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The novel zinc cluster regulator Tog1 plays important roles in oleate utilization and oxidative stress response in *Saccharomyces cerevisiae*

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ABSTRACT

Many zinc cluster proteins have been shown to play a role in the transcriptional regulation of glucose-repressible genes during glucose exhaustion and diauxic shift. Here, we studied an additional member of this family called Yer184c (herein called Tog1) for transcriptional regulator of oleate. Our results showed that a Δ*tog1* strain displays impaired growth with several non-fermentable carbons. Tog1 is also implicated in oxidative stress tolerance. Importantly, during the glucose–oleate shift, combined results from quantitative real time-PCR and chromatin immunoprecipitation (ChIP) experiments showed that Tog1 acts as a direct activator of oleate utilizing genes, encoded key enzymes in β-Oxidation and NADPH regeneration (*POX1*, *FOX2*, *POT1* and *IDP2*), the glyoxylate shunt (*MLS1* and *ICL1*), and gluconeogenesis (*PCK1* and *FBP1*). A transmission electron microscopy (TEM) analysis of the Δ*tog*1 strain assayed with oleate also revealed a substantial decrease in peroxisome abundance that is vital for fatty acid oxidation. Overall, our results clearly demonstrated that Tog1 is a newly characterized zinc cluster regulator that functions in the complex network of non-fermentable carbon metabolism in *Saccharomyces cerevisiae*.

1. Introduction

Glucose is the preferred carbon source for *Saccharomyces cerevisiae* while other carbon sources such as glycerol, ethanol, acetate, lactate or fatty acids can alternatively be used [1–3]. In response to glucose depletion, a major reprogramming of gene expression is elicited via the master Snf1 kinase (a member of adenosine monophosphate (AMP)-activated serine/threonine protein kinases) [4,5]. Activation of the Snf1 kinase allows phosphorylation of some transcription factors, resulting in the deactivation of the repressor Mig1 (a member of the C_2H_2 family) as well as activation of the Zn_2Cys_6 activators Cat8 and Rds2 [2,4]. The release of phosphorylated Mig1 from promoters of various genes, including *CAT8* allows for expression of gluconeogenic and glyoxylate cycle genes as well as the *SIP4* gene that encodes a transcriptional regulator of this pathway [4,6–10]. Cat8, Sip4 and Rds2 belong to the major subfamily of zinc cluster transcriptional regulators with a well-conserved signature motif $CysX_2CysX_6CysX_{5\text{-}12}CysX_2CysX_{6\text{-}8}Cys$ in the DNA-binding domain [11].

Regarding the transcriptional regulation of non-fermentative metabolism, Cat8 and Sip4 are involved in the control of gluconeogenic and glyoxylate shunt gene expression during glucose exhaustion [8,12]. Rds2 is also found to directly control expression of gluconeogenic genes (e.g. *PCK1* and *FBP1*), some TCA cycle genes and the important regulatory gene for respiration *HAP4* during the ethanol shift [13]. Moreover, Rds2 binds to promoters of genes involved in gluconeogenesis, mitochondrial metabolism and stress adaptation during the glycerol shift [14]. In addition, the zinc cluster proteins Oaf1 and Pip2 are paired regulators that play key roles in fatty acid β-Oxidation and peroxisomal biogenesis [2]. These factors can heterodimerize and mediate gene expression through direct binding to oleate response elements (OREs) found in the promoters of β-Oxidation genes [15]. In yeast, peroxisome is the sole site for β-Oxidation and the glyoxylate cycle [16]. Strains carrying deletion of *OAF1* or *PIP2* are unable to grow on oleate as a sole carbon source. The C_2H_2 zinc finger regulator Adr1 is also shown to regulate expression of over 30 glucose-repressible genes involved in ethanol, glycerol and fatty acid utilization, as well as peroxisomal protein genes (e.g., *ADH2*, *ALD4*, *GUT1*/2, *PEX* and *FOX* genes) [8].

Here, we show that cells lacking the zinc cluster protein-encoding gene *YER184C* (*TOG1*) display defective growth on several non-fermentable carbon sources, including fatty acids. Using the Δ*tog1* strain, we perform qRT-PCR and ChIP analyses with oleate as a sole carbon source and find that Tog1 indeed directly regulates the expression of key genes, involved in oleate utilization. Deletion

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of *TOG1* also results in decreased expression of genes involved in oxidative stress response, a common event that occurs during the non-fermentative mode of growth.

