

# Utilization of glutathione as an exogenous sulfur source is independent of $\gamma$ -glutamyl transpeptidase in the yeast *Saccharomyces cerevisiae*: evidence for an alternative glutathione degradation pathway

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## Abstract

$\gamma$ -Glutamyl transpeptidase ( $\gamma$ -GT) is the only enzyme known to be responsible for glutathione degradation in living cells. In the present study we provide evidence that the utilization of glutathione can occur in the absence of  $\gamma$ -GT. When disruptions in the *CIS2* gene encoding  $\gamma$ -GT were created in *met15* $\Delta$  strains, which require organic sulfur sources for growth, the cells were able to grow well with glutathione as the sole sulfur source suggesting that a  $\gamma$ -GT-independent pathway for glutathione degradation exists in yeast cells. The *CIS2* gene was strongly repressed by ammonium and derepressed in glutamate medium, and was found to be regulated by the nitrogen regulatory circuit. The utilization of glutathione as a sulfur source was, however, independent of the nitrogen source in the medium, further underlining that the two degradatory pathways were distinct.

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## 1. Introduction

Glutathione, the most abundant non-protein thiol present in almost all eukaryotic cells, plays numerous roles within the cell, and is synthesized through the consecutive action of two enzymes,  $\gamma$ -glutamyl-cysteinyl synthetase (Gsh1p) and glutathione synthetase (Gsh2p) [1,2]. In addition to endogenous synthesis, glutathione can also be taken up into the cell through the action of specific glutathione transporters [3]. The intracellular concentrations of glutathione within the cell have been estimated to range between 0.1 and 10 mM [4]. The importance of glutathione for the cell is underlined by the fact that it is essen-

tial for the growth of yeasts [5–7], as well as mammals [8]. In the absence of endogenous (or exogenous) glutathione, cells eventually stop growing. In *Schizosaccharomyces pombe*, growth stasis occurs almost immediately, while in *Saccharomyces cerevisiae*, there is a ‘delayed growth stasis’ with cells growing for almost seven or eight generations at rates comparable to wild-type [9] before entering growth stasis. In mammals, glutathione deprivation (in *gsh1* $\Delta$  knockouts) leads to embryonic lethality.

The turnover of glutathione is known to occur through the action of the enzyme  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT), which, so far, is the only enzyme known to be involved in the degradation of glutathione [10,11].  $\gamma$ -GT or  $\gamma$ -glutamyl transferase, which is localized at the plasma membrane of mammalian cells [12] and on the vacuolar membrane in yeasts [13], catalyzes the first step in the degradation of glutathione which involves the cleavage of the  $\gamma$ -glutamyl moiety and release of cysteinylglycine, which in turn is further hydrolyzed into its constituent amino acids, cysteine and glycine, by a still unidentified gene (in yeasts) encoding the cysteine glycine dipeptidase. The  $\gamma$ -glutamyl moiety cleaved from glutathione is transferred to suitable

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acceptor amino acids and peptides. Extensive studies have been carried out on the enzymology of the mammalian and the yeast enzymes [14–17]. Despite this, several aspects of the  $\gamma$ -GT enzyme and its true roles within the cell remain unclear.

The  $\gamma$ -GT enzyme of yeast has, very recently, been shown to be encoded by the *CIS2* (*ECM38*) gene and was demonstrated to play a role in the turnover of vacuolar glutathione [18] by releasing the degraded products into the cytosol. In earlier studies, the *CIS2* gene was also identified in a screen of mutants defective in cell wall biosynthesis [19], and in a separate study the gene was also identified as a multicopy suppressor of *cik1* and *kar3* null mutants, genes involved in microtubule assembly [20]. However, the precise role of  $\gamma$ -GT in these pathways is not clear.

The possibility that in addition to  $\gamma$ -GT a second degradation pathway might exist in yeast or in mammals is an aspect of glutathione homeostasis that has not been properly addressed. The recent identification of the *CIS2* gene as encoding the  $\gamma$ -GT enzyme and the availability of different mutants in yeast thus offer several possibilities for investigating these questions.

