## Coordinated Remodeling of Cellular Metabolism during Iron Deficiency through Targeted mRNA Degradation

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#### Summary

Iron (Fe) is an essential micronutrient for virtually all organisms and serves as a cofactor for a wide variety of vital cellular processes. Although Fe deficiency is the primary nutritional disorder in the world, cellular responses to Fe deprivation are poorly understood. We have discovered a posttranscriptional regulatory process controlled by Fe deficiency, which coordinately drives widespread metabolic reprogramming. We demonstrate that, in response to Fe deficiency, the Saccharomyces cerevisiae Cth2 protein specifically downregulates mRNAs encoding proteins that participate in many Fe-dependent processes. mRNA turnover requires the binding of Cth2, an RNA binding protein conserved in plants and mammals, to specific AU-rich elements in the 3' untranslated region of mRNAs targeted for degradation. These studies elucidate coordinated global metabolic reprogramming in response to Fe deficiency and identify a mechanism for achieving this by targeting specific mRNA molecules for degradation, thereby facilitating the utilization of limited cellular Fe levels.

### Introduction

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Iron (Fe) is an essential nutrient for virtually all organisms. Fe serves as a cofactor for a wide variety of cellular processes, including oxygen transport, cellular respiration, the tricarboxylic acid (TCA) cycle, lipid metabolism, synthesis of metabolic intermediates, gene regulation, and DNA replication and repair. Despite its abundance in the earth's crust, Fe bioavailability is highly restricted due to its extreme insolubility at physiological pH. Indeed, Fe deficiency is the primary nutritional disorder in the world, estimated to affect over two billion people and resulting in iron deficiency anemia (Baynes and Bothwell, 1990). Alterations in iron homeostasis underlie many human diseases, including Friedreich's ataxia, hereditary hemochromatosis, aceruloplasminemia, Parkinson's disease, aging, microbial pathogenesis, and cancer (Hentze et al., 2004; Nittis and Gitlin, 2002; Roy and Andrews, 2001).

Elegant genetic, biochemical, and physiological studies have elucidated many of the components that function in Fe uptake, efflux, and distribution and their mech-

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anisms of action in both prokaryotic and eukaryotic cells (Escolar et al., 1999; Hentze et al., 2004; Van Ho et al., 2002). Studies with the baker's yeast Saccharomyces cerevisiae have demonstrated that, in response to Fe deprivation, cells utilize the Fe-responsive transcription factors Aft1 and Aft2 to induce expression of the socalled iron regulon (Rutherford et al., 2003; Shakoury-Elizeh et al., 2004), which includes proteins involved in Fe reduction at the plasma membrane, uptake, mobilization from intracellular stores, and utilization from heme. among others (Van Ho et al., 2002). Less attention has been dedicated to the characterization of metabolic pathways that are specifically downregulated by Fe depletion. Recent studies have shown that mRNA levels of genes involved in biotin synthesis, glutamate metabolism and heme assembly are downregulated under low Fe conditions (Lesuisse et al., 2003; Shakoury-Elizeh et al., 2004). However, the mechanisms controlling the Fe deprivation-dependent downregulation of these genes, and other global metabolic pathways altered as a consequence of Fe deficiency, have not been elucidated.

In mammals, one response to iron scarcity is posttranscriptionally controlled by the iron-regulatory proteins IRP1 and IRP2. In response to Fe deprivation, IRP1 binds to specific mRNA stem-loop structures known as ironresponsive elements (IREs). IRP1 binding to IREs in the 5' untranslated region inhibits translation of erythroid aminolevulinic acid synthase, mitochondrial aconitase, the ferroportin Fe efflux pump, and subunits of the Fe storage protein ferritin. IRP1 binding to IREs in the 3' untranslated region (3'UTR) of the transferrin receptor 1 isoform stabilizes the mRNA, thereby increasing protein levels and enhancing Fe uptake via Fe loaded transferrin (Hentze et al., 2004; Theil, 2000). A posttranscriptional downregulation of Fe-dependent pathways, which depends on small antisense RNAs, has recently been described in bacteria (Masse and Gottesman, 2002; Wilderman et al., 2004).