2. Materials and methods

2.1. Yeast strains and media

The wild-type *S. cerevisiae* strain used for phenotypic, TEM and ChIP analyses was FY73 (MAT *his3*-Δ200; *ura3-52*) [17]. The Δ*tog1* (MAT *his3*-Δ200; *ura3-52*, tog1::*HIS3*), carrying a deletion of zinc cluster motif located in the DNA-binding domain by the PCR method as previously described [18,19]. Yeast wild-type BY4742 (MAT *his3Δ1*; *leu2Δ0*; lys2Δ*0*; *ura3Δ0*) and the Δ*tog1* (MAT *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*, yer184c::KANR) strains were used for gene expression analysis. The *TOG1* ORF was N-terminally tagged at its natural chromosomal location with a triple Myc-epitope. The tagging cassette was obtained by PCR using the oligonucleotides PET*TOG1*-F: 5′-AAGCGCATTAGGTTAACGACATTATTGTTG TTTAAATTTTAAGCTTTTTA-3′ and PET*TOG1*-R: 5′-TTAATCTTTTTCC TATGGCATCTATCACAGGCCTTGGTTACCCTA GAT TT-3′ with the plasmid p3XMyc as a template. The yeast extract-peptone (YP) plates contained 1% yeast extract, 2% peptone, 2% agar, 0.5% potassium phosphate (buffered at pH 6.0) and 0.5% Tween 80 (only for fatty acids), supplemented with different carbon sources either 2% glucose, 3% ethanol, 3% acetate, 3% lactate, 3% glycerol, 0.125% palmitic acid, 0.125% oleic acid (oleate), 0.125% linoleic acid or 0.005% carnitine.

2.2. Phenotypic analysis

Wild-type FY73 and the Δ*tog1* deletion strains were grown overnight in YP-dextrose (YPD) medium. Cells were then spun, washed with water, and serially diluted prior to be spotted on appropriate plates containing different carbon. For sensitivity to oxidative stress, cells were spotted on YPD plates, supplemented with 3 mM H_2O_2 or 0.4 mM diamide (1,1′-azobis [*N*,*N*-dimethylformamide]). For menadione treatment, mid-log phase cells were exposed to 0.6 mM menadione (solubilized in dimethyl sulfoxide) for 1 h. at 30 °C and then spotted onto YPD plates. The plates were then incubated at 30 °C for 3 days.

2.3. Gene induction condition

For gene induction, cells were grown overnight in YPD medium containing 2% glucose overnight, diluted to OD_{600} of 0.1 and regrown to mid-log phase (OD_{600} of 0.8–1.0). Cells were then spun, washed twice with water, and transferred to fresh YP medium containing either 0.125% oleic acid or 2% glucose (control) and grown for an additional 3 h.

2.4. Quantitative RT-PCR analysis (qRT-PCR)

The wild-type and Δ*tog1* strains were grown as described above. Total RNA was isolated by the hot acid phenol method and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was done by using a Super Script™ III first-strand synthesis kit (Life Technologies, Carlsbad CA, USA). qRT-PCR was performed using a Mx3005P QPCR system (Agilent Technologies, Santa Clara CA, USA) with MxPro QPCR software for analysis. The reaction mixture contained Brilliant II SYBR Green QPCR Mix (Kapabiosystem). DNA sequences of the oligonucleotides used for qRT-PCR analysis are given in Table 1. The relative quantification of each transcript was calculated by the $2^{-\Delta\Delta C_t}$ method [20] using the *ACT1* (actin) gene as normalizer.

2.5. ChIP analysis

ChIP assays were performed as described by Larochelle et al. with some minor modifications [21]. Cells from the wild-type untagged and Myc-Tog1 strains were grown overnight in YPD medium, inoculated and regrown until reaching the approximated OD_{600} of 0.7. Then, cells were washed twice in water, transferred to YP media containing either fresh 2% glucose or 0.125% oleic acid as a sole carbon source, and regrown for addition of 3 h. Sequences of oligonucleotides used are given in Table 1.