In the present study we have tried to gain further information on the true role of  $\gamma$ -GT in the cell and also tried to address the question as to whether glutathione turnover and degradation can occur even in the absence of  $\gamma$ -GT. Our results demonstrate, for the first time, evidence for the presence of a  $\gamma$ -GT-independent pathway for the degradation and utilization of exogenous glutathione, and also indicate that the primary role of the vacuolar  $\gamma$ -GT enzyme is restricted to mobilization of the vacuolar stores of glutathione under specific nutritional stress conditions.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals used were of analytical reagent grade. All

the reagents were obtained from Sigma-Aldrich or Hi Media (India). Medium components were purchased either from Hi Media (India), or from Difco. Restriction enzymes and Vent DNA polymerase were from New England Biolabs. Oligonucleotides were purchased from Bioscience (Canada).

### 2.2. Yeast strains and growth conditions

The list of yeast strains used in this study is shown in Table 1. Yeast cells were routinely grown at 30°C in YPD medium. The minimal medium (MM) contained yeast nitrogen base, glucose and ammonium sulfate supplemented with the required amino acids and bases. Glutathione, methionine and cysteine when added were used at concentrations of 250  $\mu$ M.  $\gamma$ -GT induction medium was made by using glutamate in place of ammonium sulfate at a concentration of 1 mg ml<sup>-1</sup>.

### 2.3. Yeast DNA isolation and yeast transformation

Yeast chromosomal DNA was isolated by the glass bead lysis method and yeast transformations were carried out using the lithium acetate method [21].

### 2.4. Construction of strains

The *CIS2* gene disruption was created in different backgrounds (Table 1) using the one-step polymerase chain reaction (PCR) disruption method as described earlier using the KanMX2 module [22]. The primers used for the disruption were *CIS2*-DEL1, 5'-CA AGACTTTAAATACCTGTTTTATACTGCATATATTCACGCTGAAGCTTCGTACGC-3' and *CIS2*-DEL2, 5'-CTCACCGCATGCCATTCTCCCCTGACATTGCGTATAGCATAGGCCACTAGTGGATC-3'. Disruptions were confirmed by the *CIS2*-FOR and *CIS2*-REV primers described below. The construction of ABC1195, ABC1196 and ABC1257 was carried out by transformation of *S. cerevisiae* strains ABC734, ABC1066 and ABC1083 respectively, with a *gsh1* $\Delta$ ::*LEU2* plasmid [23].

Table 1  
List of strains used in this study

Strain	Genotype	Source
ABC154 (YPH499)	Mata <i>ura3-52 leu2-<math>\Delta</math>1 lys2-801 his-<math>\Delta</math>200 trp1-<math>\Delta</math>63 ade2-101</i>	K. Kuchler
ABC1069	Mata <i>ura3-52 leu2-<math>\Delta</math>1 lys2-801 his-<math>\Delta</math>200 trp1-<math>\Delta</math>63 ade2-101 cis2<math>\Delta</math>::KanMX2</i>	This study
ABC591	Mata <i>ura3-52 leu2-<math>\Delta</math>1 lys2-801 his3-<math>\Delta</math>200 trp1-<math>\Delta</math>63 ade2-101 gsh1<math>\Delta</math>::LEU2</i>	This lab
ABC734 (BY4742)	Mata $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	J. Boeke
ABC1066	Mata $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 cis2<math>\Delta</math>::KanMX2</i>	This study
ABC1195	Mata $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 gsh1<math>\Delta</math>::LEU2</i>	This study
ABC1196	Mata $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 cis2<math>\Delta</math>::KanMX2 gsh1<math>\Delta</math>::LEU2</i>	This study
ABC1094	Mata $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 gln3<math>\Delta</math>::KanMX4</i>	Euroscarf
ABC1113	Mata $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 ure2<math>\Delta</math>::KanMX4</i>	Euroscarf
ABC733 (BY4741)	Mata <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 met15<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	J. Boeke
ABC1083	Mata <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 met15<math>\Delta</math>0 ura3<math>\Delta</math>0 cis2<math>\Delta</math>::KanMX2</i>	This study
ABC1257	Mata <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 met15<math>\Delta</math>0 ura3<math>\Delta</math>0 cis2<math>\Delta</math>::KanMX2 gsh1<math>\Delta</math>::LEU2</i>	This study

