ZAP1-mediated modulation of triacylglycerol levels in yeast by transcriptional control of mitochondrial fatty acid biosynthesis

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Summary

The transcriptional activator Zap1p maintains zinc homeostasis in Saccharomyces cerevisiae. In this study, we examined the role of Zap1p in triacylglycerol (TAG) metabolism. The expression of ETR1 is reduced in zap1Δ. The altered expression of ETR1 results in reduced mitochondrial fatty acid biosynthesis and reduction in lipoic acid content in zap1Δ. The transcription factor Zap1 positively regulates ETR1 expression. Deletion of ETR1 also causes the accumulation of TAG, and the introduction of ETR1 in zap1Δ strain rescues the TAG level. These results demonstrated that the compromised mitochondrial fatty acid biosynthesis causes a reduction in lipoic acid and loss of mitochondrial function in zap1Δ. Functional mitochondria are required for the ATP production and protect in mitochondria slow down the process which may channeled carbon towards lipid biosynthesis and stored in the form of TAG.

Introduction

Zinc is a principal trace element that is present in all organisms because of its catalytic and structural roles for hundreds of proteins (Vallee and Falchuk, 1993). Recent studies have suggested that zinc may also serve as a secondary messenger in various signal transduction pathways (Hirano, 2008). Zinc serves as a cofactor for alcohol dehydrogenase, carbonic anhydrase, proteases and superoxide dismutase (Vallee and Falchuk, 1993) and a structural constituent of proteins such as chaperones, lipid-binding proteins and transcription factors (Schwabe and Klug, 1994; Ellis et al., 2004). Transcription factors belong to the largest class of zinc-dependent structural proteins and contain zinc-dependent DNA-binding motifs, such as zinc fingers and zinc clusters, which are essential for their biological functions (Schwabe and Klug, 1994). Almost 5% of the total yeast proteins require zinc to perform its function in Saccharomyces cerevisiae (Schjerling and Holmberg, 1996; Bohm et al., 1997). Although zinc is an essential nutrient for biological systems, an excess of zinc is toxic to cells. The precise reason for the metal toxicity is not clear. Depending on the extracellular metal availability, the cells must maintain zinc homeostasis to meet cellular metal demand. There are several mechanisms cells may use to maintain the optimum cellular zinc level; these include binding of the metal by metallothioneins (Hamre, 1986), storage in intracellular compartments and transport of the metal either in or out of the cell (Palmiter and Findley, 1995).

There are 17 zinc-regulated genes (ZRGs) are present in S. cerevisiae. Of the 17 ZRGs 1, 5, 7, 8, 10, 11, 12, 13, 15, 16 and 17 are induced under low-zinc conditions, whereas ZRG6 and ZRG14 are induced under conditions of excess zinc (Yuan, 2000). Among the 17 members, ZRG16 is an essential gene and the deletion leads to lethality. There are different mechanisms available to regulate zinc levels; however, zinc homeostasis is primarily regulated at the transcriptional level in yeast by the zinc-responsive transcription factor protein ZAP1 (also known as ZRG10). Zap1 directly controls the expression of number of genes in response to zinc levels (Zhao and Eide, 1997). Zap1p is 880 amino acids long and contains a DNA-binding domain consisting of five zinc fingers at the carboxyl terminus (Bird et al., 1997). Via an activation domain, Zap1p contains two independent activation domains, which are designated as AD1 and AD2 and mediate the transcription of target genes
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of transcription factor ZAP1

Among the identified zinc-regulated genes, the deletion genes (phosphate conditions (Yadav et al., 2004). In addition, the Kennedy pathway enzymes, which include ethanolamine kinase (EK11) and choline kinase (CK11), show increased activities under zinc-limited conditions (Soto and Carman, 2008; Kersting and Carman, 2006). In yeast, phospholipid metabolism and its regulation at the transcriptional level has been well studied. A number of genes involved in phospholipid metabolism are regulated by three main transcription factors: INO2, INO4 and ZAP1 (Ballis et al., 1992; Greenberg and Lopes, 1996; Henry and Patton-Vogt, 1998; Iwanyshyn et al., 2004).