While several dozen metabolic enzymes require Fe for catalysis in eukaryotic cells, little is known about global reprogramming and regulatory mechanisms governing this process in response to Fe deficiency. We have discovered a mechanism that mediates global posttranscriptional control of multiple components of Fe-dependent pathways to respond in a concerted fashion to Fe deficiency. The Fe-regulated protein Cth2 coordinates this process by binding to and targeting specific mRNA molecules for degradation under Fe deficiency, thereby facilitating the utilization of limited available Fe for normal growth.

### Results

Genome-Wide Response of Saccharomyces cerevisiae to Iron Deprivation

Although Fe plays a crucial role in a wide array of cellular processes, little is known about how Fe deprivation affacts metabolic pathways on a global scale in eukarvotic



Figure 1. Response of Fe-Dependent Processes to Fe Depletion in Yeast

B4741 wild-type cells were grown in SC containing 300 µM Fe or 100 µM BPS, and RNA was analyzed with DNA microarrays as detailed in Experimental Procedures. Only components of multiple Fe-dependent pathways with a fold change greater than two have been represented. A list of the genes grouped in each functional family is shown in Supplemental Tables S2 and S3.

deprivation, we compared the mRNA expression profile of wild-type cells grown under Fe-replete conditions to cells grown under Fe scarcity achieved by addition of the Fe(II) chelator bathophenantholine disulfonic acid (BPS). We observed that, in addition to changes in other processes (data not shown), key components of multiple Fe-dependent metabolic pathways are significantly altered by Fe availability (Figure 1 and see Supplemental Tables S2 and S3 at http://www.cell.com/cgi/content/ full/120/1/99/DC1/). In addition to the induction of the Aft1/2-dependent Fe regulon previously described (Blaiseau et al., 2001; Rutherford et al., 2003; Shakoury-Elizeh et al., 2004; Yamaguchi-Iwai et al., 1996), genes involved in sterol biosynthesis (ERG genes) and the fatty acid desaturase OLE1 are induced under Fe deprivation. In addition, key components of multiple Fe-dependent pathways and proteins including (1) the TCA cycle; (2) the mitochondrial electron transport chain; (3) Fe-S cluster, di-Fe-tyrosyl, and heme-containing proteins; and, (4) as recently described (Lesuisse et al., 2003; Shakoury-Elizeh et al., 2004), HEM15 encoding ferrochelatase, the last step in heme biosynthesis, and two enzymes involved in biotin synthesis are coordinately downregulated by Fe depletion (Figure 1 and Supplemental Table S3). Taken together, these results demonstrate that mRNA levels of multiple components of Fe-dependent metabolic pathways in S. cerevisiae are coordinately regulated in response to Fe deprivation.

## The Aft1-Aft2 Target CTH2 Is Important for Growth under Fe Limitation

Previous DNA microarray experiments strongly suggest that the *CTH2* gene, which encodes a protein related to the mammalian tandem zinc finger (TZF) protein tristetraprolin or TTP (Figure 2A), is transcriptionally induced under Fe limitation (Foury and Talibi, 2001; Rutherford et al., 2003; Shakoury-Elizeh et al., 2004). As shown in Figures 2B and 2C, the steady-state levels of *CTH2* mRNA and a functional FLAG epitope-tagged Cth2 protein under Fe-adequate conditions are very low but dramatically increase in response to Fe deprivation. Fur-

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thermore, *CTH2* expression under low Fe conditions is significantly decreased in an *aft1* strain and is undetectable under either condition in the *aft1aft2* double mutant. Mutagenesis of two putative Aft1-Aft2 binding sites (Yamaguchi-lwai et al., 1996) from the *CTH2* upstream sequence indicates that both sites cooperate in the activation of *CTH2* by Fe starvation, although these experiments do not exclude the participation of other *cis*regulatory sequences in *CTH2* regulation by Fe (see Supplemental Figure S2 on the *Cell* web site).