### 2.5. Cloning of *CIS2*

The *CIS2* gene was cloned by PCR from genomic DNA isolated from BY4742(ABC734) using the primers CIS2-FOR, 5'-TAGCGTTCTAGACTTACAGTTATGCTGTG-3', and CIS2-REV, 5'-TTACCCCTCGAGTTAGTA-TACGGAGGAGATTCCTC-3'. PCR was carried out using Vent polymerase. The PCR product was purified, digested with *Xba*I and *Xho*I and cloned downstream of the TEF promoter in the centromeric expression vector p416-TEF [24]. The sequence of the *CIS2* gene was confirmed by sequencing.

### 2.6. $\gamma$ -GT assay

Cells were harvested at exponential phase in the respective medium, washed and finally resuspended in 0.1 M Tris-HCl buffer, pH 9.0. Cells were broken by glass bead lysis, the glass beads and cell debris removed by low speed centrifugation, and the supernatant used as enzyme source. The entire procedure was carried out at 4°C. The enzyme was assayed using L- $\gamma$ -glutamyl-*para*-nitroanilide as donor and glycyglycine as acceptor according to the method described earlier [25]. The activity (U) was expressed as nmol of *p*-nitroaniline released per minute and specific activity as U mg<sup>-1</sup> of protein. Protein was estimated by Bradford reagent using bovine serum albumin as the standard.

### 2.7. Glutathione estimation

Total glutathione was estimated by the DTNB-glutathione reductase coupled assay [26], as described earlier [7].

### 2.8. Growth curve experiments

Growth experiments for delayed growth stasis in *gsh1Δ* and *gsh1Δ cis2Δ* strains were essentially carried out as described previously [9] except that glutamate was used in place of ammonium sulfate as the nitrogen source.

Growth experiments to see the effect of glutathione and methionine as exogenous sulfur sources were carried out in strains ABC733 (*met15Δ*) and ABC1083 (*met15Δ cis2Δ*). Both strains were grown overnight in YPD, and reinoculated into minimal medium supplemented with ammonium sulfate and methionine and grown for about 20 h. After growth, the cells were washed as described above and reinoculated at an OD<sub>600</sub> of about 0.1 in minimal media having ammonium or glutamate as nitrogen source and either methionine or glutathione as organic sulfur source. The growth was monitored by recording absorbance at 600 nm at regular intervals.

### 2.9. Plasmid construction

pAB1118 (2  $\mu$ m *URA3 P<sub>CIS2</sub>-lacZ*), which contains 634

bp of *CIS2* promoter fused in-frame to the *lacZ* gene, was constructed by PCR amplifying these 634 bases present upstream of *CIS2* gene from the genomic DNA isolated from BY4742 using primers CIS2-FUSX, 5'-CCGTTACTCGAGCACCTTCATCGCCTACTAGGAG-3', and CIS2-FUSB, 5'-ACACAAGGATCCCAGCATAACTGTAAGTCTAGTACGC-3'. The amplified fragment was gel purified, digested with *Xho*I-*Bam*HI and cloned into the *Xho*I-*Bam*HI sites of the  $\beta$ -galactosidase ( $\beta$ -gal) reporter plasmid, pLG699Z [27]. The promoter sequence and the resultant in-frame fusions were confirmed by sequencing.

### 2.10. $\beta$ -Gal assays/induction conditions

The fusion plasmid constructed above was transformed into the different strains and the fresh transformants were inoculated and grown in minimal medium+supplements for several hours followed by re-inoculation into the induction medium. After the growth of cells for 6–8 h, the cells were harvested, washed, resuspended in Z buffer, and assayed for  $\beta$ -gal activity by the permeabilized assay as described earlier [28]. Measurements were carried out in triplicate, and activity was expressed as  $\beta$ -gal units per OD<sub>600</sub> unit of cells. In the case of *gln3Δ* strains (ABC1094), cell growth was slow, therefore growth in induction medium was carried out for 12–14 h.