2.6. TEM analysis

The peroxisomal morphology of the wild-type and the Δ*tog1* cells during oleate induction were examined using the JEM-1230 transmission electron microscopy (Tokyo, Japan). Yeast cells were prepared as done under gene induction. Cell pellets were prepared and examined as previously described [22], by at least two independent experiments. Average numbers of peroxisome were obtained from examination of 100 yeast cells grown in glucose (control) or oleate. Peroxisome size was averaged from a total of 10 yeast cells.

3. Results

3.1. Deletion of the TOG1 gene results in impaired growth on non-fermentable carbon sources and sensitizes cells to oxidative stress

Akache and co-workers previously showed that a strain with a deletion in the *YER184C* (*TOG1*) gene displays impaired growth on glycerol and lactate [19]. Here, we further tested the ability of Δ*tog1* strain to grow on additional carbon sources. Interestingly, our phenotypic analysis revealed that the Δ*tog1* strain grows poorly when ethanol, glycerol, acetate, carnitine or fatty acids (namely oleic, linoleic and palmitic acids) were used as the sole carbon source (Fig. 1), suggesting a role for Tog1 in non-fermentable carbon utilization. Notably, aerobic metabolism during non-fermentative growth also produces oxidative stress within the cells, resulting in reduced growth rate and cell viability. Thus, the effect of *TOG1* deletion on cellular response to oxidative stress was also tested with some oxidative agents such as H_2O_2, diamide and menadione. Our spot test showed an increased sensitivity of the Δ*tog1* strain to these agents (Fig. 1), suggesting an important function of Tog1 during respiratory mode of growth and in cellular defense against oxidative stress.

3.2. Reduced expression of oleate utilizing and oxidative stress genes in the Δtog1 strain

The inability of the Δ*tog1* strain to grow on non-fermentable carbons (Fig. 1) suggested for defective activation of genes related to poor carbon source utilization and oxidative stress. To address this possibility, the expression of some genes, encoding key enzymes and transporters of these pathways was examined by qRT-PCR analysis during a glucose-oleate shif. In wild-type cells, it was found that the expression of glyoxylate cycle and gluconeogenic genes was greatly induced in the absence of glucose as expected (data not shown, [5,23]). Importantly, deletion of *TOG1* resulted in decreased expression of oleate utilizing genes in the β-Oxidation, including *POX1*, *FOX2* and *POT1* genes by at least 2-folds (Fig. 2). These genes encode a peroxisomal fatty acyl-coenzyme A oxidase (Pox1), a multifunctional enzyme with 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities (Fox2 or Pox2) and a 3-ketoacyl-CoA thiolase (Pot1 or Fox3), required for conversion of oleic acid to acyl-CoA and acetyl-CoA.

Table 1
Oligonucleotides used for gene expression analysis via qRT-PCR and standard chromatin immunoprecipitation (ChIP).

Oligonucleotide	DNA sequence (5′–3′)
Gene expression analysis (qRT-PCR)	
ACT1	ATTATATGTTTAGAGGTTGCTTTGG and CAATTCGTTGTAGAAGGTATGATGCC
POX1	GCGAAGATGGGTCGTGACGG and GCGAATTGCTGACGACCAAC
FOX2	GGAAGAAGGGTTATTGGCCA and CTTTGCTTGTGCATCCAAGTCC
POT1	TCAATGTTGGAGCCGGTGCT and GCAGCTCTTCAGAGGAGATC
IDP2	AACATGGTGGGGTTGCCATG and CTGATTCTACGTCACCGTCG
MLS1	AGGTCACCCCAGAATTAACC and CGTGTACATTCACAATTTGC
ICL1	TTCCCTGACCAATGG and TCCCATCCTTGGCAAAGTCA
PCK1	GAAGTCACCACCATCAGACG and ATGCATCGACAATATAAATG
FBP1	GAAGAATGAATGCCAACTC and TTCATCACCTAGAACGTCCT
Chromatin immunoprecipitation (ChIP) analysis	
ACT1	GTATGTTCTAGCGCTTGCACCATC and TACCGACGATAGATGGGAAGACAG
GND1	AGAGAGACCTAAACGTAAGAG and AGCTCAGGAACAATACTGCAG
POX1	GCGACGAAAAATGCGAGATC and CTTTAAGGCCTGTTCAAGT
FOX2	GCTTCCCTTCTGATTCTCCC and GAGTAAAGCCAGAAACG
POT1	GTGTCACGATCATCATCACT and CACTCTGTACTCAGAGCCAC
IDP2	ATGCGTCACTTACGACGGCA and TACTCCACCCCCACTATTCC
MLS1	CGTGCTTAGTGATGTCTCAA and AATCGTCGTAAGGCTGACAC
ICL1	GGTTTTGCTACTCGTCATCC and GGACTTTGGACTGACTTATGC
PCK1	TATCCCACACGATCCACCGG and AACGTACCATTGTCCAACCA
FBP1	ACGCTCTACCAACTGAGCTA and TGATATGTGGGAATACCAGG

