Resnick. 2001. Chemistry and biology of eukaryotic iron metabolism. *Int. J. Biochem. Cell Biol.* **33:** 940–959.
2. Anderson, G. A., A. Dancis, D. G. Roman, and R. D. Klausner. 1994. Ferric iron reduction and iron uptake in eukaryotes: Studies with the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, pp. 81–89. *In* C. Hershko, A. M. Konijn, and P. Aisen (eds.), *Progress in Iron Research.* Plenum Press, New York.
3. Babcock, M., D. deSilva, R. Oaks, S. Davis-Kaplan, S. Jiralerspong, L. Montermini, M. Pandolfo, and J. Kaplan. 1997. Regulation

of mitochondrial iron accumulation by Yfh1p, a putative homology of frataxin. *Science* **276:** 1709–1712.
4. Bauminger, E. R., A. Treffry, A. J. Hudson, D. Hechel, N. W. Hodson, S. C. Andrews, *et al.* 1994. Iron incorporation into ferritins: Evidence for the transfer of monomeric Fe (III) between ferritin molecules and for the formation of an unusual mineral in the ferritin of *Escherichia coli*. *Biochem. J.* **302:** 813–820.
5. Briones, C. and J. P. Ballesta. 2000. Conformational changes induced in the *Saccharomyces cerevisiae* GTPase-associated rRNA by ribosomal stalk components and a translocation inhibitor. *Nucleic Acids Res.* **28:** 4497–4505.
6. Clark, M. W., M. L. R. Yip, J. Compbell, and J. Abelson. 1990. SSB1 of the yeast *Sacchromyces cerevisiae* is a nuclear-specific, silver binding protein that is associated with the snR10 and snR11 small nuclear RNA. *J. Cell Biol.* **111:** 1741–1751.
7. Decatur, W. A. and M. J. Fournier. 2002. rRNA modifications and ribosome function. *Trends Biochem. Sci.* **27:** 344–351.
8. Denis, V. and B. Dignan-Fornier. 1998. Synthesis of glutamine, glycine and 10-formyl tetrahydrofolate is coregulated with purine biosynthesis in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **259:** 246–255.
9. Ellgaard, L. and A. Helenius. 2003. Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell. Biol.* **4:** 181–191.
10. Freitas, J. D., H. Wintz, J. H. Kim, H. Poynton, T. Fox, and C. Vulpe. 2003. Yeast, a model organism for iron and copper metabolism studies. *Biometals* **16:** 185–197.
11. Görg, A., C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, and W. Weiss. 2000. The current state of two-dimensional electrophoresis with immobilized pH gradient. *Electrophoresis* **21:** 1037–1053.
12. Harrison, P. M. and P. Arosio. 1996. The ferritins; molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta* **1275:** 161–203.
13. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153:** 163–168.
14. Kim, K.-S., Y.-J. Chang, Y.-J. Chung, C.-U. Park, and H.-Y. Seo. 2007. Enhanced expression of high-affinity iron transporters *via* H-ferritin production in yeast. *J. Biochem. Mol. Biol.* **40:** 82–87.
15. Molly, M. P., B. R. Herbert, B. J. Walsh, M. I. Tyler, M. Traini, J. C. Sanchez, D. F. Hochstrasser, K. L. Williams, and A. A. Gooley. 1998. Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. *Electrophoresis* **19:** 837–844.
16. Myers, P. L., R. C. Skvirsky, M. L. Greenberg, and H. Greer. 1986. Negative regulatory gene for general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 6: 2150–2155.
17. Oexle, H., E. Gnaiger, and G. Weiss. 1999. Iron-dependent changes in cellular energy metabolism: Influence on citric acid cycle and oxidative phosphorylation. *Biochim. Biophys. Acta* **1413:** 99–107.
18. Okamura, K., Y. Kimata, H. Higashio, A. Tsuru, and K. Kohno. 2000. Dissociation of Kar2p/BiP from an ER sensory molecule, Ire1p, triggers the unfolded protein response in yeast. *Biochem. Biophys. Res. Commun.* **279:** 445–450.
19. Oppenheim, E. W., I. M. Nasrallh, M. G. Mastri, and P. J. Stover. 2000. Mimosine is a cell-specific antagonist of folate metabolism. *J. Biol. Chem.* **275:** 19268–19274.
20. Planta, R. J. and W. H. Mager. 1998. The list of cytoplasmic ribosomal proteins of *Saccharomyces cerevisiae*. *Yeast* **14:** 471–477.
21. Portnoy, M. E., X. F. Liu, and V. C. Culotta. 2000. *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the Nramp family of metal transporters. *Mol. Cell. Biol.* **20:** 7893–7902.
22. Puig, S., E. Askeland, and D. J. Thiele. 2005. Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. *Cell* **120:** 99–110.
23. Radisky, D. C. and J. Kaplan. 1999. Regulation of transition metal transport across the yeast plasma membrane. *J. Biol. Chem.* **274:** 4481–4484.
24. Rutherford, J. C., S. Jaron, and D. R. Winge. 2003. Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. *J. Biol. Chem.* **278:** 27636–27643.
25. Sato, M., K. Sato, and A. Nakano. 2004. Endoplasmic reticulum quality control of unassembled iron transporter depends on Rer1p-mediated retrieval from the Golgi. *Mol. Biol. Cell* **15:** 1417–1424.
26. Seo, H.-Y., Y.-J. Chung, S.-J. Kim, C.-U. Park, and K.-S. Kim. 2003. Enhanced expression and functional characterization of the human ferritin H- and L-chain genes in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotech.* **63:** 57–63.
27. Shakoury-Elizeh, M., J. Tiedeman, J. Rashford, T. Ferea, J. Demeter, E. Garcia, *et al.* 2004. Transcriptional remodeling in response to iron deprivation in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **15:** 1233–1243.
28. Tosco, A., R. A. Siciliano, G. Cacace, M. F. Mazzeo, R. Capone, A. Malorni, A. Leone, and L. Marzullo. 2005. Dietary effects of copper and iron deficiency on rat intestine: A differential display proteome analysis. *J. Proteome Res.* **4:** 1781–1788.
29. Yamaguchi-Iwai, Y., A. Dancis, and R. D. Klausner. 1995. AFT1, a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *EMBO J.* **14:** 1231–1239.