## 3. Results

### 3.1. Delayed growth stasis upon glutathione depletion is independent of the presence of $\gamma$ -GT activity

During our investigations of the phenomenon of delayed growth stasis, *S. cerevisiae gsh1Δ* cells were found to be able to grow for an additional seven or eight generations after transfer to glutathione-free medium [9]. These investigations were carried out in the YPH499 strain background. YPH499 is a widely used strain of *S. cerevisiae* that is 'congenic' to S288C [29]. Further investigations of this phenomenon, however, revealed that the YPH499 strain background being used lacked any  $\gamma$ -GT activity even when grown under conditions known to increase  $\gamma$ -GT activity in the cell (Table 2).

Since  $\gamma$ -GT is the only enzyme known to be involved in glutathione turnover it was possible that the prolonged growth in the absence of glutathione that we were observing in YPH499 backgrounds was a result of the absence of the enzyme in this background. We therefore switched to BY4742 (another S288C background) and found that this strain had a functional  $\gamma$ -GT. The differences between the two S288C strains in relation to  $\gamma$ -GT were found to be the result of certain polymorphisms, the origin of which has now been investigated in detail (C. Kumar, R. Sharma and A.K. Bachhawat, submitted for publication). All subsequent studies were carried out in the BY4742 background.

Table 2  
 $\gamma$ -GT activity in wild-type and *cis2* $\Delta$  strains and strains overexpressing the *CIS2* gene from the TEF promoter under different growth conditions

Strain	Growth conditions	Specific activity of $\gamma$ -GT (nmol min <sup>-1</sup> mg <sup>-1</sup> protein)
ABC154 (YPH499)	ammonium sulfate	nd <sup>a</sup>
ABC154 (YPH499)	glutamate	nd
ABC591 ( <i>gsh1</i> $\Delta$ in YPH499)	ammonium sulfate	nd
ABC591 ( <i>gsh1</i> $\Delta$ in YPH499)	glutamate	nd
ABC734 (BY4742)	ammonium sulfate	nd
ABC734 (BY4742)	glutamate	20.5 $\pm$ 1.9
ABC1066 ( <i>cis2</i> $\Delta$ in BY4742)	ammonium sulfate	nd
ABC1066 ( <i>cis2</i> $\Delta$ in BY4742)	glutamate	nd
ABC734 (pTEF- <i>CIS2</i> )	ammonium sulfate	148 $\pm$ 10.53

<sup>a</sup>nd: not detectable.

To investigate the role of  $\gamma$ -GT we created disruptions in the *CIS2* gene encoding the  $\gamma$ -GT enzyme in a *gsh1* $\Delta$  strain background, and investigated how the presence or absence of  $\gamma$ -GT affected the total glutathione pools (*gsh1* $\Delta$  cells are deleted for endogenous glutathione biosynthesis and cells were grown in glutamate medium known to increase  $\gamma$ -GT activity). As expected, cells lacking  $\gamma$ -GT (*gsh1* $\Delta$  *cis2* $\Delta$ ) had an almost three-fold higher glutathione content than the *gsh1* $\Delta$  cells containing  $\gamma$ -GT (*gsh1* $\Delta$  *CIS2*) (Table 3). We examined these strains for the delayed growth stasis phenomenon to determine if there was an increased delayed stasis in *cis2* $\Delta$  deletion strains as compared to *CIS2* strains and observed that the strains *gsh1* $\Delta$  *CIS2* and *gsh1* $\Delta$  *cis2* $\Delta$  were identical in the response to glutathione deprivation (data not shown) and showed the same delayed growth stasis behavior seen in the *gsh1* $\Delta$  strains in the YPH499 background. The presence or absence of a functional *CIS2* gene (encoding  $\gamma$ -GT) did not affect either the growth patterns or the delayed growth stasis seen in these strains, and indicated that the  $\gamma$ -GT enzyme did not affect to any significant extent the glutathione pools responsible for cell growth.