Likewise, *CTA1* and *IDP2* expression was reduced by an approximately 2–3-folds upon the removal of *TOG1* (Fig. 2). *CTA1* encodes an enzyme involved in the breakdown of H_2O_2, generated during the first and the rate limiting Pox1-dependent step of fatty acid oxidation. *IDP2* encodes a cytosolic NADP-specific isocitrate dehydrogenase. Both gene products play a crucial role in provision of cellular adaptation to oxidative stress, generated during the fatty acid breakdown [24]. Indeed, the expression of many antioxidant-defensive genes is repressed by glucose and induced during aerobic respiration [25]. The failure of the Δ*tog1* strain to fully transcribe the above genes provides an explanation for its inability to grow on oleate and in the presence of oxidative agents.

Regarding the glyoxylate pathway, expression of *MLS1* and *ICL1* genes was reduced by approximately 2–3-folds upon the deletion of *TOG1* (Fig. 2). Mls1 is a peroxisomal malate synthase while Icl1 is a cytosolic isocitrate lyase that catalyzes the formation of succinate and glyoxylate. Activation of glyoxylate genes has been shown to enhance the biosynthesis of pre-gluconeogenic carbons (e.g., citrate and malate) and increase the carbon flux from peroxisomes to mitochondria for generation of ATPs. Alternate to glyoxylate cycle, the acetyl-CoA can also combine with peroxisomal carnitine to yield acetyl-carnitine via specific acrylcarnitine transferase system, involving *YAT2* [26]. In addition, the exogenous carnitine can also be taken up into the cells via a plasma membrane carnitine transporter Agp2 [27]. The expression of *YAT2* was modestly affected by deletion of *TOG1* while *AGP2* expression was reduced by approximately 2-folds (Fig. 2). Importantly, Tog1 was also responsible for the up-regulated expression of genes that encode the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (Pck1) and fructose-1,6-bisphosphatase (Fbp1) of gluconeogenic pathway, required for synthesis of important metabolites and cellular components. Approximately 2-folds decrease in *PCK1* and *FBP1* expression was depended on Tog1 (Fig. 2).

3.3. Activator Tog1 directly binds to promoters of genes involved in β-Oxidation, NADPH regeneration, gluconeogenesis and glyoxylate cycle

To show that Tog1 is a direct activator of some genes required for utilization of oleate, a standard ChIP assay of Tog1 was performed. For this purpose, Tog1 is N-terminally tagged with a triple Myc-epitope prior to be tested for its binding ability in oleate. The strongest binding of Tog1 was observed for the glyoxylate cycle gene *MLS1* with the binding enrichment of over 30-folds in comparison with the untagged protein (Fig. 3). Tog1 was bound to *MLS1* promoter at location between −277 and −670 bp upstream of the start codon ATG (Fig. 3). However, almost no Tog1 binding enrichment was observed for the negative controls *ACT1* or *GND1* regions (Fig. 3). Given the interaction of Tog1 with the promoter of *MLS1* gene, we further checked for its binding on promoters of other oleate utilizing genes. Similarly, Tog1 was also bound to *ICL1* promoter at location between −189 and −450 bp and to *IDP2* promoter within the −193 to −420 bp region from the ATG codon (Fig. 3). For gluconeogenic genes, binding of Tog1 was identified at the promoter regions between −320 to −600 bp for *PCK1* and −340 to −610 for *FBP1*, upstream of the ATG (Fig. 3).