In contrast, the regulation of neutral lipid metabolism at the transcriptional level in yeast is not well studied. However, recently it has been showed that TAG metabolism is regulated by Pho4 transcription factor under low phosphate conditions (Yadav et al., 2015). The present study aims to investigate the effect of zinc regulated genes (ZRG) deletion on neutral lipids in S. cerevisiae. Among the identified zinc-regulated genes, the deletion of transcription factor ZAP1 showed a significant increase in triacylglycerol (TAG). We found that the expression of ETR1, an enzyme involved in the mitochondrial fatty acid synthesis (mFAS) pathway, is downregulated in zap1Δ strain, and the deletion of ETR1 results in a significant increase in neutral lipids. The ETR1 catalyzes the NADPH dependent reduction of 2-enoyl thioesters. The mFAS pathway is a conserved in eukaryotes and produces octanoic acid, which is a precursor for the cofactor lipoic acid (LA). LA is indispensable for the function of several mitochondrial enzymes (Schonauer et al., 2009; Hiltunen et al., 2010). Previous studies showed that deletion or changes in the expression of any gene involved in mFAS resulted in rudimentary mitochondria that were deficient in respiratory competence (Hiltunen et al., 2009). MIFAS pathway genes in zap1Δ showed compromised expression level. The ETR1, like other Zap1p-regulated target genes, has a ZRE sequence in its promoter that moderately responds to changes in zinc conditions. The regulation of the mFAS pathway genes at the transcriptional level is not well understood. In this study, we show that ZAP1 affects mitochondrial function through the transcriptional regulation of ETR1. A reduction in the expression level of ETR1 in the absence of Zap1p causes a decrease in respiratory competence due to alterations in mitochondrial morphology and functions, which in turn leads to an accumulation of TAG in the zap1Δ strain.

Results

Growth of wild-type yeast on low-zinc medium

To understand the effect of zinc-regulated genes deletion on TAG metabolism in yeast, we first observed the effect of zinc concentration on the growth of wild-type (BY4741) cells. Cells were grown in a synthetic medium supplemented with varying concentrations of zinc. We observed that cells grown in media without zinc and in 50 nM zinc showed slow growth compared with cells grown in synthetic medium containing 2 μM zinc (Supporting Information Fig. S1A and B). Further studies were carried out with 50 nM (low) and 2 μM zinc (high). To confirm, the synthetic media had the expected low-zinc concentration, wild-type cells were grown in synthetic media containing low and high zinc and were stained with intracellular zinc-stain zinquin ethyl ester, which binds to zinc at nanomolar concentrations and fluoresces. A significant reduction in fluorescence was observed in the cells grown in low-zinc medium (Supporting Information Fig. S1C). The level of zinc in wild-type cells grown in low and high zinc synthetic media by atomic absorption spectroscopy was estimated and found that conditions were maintained (Supporting Information Fig. S1D). The expression levels of zinc-regulated genes were studied in addition and found that several ZRG genes were highly expressed under low-zinc conditions (Supporting Information Fig. S1E). Among the ZRGs, ZRG10/ZAP1 showed the highest expression. These results indicated that the level of zinc was maintained.

Deletion of ZAP1 caused an increase in triacylglycerol

To determine the effect of ZRG mutants on lipid metabolism, wild-type (BY4741) and deletion strains were grown on low-zinc and zinc-supplemented synthetic media plates. We found that wild-type and deletion strains grew well on zinc-supplemented media.
However, the zap1Δ mutant was unable to grow on low-zinc medium, whereas other deletion strains showed slow growth at the early stage but later resumed the growth (Supporting Information Fig. S2A and B). Wild-type and deletion strains were grown in complete synthetic medium in the presence of \([^{14}\text{C}]\text{acetate}\) until reaching stationary phase. We found that zap1Δ and zrg15Δ showed significant increase in TAG (Fig. 1A).

We chose to focus our study on the zap1Δ mutant. When wild-type and zap1Δ cells were grown in a complete synthetic medium in the presence of \([^{14}\text{C}]\text{acetate}\) and the levels of neutral lipids were grown in synthetic media with 2% galactose in the presence of \([^{14}\text{C}]\text{acetate}\) and the levels of neutral lipids were grown in complete synthetic media in the presence of \([^{14}\text{C}]\text{acetate}\) until reaching stationary phase. We found that zap1Δ and zrg15Δ showed significant increase in TAG (Fig. 1A). We chose to focus our study on the zap1Δ mutant. When wild-type and zap1Δ cells were grown in a complete synthetic medium in the presence of \([^{14}\text{C}]\text{acetate}\) and the levels of neutral lipids were grown in complete synthetic media in the presence of \([^{14}\text{C}]\text{acetate}\) until reaching stationary phase. We found that zap1Δ and zrg15Δ showed significant increase in TAG (Fig. 1A).

To validate that the increase in TAG observed in zap1Δ was due to ZAP1, ZAP1 was cloned into the galactose-inducible expression vector pYES2-NT/C. The wild-type and zap1Δ strains were transformed with pYES2-NT/C and pYES2-NT/C-ZAP1. Transformed cells were grown in synthetic media with 2% galactose in the presence of \([^{14}\text{C}]\text{acetate}\) and the levels of neutral lipids were grown in synthetic media were stained with BODIPY493/503 and observed under confocal microscope. An increase in the size and number of lipid droplets were observed in the zap1Δ strain (Fig. 1D).