### 3.2. Overexpression of $\gamma$ -GT in yeast restores glutathione levels in deleted strains to wild-type levels, but does not lead to enhanced turnover rates

The surprising observations described above prompted us to further investigate the role of  $\gamma$ -GT. We overexpressed  $\gamma$ -GT enzyme by expressing it downstream of a strong constitutive promoter. As seen in Table 3, we ob-

served that pTEF-*CIS2*-containing strains, which had many-fold higher activity of  $\gamma$ -GT enzyme, restored the GSH pools from 23.05  $\pm$  2.47 nM observed in *cis2* $\Delta$  cells to 9.4  $\pm$  0.28 nM, levels seen in *CIS2* cells. However, surprisingly, the presence of an overproduced  $\gamma$ -GT did not display any toxicity, or decreased growth as seen from growth curve experiments (data not shown), which may perhaps be a result of the fact that the cellular glutathione levels are not completely depleted even when the  $\gamma$ -GT levels in the cell are significantly high.

We also attempted to evaluate the glutathione turnover rates in *CIS2* and *cis2* $\Delta$  strains in the *gsh1* $\Delta$  background. Although the glutathione/cell number ratio decreases with time, this results primarily from the increase in cell number. The apparent glutathione half-life determined on the basis of the glutathione content per cell OD was 136  $\pm$  7.35 min<sup>-1</sup> in *CIS2* cells and 132  $\pm$  13.8 min<sup>-1</sup> in *cis2* $\Delta$  cells. However, as there is a concurrent increase in cell number with a generation time of 153  $\pm$  2.82 min in the case of *CIS2* cells and 144  $\pm$  1.41 min in the case of *cis2* $\Delta$  cells, the effective glutathione turnover is exceedingly slow or negligible in both *CIS2* and *cis2* $\Delta$  cells.

### 3.3. Ability of *met15* $\Delta$ strains which are organic sulfur auxotrophs to utilize glutathione as exogenous sulfur source is not affected after deletion of the *CIS2* gene

The evidence with *gsh1* $\Delta$  strains described above clearly indicated that the total glutathione turnover rate was negligible in *gsh1* $\Delta$  strains. We decided to examine the issue of glutathione turnover through an alternative genetic strat-

Table 3  
 Effect of  $\gamma$ -GT on the glutathione content of different yeast strains

Strain	Relevant genotype	GSH levels at time <sup>a</sup> (nmol GSH 10 <sup>-8</sup> cells)		
		0 h	4 h	8 h
ABC1195	( <i>gsh1</i> $\Delta$ )	9.4 $\pm$ 0.28	2.44 $\pm$ 0.17	1.96 $\pm$ 0.28
ABC1196	( <i>gsh1</i> $\Delta$ <i>cis2</i> $\Delta$ )	23.05 $\pm$ 2.47	6.92 $\pm$ 1.86	2.68 $\pm$ 0.17
ABC1196 (pTEF)	( <i>gsh1</i> $\Delta$ <i>cis2</i> $\Delta$ )	29.2 $\pm$ 2.82	9.4 $\pm$ 3.45	4.58 $\pm$ 1.16
ABC1196 (pTEF- <i>CIS2</i> )	( <i>gsh1</i> $\Delta$ <i>cis2</i> $\Delta$ )	9.32 $\pm$ 0.62	3.72 $\pm$ 0.96	2.68 $\pm$ 0.06

<sup>a</sup>The total intracellular glutathione levels were determined in these strains at different time intervals. Cells were grown in minimal medium having glutamate supplemented with glutathione, washed and then resuspended in glutathione-free medium (time 0). All the values here are average values of three independent experiments.