Given that CTH2 mRNA levels are tightly regulated by Fe availability and the Aft1-Aft2 Fe-responsive transcription factors, we assayed growth of cth2 deletion mutant cells under Fe deprivation conditions. cth2 cells exhibited a growth defect compared to wild-type cells in the presence of the intracellular Fe-specific chelator ferrozine (Figure 3A). The cth2 growth defect on ferrozine was reversed by addition of Fe (Figure 3A), demonstrating that the growth defect of cth2 cells occurs in response to Fe deprivation rather than to ferrozine administration. The yeast genome harbors a gene encoding a protein similar to Cth2, Cth1 (Thompson et al., 1996), whose transcription is independent of Fe levels (Figure 2B). Although cth1 cells did not display a growth defect under Fe deprivation conditions, cells lacking both CTH1 and CTH2 exhibited a more severe growth defect than those lacking only CTH2 (Figure 3A). Similarly, the cth1cth2 growth defect in the presence of ferrozine was partially suppressed by CTH1 and completely recovered by coexpression of both CTH1 and CTH2 (Figure 3B). These results demonstrate that CTH2 is important for growth under Fe deprivation induced by the membrane permeable Fe chelator ferrozine and suggest that Cth1 function in yeast cells may partially overlap with Cth2.

### CTH2 Coordinates the Downregulation of Multiple Fe-Dependent Pathways under Fe Deprivation

A prominent feature of Cth2 is the presence of a  $Cx_8Cx_5Cx_3H$  tandem zinc finger (TZF) domain near the carboxyl terminus of the protein (Figure 2A and Supplemental Figure S1). This TZF motif is present in a family of



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Figure 2. Expression of CTH2 upon Fe Depletion Is Dependent on Both Aft1 and Aft2 Transcription Factors

(A) Model for the primary structure of S. cerevisiae Cth2 and Cth1 and human tristetraprolin (hTTP) protein. TZF, tandem zinc finger. (B) CM3260 wild-type, *aft1, aft2,* and *aft1aft2* cells were grown in SC containing 100  $\mu$ M Fe (Fe +) or 100  $\mu$ M BPS (Fe -) and RNA extracted and analyzed by RNA blotting. The Aft1 target *FET3* was used as a control for Fe-regulated expression.

(C) cth1cth2 cells transformed with pRS416-FLAG2-CTH2 or pRS416 (vector) were grown in SC-Ura containing 300  $\mu$ M Fe (Fe +) or 100  $\mu$ M BPS (Fe -) and protein extracted and analyzed by immunoblotting. Phosphoglycerate kinase (Pgk1) was used as a loading control.

RNA binding proteins typified by the mammalian protein tristetraprolin (TTP). TTP mediates the targeted destabilization of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), cyclooxygenase-2, interleukin-3, and granulocyte/macrophage colony-stimulating factor (GM-CSF) mRNAs (Blackshear, 2002; Carballo et al., 1998; Sawaoka et al., 2003; Stoecklin et al., 2001). An alignment of yeast Cth1 and Cth2 with human TTP shows that, while Cth1 and Cth2 proteins share 46% identity, hTTP homology to Cth1 and Cth2 is restricted to the TZF domains (Supplemental Figure S1). Despite little homology in the rest of the protein, we hypothesized that Cth2 could be involved in posttranscriptional regulation of specific mRNAs under Fe deprivation. To test this hypothesis, we ascertained the effect of Cth2 on multiple mRNAs we observed in our microarray to be downregulated by Fe deficiency. As shown in Figure 3C, genes encoding proteins involved in the TCA cycle (SDH4), heme synthesis (HEM15), Fe-S cluster assembly (ISA1), vacuolar Fe accumulation (CCC1), and Fe-S proteins (LIP5) are dramatically downregulated under Fe starvation in a wild-type cleotide reductase, are only modestly decreased by Fe deprivation (Figure 3C). Interestingly, this coordinated mRNA downregulation does not occur in the absence of *CTH2* (Figure 3C, wt versus *cth2* mutant). While mRNA levels in *cth1* cells did not change significantly with respect to wild-type cells, the *cth1cth2* mutant displayed reduced mRNA downregulation, suggesting that Cth1 directly or indirectly influences in this process. These results demonstrate that Cth2 functions in the downregulation of specific mRNAs under conditions of Fe deprivation.