Interestingly, Tog1 was bound in the vicinity regions of the cis-acting DNA elements called carbon source responsive elements (CSREs), required for induction of many glucose-repressible genes [28–30]. In response to oleate induction, Tog1 was also found to localize at the promoters of β-Oxidation genes, including *POX1* (between −112 and −469 bp), *FOX2* (between −102 and −430) and *POT1* (−23 to −346), relative to the ATG (Fig. 3). In summary, the combined results of qRT-PCR and ChIP experiments clearly demonstrate that Tog1 directly activates the expression of *POX1*, *POT1*, *FOX2*, *IDP2*, *PCK1*, *FBP1*, *MLS1* and *ICL1* genes during the oleate shift (Figs. 2 and 3 and Supplementary Table S1).

3.4. Morphological characterization of peroxisomes in the Δtog1 strain during the glucose-oleate shift

Given that the peroxisomes are the sole sites for fatty acid oxidation in yeast, the peroxisomal content was then examined via TEM analysis. For both the wild-type and the Δ*tog1* cells, the peroxisomes were rarely detected when cells were grown in glucose (Fig. 4(A) and (B)). However, for the oleic acid culture, the peroxisome numbers of the wild-type cells were greatly increased to the average of four to five peroxisomes/cell and found near the proximity of the plasma membranes with approximate diameters of 0.3 μm (Fig. 4(C)). The high abundance of peroxisomes of the wild-type cells during oleate induction (Fig. 4(C)) agreed well with other previous reports [31,32]. In contrast, strikingly for the Δ*tog1* cells grown in oleic acid, peroxisome content was quite low as observed for the uninduced cells grown under glucose condition. They displayed an average of less than one peroxisome/cell with approximate diameters of 0.2 μm (Fig. 4(D)). Thus, our TEM data