egy in a *met15Δ* strain, BY4741, a strain in the BY4742 background which had a functional  $\gamma$ -GT enzyme (data not shown). Strains defective in *MET15* are organic sulfur auxotrophs as they lack the enzyme for the reduction of sulfate [30]. These strains are able to grow on either methionine, glutathione or cysteine as organic sulfur source but are unable to grow in the absence of organic sulfur. If the presence of  $\gamma$ -GT was indeed mandatory for glutathione turnover and degradation in the cell, we argued that in a *met15Δ cis2Δ* background, these cells would be able to grow in methionine as the sole organic sulfur source, but not with glutathione as the sole sulfur source. We created a *cis2Δ* in a *met15Δ* background and examined the growth of these cells in glutathione and methionine. The experiments were carried out in ammonium sulfate medium as well as in glutamate medium where  $\gamma$ -GT activity is known to be high. The fact that glutathione is actually transported inside through a specific glutathione transporter (Hgt1p) prior to utilization has been demonstrated by us earlier since *met15Δ* is lethal in a *hgt1Δ* background [3]. Both in plates as well as in liquid medium we could not see any differences in growth (and growth rates) for the *met15Δ* cells as compared to the *met15Δ cis2Δ* cells on glutathione (Fig. 1). Furthermore, the growth rates were almost comparable to those on methionine and there was also no significant difference in growth rate between the use of ammonium sulfate or glutamate as the nitrogen source. This clearly demonstrates that there is no essential role of  $\gamma$ -GT in the utilization of GSH as an exogenous source of sulfur.

To further examine if this  $\gamma$ -GT-independent utilization of GSH could also occur in a *gsh1Δ* background (where GSH has an essential growth function), we introduced a deletion in the *GSH1* gene in a *met15Δ cis2Δ* background. We observed that in these *gsh1Δ met15Δ cis2Δ* strains (where glutathione is essential for growth as a redox requirement and as a sulfur source), the utilization of glutathione occurs very efficiently in a manner similar to that of *met15Δ cis2Δ GSH1* strains (data not shown). These results indicate that even in a *gsh1Δ* background efficient turnover and utilization of glutathione can occur.

### 3.4. The *CIS2* gene is regulated by nitrogen- and sulfur-derepressing conditions and is not induced by addition of glutathione

Previous studies (at the enzyme activity level) have indicated reduced  $\gamma$ -GT enzyme activity in cells grown in ammonium [11]. Furthermore, it has also been shown that  $\gamma$ -GT is derepressed in *gsh1Δ* strains which was most probably due to an alteration of the endogenous thiol status [31]. We decided to undertake a more detailed investigation of the regulation of  $\gamma$ -GT enzyme, by examining the transcriptional regulation of *CIS2*, to get some further insights into its true roles within the cell, relative to a putative GSH degradation pathway that might be inde-