We used DNA microarrays to ascertain which mRNAs exhibit CTH2-dependent changes on a genome-wide scale by comparing the gene expression profiles under Fe deficiency of cth1cth2 cells expressing a plasmidborne CTH2 gene or transformed with vector alone. Messenger RNAs corresponding to 84 genes were significantly upregulated in the absence of CTH2 (Table 1). Interestingly, 54% (45 of 84) of the upregulated genes are involved in obvious Fe-dependent processes, 14% (12 of 84) have other functions, and 32% (27 of 87) are genes of unknown function. Among the 45 Fe-related genes whose expression is increased in cth2 mutants under low Fe conditions compared to wild-type cells, we find (1) three members of the Fe regulon (FIT1, FIT2, and HMX1); (2) genes encoding key enzymes involved in heme biosynthesis (HEM15); (3) two genes encoding proteins involved in Fe-S cluster assembly (ISA1 and NFU1); (4) eight genes encoding enzymes that participate in the TCA cycle including aconitase (ACO1), succinate dehydrogenase subunits SDH2 and SDH4, a-ketoglutarate dehydrogenase (KGD1), and dihydrolypoyl transsuccinylase (KGD2); (5) 15 genes encoding proteins that participate in the electron transfer chain that include four subunits of the cytocrome c oxidase (COX4, COX6, COX8, COX9) and six subunits of the ubiquinol cytochrome c reductase complex (COR1-5 and RIP1); (6) eight members of the sterol and unsaturated fatty acid synthesis and metabolism pathways (ERG genes and OLE1); (7) ribonucleotide-diphosphate reductase subunits (RNR4); and (8) genes encoding additional Fe-S cluster-containing proteins (LIP5, encoding lipoic acid synthase; LEU1, required for leucine biosynthesis; and RLI1, related to RNase L inhibitor). Taken together, these results demonstrate that Cth2 functions in the coordinated downregulation of multiple Fe-dependent metabolic pathways, and potentially other as yet uncharacterized pathways, in yeast under conditions of Fe deficiency.

### A Conserved RNA Binding Motif Is Required for Cth2-Mediated mRNA Downregulation

Studies with TTP in mammalian systems have demonstrated that the integrity of the zinc finger domains is required for binding and destabilization of specific mRNAs (Blackshear, 2002; Lai et al., 1999, 2003). We tested the role of the CCCH zinc fingers in Cth2-dependent mRNA downregulation by mutagenizing conserved cysteine residues, located in both zinc finger motifs, to arginine. First, cells expressing *CTH2-C190R* or *CTH2-C213R* mutant alleles displayed a growth defect in the

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Figure 3. CTH2 Is Required for Fe-Limited Growth and Fe Deficiency-Dependent mRNA Downregulation

(A) BY4741 wild-type, *cth1*, *cth2*, and *cth1cth2* cells were assayed for growth on SC (Complete) and SC containing 750 µM ferrozine without or with 300 µM Fe (+ Fe).

(B) cth1cth2 cells cotransformed with pRS416 plus pRS415 (vector), pRS416-CTH1 plus pRS415 (C7H1), pRS416 plus pRS415-CTH2 (C7H2), and pRS416-CTH1 plus pRS415-CTH2 (C7H1 + C7H2) were assayed on SC-Ura-Leu (--Ura - Leu) and SC containing 750  $\mu$ M ferrozine. (C) C7H2 is essential for Fe deficiency-dependent mRNA downregulation. Wild-type cth2, cth1, and cth1cth2 cells were grown in SC media containing 300  $\mu$ M Fe (Fe +) or 100  $\mu$ M BPS (Fe -) and RNA extracted and analyzed by RNA blotting.

(D) Schematic representation of the CCCH TZF domain in Cth2 protein. Cysteine residues 190 and 213 (white characters) were mutagenized to arginine.

(E) The Cth2 TZF domains are essential for growth in the presence of ferrozine. BY4741 wild-type and cth2 cells transformed with vector alone or expressing CTH2, CTH2-C190R, and CTH2-C213R alleles were assayed for growth on ferrozine plates.

(F) The Cth2 CCCH TZF motifs are essential for mRNA downregulation. cth1cth2 cells containing vector or expressing CTH2, CTH2-C190R, and CTH2-C213R alleles were analyzed by RNA blotting as described for (C).

LIP5, COX6, and other mRNAs (data not shown) was abrogated in both Cth2 mutants (Figure 3F). Similar results were obtained when cysteine residues 190 and 213 were mutagenized to alanine (data not shown). Control experiments showed that the cysteine mutant proteins are properly expressed (data not shown). Taken together, these results demonstrate that the integrity of both CCCH zinc finger motifs is essential for Cth2 function in coordinated mRNA downregulation in response to Fe deprivation.