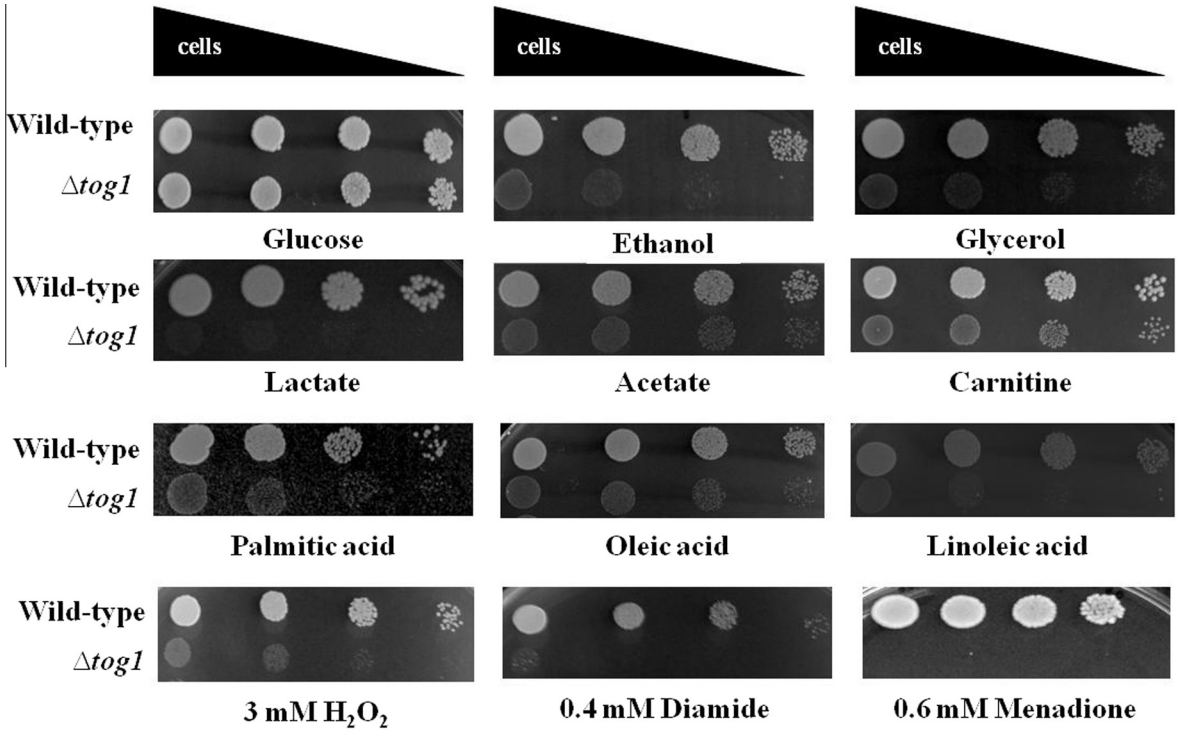

Fig. 1. Defective phenotypes of the Δ*tog1* strain on plates containing different non-fermentable carbon sources. Wild-type and Δ*tog1* cells were spotted on YP plates, containing different carbon sources or YPD plates supplemented with 3 mM H_2O_2, 0.4 mM diamide or pretreatment with 0.6 mM menadione as shown.

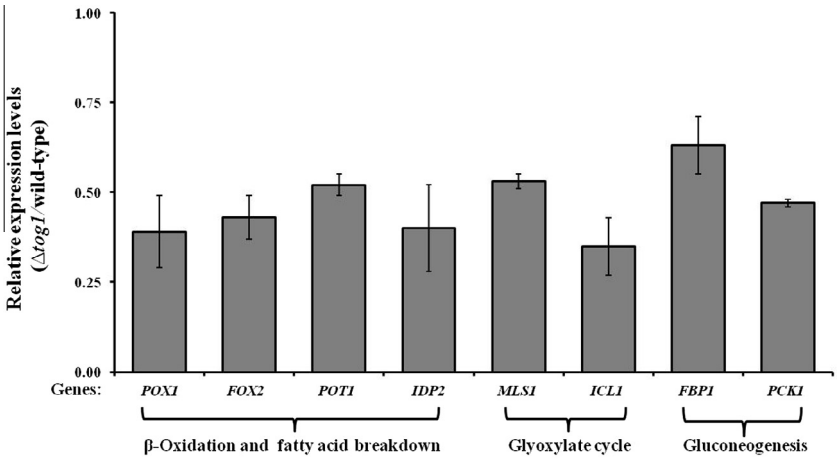

Fig. 2. Expression of Tog1 target genes in *S. cerevisiae* during the glucose–oleate shift. Analysis of gene expression was performed by qRT-PCR from three independent experiments done in triplicates; the comparative C_t method was used for quantification. Values are expressed as relative expression for each tested gene in the deletion Δ*tog1* strain versus the wild-type strain during the glucose–oleate shift, after normalization with the endogenous reference *ACT1* gene.

showed that deletion of *TOG1* results in reduced peroxisomal content when oleate was used as a sole carbon source (Fig. 4 and Table 2).

4. Discussion

Glucose catabolite repression is a widely studied phenomenon in both prokaryotes and eukaryotes [33]. In this study, Tog1 is shown to act as a direct transcriptional activator of genes involved in fatty acid utilization. Our results of qRT-PCR and ChIP analyses showed that deletion of *TOG1* reduces the expression of genes in at least 3 functional categories: (1) the peroxisomal β-Oxidation pathway and fatty acid breakdown; (2) the glyoxylate shunt; (3) gluconeogenesis as shown in (Supplementary Fig. S1) for a model of Tog1 roles and regulation. Our data strongly indicated that Tog1 directly activates functionally-related genes in a variety of pathways whose expression is induced and co-expressed during the glucose-oleate shift.

β-Oxidation is an essential pathway for fatty acid utilization and other processes such as phospholipid biosynthesis [3]. Regulation of β-Oxidation has previously been shown to be under the control Oaf1 and Pip2 as well as Adr1, suggesting a common regulatory role in fatty acid utilization. Here, we showed by ChIP that Tog1 binds within regions, containing the UAS and OREs important for oleate induction of *POX1*, *FOX2* and *POT1* genes which are shared targets of Oaf1, Pip2 and Adr1 regulators. In addition, Tog1 also have common targets with the gluconeogenic regulators Cat8, Sip4 and Rds2 (Supplementary Table S1). Functional redundancy