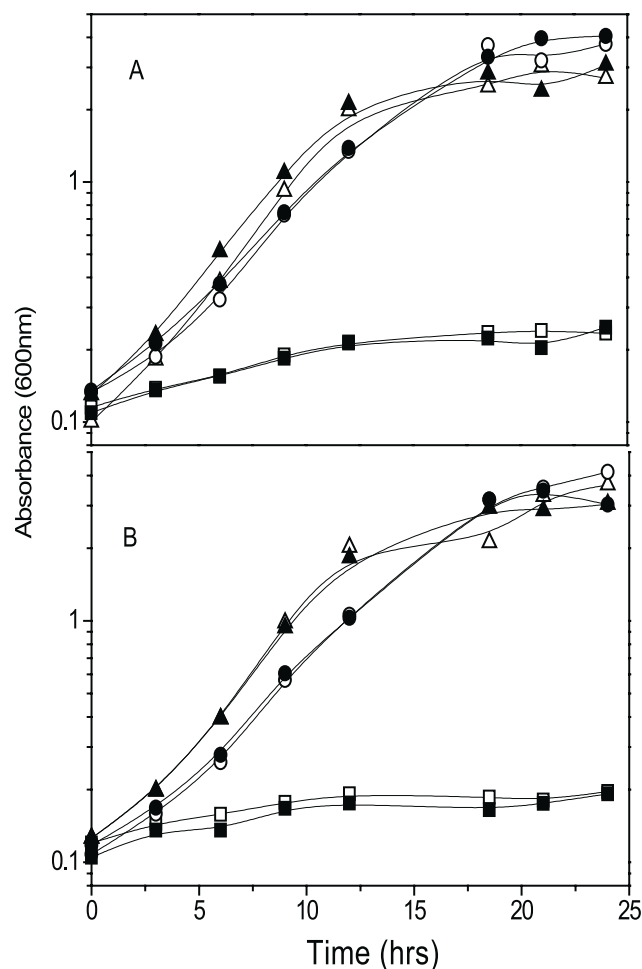


Fig. 1. Effect of glutathione and methionine as sulfur sources on the growth of *met15Δ* and *met15Δ cis2Δ* strains. Strains ABC733 (*met15Δ*) and ABC1083 (*met15Δ cis2Δ*) (Table 1) grown in (A) ammonium sulfate, (B) glutamate. Open shapes, *met15Δ*; filled shapes, *met15Δ cis2Δ*. Squares, no additions; circles, glutathione (250 μM); triangles, methionine (250 μM).

pendent of  $\gamma$ -GT. Studies on the regulation of the *CIS2* gene (using promoter- $\beta$ -gal fusions) indicated that it was primarily under nitrogen regulation and growth in derepressing nitrogen sources (glutamate) led to a very significant fold induction in activity (Table 4). Relatively milder repression was observed with methionine. In the presence of glutathione, we did not find any increase or decrease in  $\gamma$ -GT activity. The fact that *CIS2* is not regulated transcriptionally by glutathione is not surprising considering the fact that other genes involved in glutathione biosynthesis, such as *GSH1*, are also not regulated by GSH levels [32]. Considering that the  $\gamma$ -GT enzyme has earlier been shown to localize to the yeast vacuole [13], the  $\beta$ -gal reporter studies described above suggest that the enzyme has a specialized role for utilizing vacuolar stores of glutathione during nitrogen starvation (and, to a lesser extent, sulfur starvation) conditions [31,33].

The expression of most nitrogen catabolic genes in *S. cerevisiae* is regulated at the level of transcription in re-

Table 4  
Transcriptional regulation of *CIS2* under different induction conditions and strain background

Strain	Relevant genotype	Growth conditions	$\beta$ -Galactosidase activity (U $\beta$ -gal per OD <sub>600</sub> cells)
ABC734 (BY4742)	WT	ammonium sulfate	1.9 $\pm$ 0.2
ABC734 (BY4742)	WT	glutamate	17 $\pm$ 2.3
ABC734 (BY4742)	WT	glutamate+methionine	5.5 $\pm$ 0.7
ABC734 (BY4742)	WT	glutamate+cysteine	11.8 $\pm$ 1.5
ABC734 (BY4742)	WT	glutamate+glutathione	12.2 $\pm$ 2.1
ABC1094	<i>gln3</i> $\Delta$	ammonium sulfate	0.4 $\pm$ 0.1
ABC1094	<i>gln3</i> $\Delta$	glutamate	0.37 $\pm$ .04
ABC1113	<i>ure2</i> $\Delta$	ammonium sulfate	7.2 $\pm$ 3.4
ABC1113	<i>ure2</i> $\Delta$	glutamate	21.4 $\pm$ 9.0

sponse to the quality of the nitrogen source available. This regulation is accomplished through four GATA family transcription factors: two positively acting factors capable of transcriptional activation (Gln3p and Gat1p) and two negatively acting factors capable of down-regulating Gln3p- and/or Gat1p-dependent transcription (Dal80p and Deh1p) [34]. These regulatory proteins bind to motifs in the promoter region having consensus sequence 5'-GATA-3'. In addition to the above four GATA family transcription factors, nitrogen catabolic gene expression is negatively regulated by Ure2p and one or more unidentified proteins which appear to function analogously [35,36]. We found it worth examining to check if  $\gamma$ -GT is also under nitrogen catabolite repression mediated by these factors. When we examined expression in a *gln3* $\Delta$  strain the activity was significantly reduced even in glutamate medium, and in a *ure2* $\Delta$  strain, the activity was constitutively activated even in ammonium sulfate medium, indicating that the genes was under the classical nitrogen catabolite repression.