Downregulation of Specific mRNAs by Fe Deprivation Requires AU-Rich Elements

Human TTP binds to AU-rich elements (AREs) within the 3'UTR of target mRNAs and induces RNA degradation (Blackshear et al., 2003; Lai et al., 1999). Interestingly, in silico analysis and visual inspection reveals that approximately 80% of the mRNAs upregulated in *cth2* cells

under low Fe conditions contain one or more putative AREs, defined as 5'-UAUUUAUU-3' and 5'-UUAUU UAU-3' octamer sequences, located within 500 nucleotides after the translation termination codon (Table 1). To test whether Cth2-dependent mRNA downregulation during Fe deficiency occurs via AREs located within the 3'UTR, we used the mRNA encoding the membraneanchored heme-containing subunit of the succinate dehydrogenase complex in mitochondria, SDH4, which is downregulated under Fe deprivation in a manner completely dependent on Cth2 (Figures 3C and 3F). The SDH4 3'UTR contains three 5'-UUAUUUAUU-3' seguences beginning at 125, 135, and 158 nucleotides after the translation termination codon (Table 1 and Figure 4A). The adenine nucleotides 127, 134, 141, and 160 were mutated to cytosine in a plasmid-borne copy of the SDH4 gene (Figures 4A and 4B, SDH4-AREmt2) and mRNA levels assessed under high and low Fe conditions

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Table 1. Genes Upregulated in cth2 versus Wild-Type Cells under Fe-Limiting Conditions