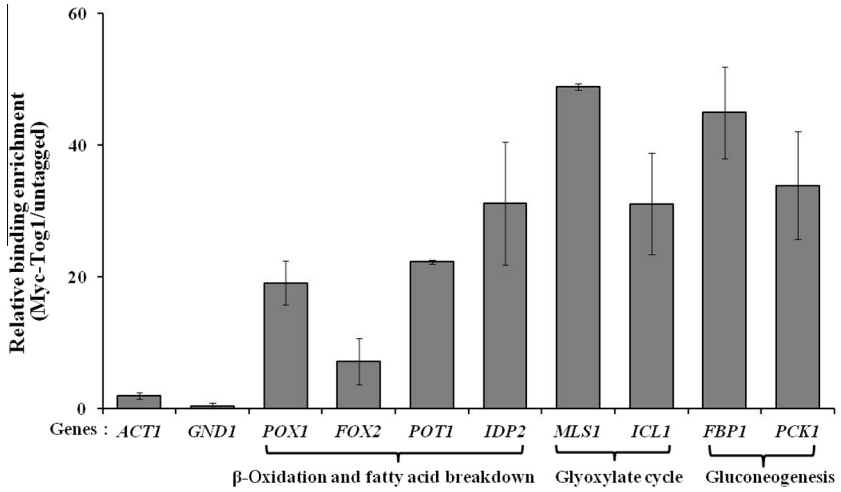

Fig. 3. Tog1 binds to promoters of some β-Oxidation, gluconeogenic and glyoxylate cycle genes during the oleate induction. Standard ChIP assays were performed with strains expressing untagged or Myc-tagged Yer184c, grown in rich medium containing oleate as a sole carbon source.