#### 4. Discussion

The results described in this report clearly demonstrate that the utilization of glutathione as a sulfur source in *met15* $\Delta$  strains is independent of the presence or absence of a functional  $\gamma$ -GT enzyme, and demonstrate for the first time that an alternative pathway for glutathione degradation which is independent of  $\gamma$ -GT exists in these cells. Prior to this report, the only enzyme known to be involved in the degradation of glutathione in all living cells was  $\gamma$ -GT [1].

The observations that the absence of a functional  $\gamma$ -GT enzyme in the cell affected neither the delayed growth stasis upon glutathione depletion, nor the rate of glutathione turnover in cells to any significant extent are quite intriguing especially when one considers that the total pools are increased in a  $\gamma$ -GT-deficient background. These observations point to the possibility that the  $\gamma$ -GT enzyme, which localizes to the vacuole [13], is restricted to perhaps mobilizing and utilization of these stores under specific nutritional deprivation conditions. Thus, while the vacuo-

lar pools of glutathione are mobilized in the vacuole by  $\gamma$ -GT to yield degraded products of glutathione, these degraded products need to be recycled into glutathione to regenerate the cytosolic pools of glutathione (via the glutathione biosynthetic route of Gsh1p and Gsh2p). This is not possible in a *gsh1* $\Delta$  background. This explains why, although the total glutathione pools are lower in *CIS2* cells, the cells still display the phenomenon of delayed growth stasis to the same extent as *cis2* $\Delta$  strains. Furthermore, mobilization by  $\gamma$ -GT itself cannot be a limiting factor for exogenous GSH utilization, since the utilization occurs in the absence of a functional  $\gamma$ -GT.

The importance of  $\gamma$ -GT for primarily generating nitrogen sources for the cell is reflected in the strong transcriptional repression by repressing nitrogen sources and the regulation of the *CIS2* gene by the nitrogen regulatory circuit (and the involvement of Gln3p and Ure2p). In addition the promoter of *CIS2* contains seven putative elements that were possible GATA elements but lacked the *cis* elements seen in most of the sulfur-regulated enzymes.

The strong regulation of  $\gamma$ -GT by nitrogen was in contrast to the alternative degradation pathway that was independent of the nitrogen source in the medium. Both in ammonium sulfate medium and in glutamate medium the rate of growth on glutathione as the sole sulfur source was comparable to that of methionine. The utilization of glutathione as the sulfur source was thus independent of both the  $\gamma$ -GT activity and the nitrogen source of the medium, underlining the presence of a distinct,  $\gamma$ -GT-independent pathway for glutathione degradation in yeast cells. Considering that  $\gamma$ -GT is the only enzyme known to be responsible for glutathione degradation, we examined the possibility that a second  $\gamma$ -GT with limited homology was present in *S. cerevisiae*. This was carried out by PSI-BLAST analysis. However, no other proteins were revealed even after repeated iterations (data not shown), which is also in agreement with the observation that no detectable transpeptidase activity could be seen in *cis2* $\Delta$  strains.

Although these studies are restricted to yeasts, it is quite likely that the proposed pathway might exist in mammalian cells too. Investigating glutathione turnover and utilization in yeasts has been greatly facilitated by the ability

to completely knock out endogenous synthesis of glutathione through deletion mutants thereby allowing one to more accurately determine the intracellular turnover rates of glutathione. In mammalian cells, considering that  $\gamma$ -GT is localized at the plasma membrane, it is more likely that the  $\gamma$ -GT enzyme is involved in the extracellular turnover of glutathione while the intracellular, cytosolic turnover of glutathione might be independent of  $\gamma$ -GT, using the still uncharacterized pathway that we have proposed above. These studies which have thrown a completely new light on glutathione turnover should trigger an intense search for the precise peptidases involved in the turnover of intracellular glutathione, putative enzymes that should play a vital role in the homeostasis of this important metabolite.

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