ORF	Gene	Function	Fold ± SD	Putative AREs
in the second seco				
VDD524C	E171	Cell wall mannoprotein involved in sideronhore-Fe uptake	$2.0 \pm 0.2$	263
VOR382W	FIT2	Cell wall mannoprotein involved in siderophore-Fe uptake	$1.6 \pm 0.1$	255
VI R205C	HAAYI	Herne binding peroxidase involved in reutilization of heme Fe	$2.0 \pm 0.4$	
VDD044W/	WEARIS	Conreportingen III oxidase, oxygen-requiring enzyme	$1.5 \pm 0.3^{\circ}$	68, 89
VOR176W	HEM15	Ferrochelatase catalyzes insertion of Fe(II) into	$2.2 \pm 0.2$	43, 99
10/11/000	1 3 1 9 3 3	protonorphyrin IX		
For Schutzenbiogonacia				
VICI MARC	NELI	Nifl Llike protein	$2.0 \pm 0.2$	191, 203
VI 1.027W	ISA1	Member of Fe-S cluster biosynthesis machinery	$1.9 \pm 0.1$	46, 62
TCA cycle	10mi			
YNB001C	CIT1	Citrate synthese	$2.0 \pm 0.2$	
YPB001W	CIT3	Mitochondrial isoform of citrate synthase	1.7 ± 0.4	
YI B304C	ACO1	Mitochondrial aconitase, Fe-S cluster protein	$2.6 \pm 0.3$	32, 150, 177
YIL125W	KGD1	Alpha-ketoolutarate dehvdrogenase	$1.6 \pm 0.2$	193, 230
YDB148C	KGD2	Dihydrolipovl transsuccinylase	$1.8 \pm 0.2$	242
YLL041C	SDH2	Succinate dehydrogenase (ubiquinone) Fe-S cluster subunit	$2.8 \pm 0.6$	162, 309, 328
YDR178W	SDH4	Succinate dehydrogenase membrane anchor heme-binding	$3.2 \pm 0.7$	125, 135, 158
		subunit		
YPL262W	FUM1	Mitochondrial and cytoplasmic fumarase, Fe-S cluster protein	$1.6 \pm 0.2$	
Mitochondrial resolitation/electron transport chain				
Cvtochrome c oxida:	se			
YGL187C	COX4	Subunit IV of cytochrome c oxidase	$1.9 \pm 0.4$	53
YHR051W	COX6	Subunit VI of cytochrome c oxidase	$2.2 \pm 0.2$	88
YLR395C	COX8	Subunit VIII of cytochrome c oxidase	$1.9 \pm 0.2$	104
YDL067C	COX9	Subunit VIIa of cytochrome c oxidase	$1.8 \pm 0.2$	44
Ubiauinol cytochrome c reductase				
YBL045C	QCR1/COR1	Core subunit I of ubiquinol cytochrome c reductase complex	$2.0 \pm 0.2$	140
YPR191W	QCR2/COR2	Core subunit II of ubiquinol cytochrome c reductase complex	$1.6 \pm 0.2$	155
YFR033C	QCR6/COR3	Subunit VI of ubiquinol cytochrome c reductase complex	$1.7 \pm 0.3$	31
YDR529C	QCR7/COR4	Subunit VII of ubiquinol cytochrome c reductase complex	$1.9 \pm 0.2$	150, 239
YJL166W	QCR8/COR5	Subunit VIII of ubiquinol cytochrome c reductase complex	$1.8 \pm 0.3$	97, 114
YEL024W	RIP1	Rieske Fe-S protein of ubiquinol cytochrome c reductase	$2.0 \pm 0.1$	293, 355
		complex		
YOR356W		Putative mitochondrial dehydrogenase flavoprotein	$2.1 \pm 0.2$	13, 37, 81
YGR255C	COQ6	Flavin-dependent monooxygenase, ubiquinone biosynthesis	$1.7 \pm 0.3$	42
YKR066C	CCP1	Cytochrome c peroxidase	$2.8 \pm 0.5$	18, 41, 50, 59
YMR145C	NDE1	NADH dehydrogenase	$1.6 \pm 0.2$	
YBL030C	PET9/AAC2	Mitochondrial ADP/ATP carrier	$1.6 \pm 0.2$	
Sterol and fatty acid synthesis and metabolism				
YHR072W	ERG7	Lanosterol synthase	$2.0 \pm 0.3$	4, 60
YHR007C	ERG11	Lanosterol C-14 demethylase	$1.6 \pm 0.1$	174, 203, 273
YMR208W	ERG12	Mevalonate kinase	$1.6 \pm 0.1$	19
YGR060W	ERG25	C-4 methyl sterol oxidase	$1.7 \pm 0.4$	
YER044C	ERG28	ER membrane protein, may facilitate Erg26 and Erg27	$1.6 \pm 0.3$	52
		interactions		
YGL055W	OLE1	Fatty acid desaturase	$1.6 \pm 0.3$	151, 787
YMR272C	FAH1/SCS7	Hydroxylation of C-26 fatty acid in ceramide	$1.7 \pm 0.2$	89, 105
YPL170W	DAP1	Damage response protein involved in sterol synthesis	1.6 ± 0.1	18, 146
DNA replication and	repair			<u></u>
YJL026W	RNR2	Ribonucleotide-diphosphate reductase, di-re-tyrosyl coractor	1.4 ± 0.2	00 105
YGR180C	RNR4	Ribonucleotide-diphosphate reductase, 14 subunit	1.0 ± 0.4	39, 125
Other Fe-, Cu-, and	oxygen-related h	unction	4 4 / 44	DA 444
YLR220W	CCC1	Transporter that mediates vacuolar re storage	1.4 :2 0.1*	70 00
YOR196C	LIP5	Lipoic acid synthase, Fe-S cluster protein	2.0 2 0.3	10, 34
YGL009C	LEUI	Isopropyimalate isomerase, re-5 cluster protein	16 + 03	280 201
YDH091C	HLIT	HNase L Inflotor, Fe-S cluster protein	1.0 - 0.2	275 303
YKL109W	HAP4	Subunit of Hap transcriptional activator	1.7 0.3	210,000
YHR055C	CUP1-2	Copper-binding metallotnionein	1.0 0.4	
YAR020C	PAU7	Member of PAU family	1.7 0.2	
YOH394W		wender of PAU family	1.0 - 0.2	
Other functions	0.000	the second state of the standard second by second by second s	16+00	
YCR005C	CIT2	Nonmitochononal citrate synthase	19 + 00	16
YDR007W	IRP1	Phosphonbosylanthranilate isomerase	1.0 0.2	10
YDH423C	CAD1	Leucine zipper transcriptional activator	1.0 - 0.0	54
YER003C	PMI40	Mosphomannose isomerase	1.0 0.2	
YHR002W	LEU5	Mitochondhal camer protein	1.0 0.3	

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