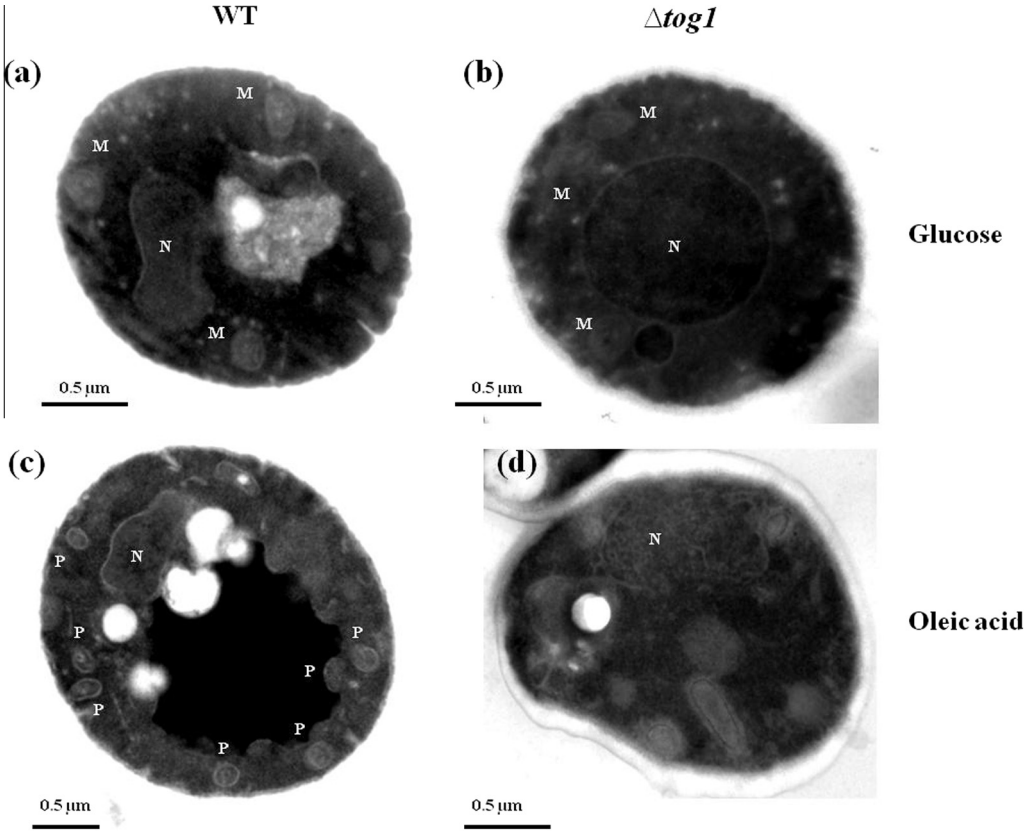

Fig. 4. Reduced peroxisomal content in the oleate-induced Δ*tog1* cells. The ultrastructure of peroxisomes of the wild-type (WT) cell (A) in glucose or (C) in oleic acid in comparison with that of the Δ*tog1* cell (B) in glucose or (D) in oleic acid. Cells were grown in YP-glucose and shifted to fresh glucose (control) or oleic acid for an additional 3 h. Peroxisomes, nuclei and mitochondria are indicated by the P, N and M characters, respectively. Bar = 0.5 μm.

and compensation among these regulators may explain modest reduction of gene expression in the single Δ*tog1* deletion strain (Supplementary Table S1). In fact, this phenomenon is quite common in yeasts. Many zinc cluster transcriptional regulators, for examples, the gluconeogenic regulators Cat8, Sip4 and Rds2 or the pleotropic drug resistance regulators Pdr1, Pdr3 and Stb5 have been shown to have overlapping target genes [2].

Table 2
Numbers and size of peroxisomes of wild-type and Δ*tog1* cells during the glucose–oleate shift or glucose (control) as examined via TEM analysis. "NV": not visible.

Strain	Peroxisome number		Peroxisome size (μm)	
	Glucose	Oleic acid	Glucose	Oleic acid
Wild-type	0.4 ± 0.5	4.7 ± 1.7	0.2 ± 0.1	0.3 ± 0.1
Δ*tog1*	NV	0.7 ± 0.8	NV	0.2 ± 0.1

Regarding peroxisomal proliferation, a significant reduction in peroxisome content, albeit little change in size, was observed with the Δ*tog1* strain (Fig. 4 and Table 2). Reduced peroxisome content and the appearance of "protoperoxisome" with vesicle or tubular irregular shapes have been reported for some yeast mutants, whose the corresponding gene products are required for proper peroxisomal biogenesis such as the Δ*pip2* and Δ*adr1* strains [34], further emphasizing common phenotypes and roles among Tog1 and other regulators in oleate utilization. Existence of multi-layers of transcription control over these metabolic genes represents a classic example of complex regulation for fine-tuning gene expression. Further characterization of Tog1 may reveal novel co-regulatory complexes and *cis*-elements, associated with oleate utilization.

Adaptation to some endogenous ROS-generated via processes of nutrient oxidation, oxidative phosphorylation and respiration is another important point to address as it is a central key to cell survival during non-fermentative metabolism. In *S. cerevisiae*, a handful of regulators have been identified, including regulators Yap1 and Yap2 of anti-oxidant genes [35], Msn2 and Msn4 of multi-stress responses [25] and the zinc cluster Stb5 of pentose phosphate pathway [21]. Interestingly, for the first time, Tog1 is shown to be involved in stress adaptation against oxidative agents (Fig. 1). The sensitivity of the Δ*tog1* strain to harmful oxidative agents may partly due to reduced expression of *CTA1* and *IDP2* genes whose products function in the catalytic inactivation of free ROS species (Fig. 2 and Supplementary Table S1). Importantly, the unviability of Δ*idp2* cells has been shown to be correlated with increased levels of intracellular ROS during metabolism of non-fermentable compounds or in the presence of exogenous H_2O_2 [36]. Thus, reduced expression of *IDP2* in the Δ*tog1* strain supports for the observed growth defects on oleate and increased sensitivity to oxidative stress, generated in the first step of β-Oxidation or by some exogenous sources (Figs. 1 and 2). Given another role of peroxisome in maintaining cellular ROS production and scavenging, the second explanation for increased oxidative stress sensitivity of the Δ*tog1* strain may be due to a significant reduction of its peroxisome content (Fig. 4 and Table 2). In conclusion, both defects in gene regulation and reduced peroxisomal content are accounted for the observed enhanced oxidative stress sensitivity of the Δ*tog1* strain.

In summary, we have identified a novel function for the less-known zinc cluster Tog1 and implicated it in the transcriptional regulatory network of non-fermentable carbon source utilization, particularly in oleate utilization in *S. cerevisiae*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.128.

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