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To understand the increase in TAG in zap1Δ strain, we searched for lipid metabolic genes with predicted Zap1p binding sites in their promoters and performed qRT-PCR on these genes. Among the genes, the expression of ETR1 and hydrolytic genes were significantly reduced (Fig. 2A). To confirm that the change in expression of ETR1 in the zap1Δ strain was specifically due to Zap1p, we checked the mRNA level of ETR1 in wild-type and zap1Δ cells grown under low zinc conditions and compared these levels to the control. The expression level of ZAP1 was used as a control. We found that expression of ETR1 was 1.5-fold higher under low zinc conditions compared with wild-type control, whereas there was no change in the expression level of ETR1 in the zap1Δ strain (Fig. 2B). These expression analyses suggest that the expression of ETR1 is regulated by Zap1p.

**Deletion of ZAP1 altered the expression of mitochondrial fatty acid synthetic pathway genes**

We found that among the lipid metabolic genes with binding sequences for Zap1p, the expression of ETR1 was significantly downregulated in the zap1Δ strain and the expression of the gene was modulated according to ZAP1 expression. ETR1 (YBR026C), a 2-enoyl thioester reductase, is involved in mitochondrial fatty acid synthesis (mtFAS) and catalyzes the reduction of 2-enoyl thioesters of the pathway in yeast. MtFAS is a conserved pathway that is essential for respiration. We observed that the expression levels of mtFAS pathway genes were lower than those in the control (Fig. 3A). To further validate the expression levels of these genes, we grew the wild-type and zap1Δ strains in synthetic media in the presence of 2% glycerol to induce mitochondrial biogenesis and function. We observed that the expression levels of mtFAS pathway genes (except MCT1) was higher in wild-type cells grown in glycerol than that in the wild-type control grown in glucose. Under the same conditions, the expression levels were higher for HFA1 (1.3-fold) and CEM1 (1.9-fold) genes, while the expression of other genes involved in the pathway remained lower in the zap1Δ strain relative to the control (Fig. 3A). MtFAS is the only source of octanoic acid, which is a required precursor for the production of the lipoic acid cofactor. We also checked the expression of genes involved in LA synthesis and attachment. We observed that expression of these genes was higher in wild-type cells grown in glycerol than in the wild-type control grown in glucose, whereas in zap1Δ the expression of these genes was lower in comparison to the control in both mutant strains grown in glucose or glycerol (Fig. 3B). Lower expression of genes involved in LA synthesis should result in a reduction in LA content. To check the LA content in zap1Δ compared with that in wild-type, lipoic acid was extracted from the cells and quantified through ESI-MS.
Direct infusion of an LA standard showed that the molecular ion (m/z 205) was partially fragmented to m/z 171 (Chen et al., 2005). Molecular ions m/z 205 and m/z 171 were found in wild-type and zap1Δ but were not detectable in etr1Δ. Mass spectrometric analysis graph showed that the level of LA in zap1Δ was reduced compared with that in wild-type (Fig. 3C). The lipoic acid content was further confirmed by western
The results suggest that, in zap1Δ, mtFAS is downregulated, leading to the alteration in mitochondrial structure and function.

**Effect of ZAP1 deletion and mitochondrial function**

To better understand the growth patterns, wild-type, zap1Δ and etr1Δ strains were spotted on synthetic agar media plates in the presence of glucose or glycerol. We observed a slower growth of deletion strains supplemented with glucose relative to that of the wild-type.

The zap1Δ showed growth defect on glycerol compared with wild-type cells. The etr1Δ did not grow on the glycerol plate (Fig. 4A). We next measured the ability of deletion strains to respire under these conditions. The rate of oxygen consumption was measured in stationary-phase cultures of wild-type, zap1Δ and etr1Δ strains in the presence of glucose or glycerol. We observed that the rate of respiration in wild-type was significantly increased (two-fold) in glycerol media compared with glucose media. The zap1Δ strain showed a reduction in respiration efficiency and there was no significant change in the respiration rate when cells were grown in glycerol media. The etr1Δ strain showed a significantly reduced respiration rate (Fig. 4B). In addition, the cytochrome c oxidase activity was measured in wild-type and mutant strains grown in galactose media. The activity of the enzyme was two-fold lower in zap1Δ than wild-type (Fig. 4C). The mitochondrial membrane potential was measured as an indicator of mitochondrial function under the same conditions. Cells were grown in synthetic media in the presence of glucose or glycerol and stained with the potential-based dye JC-1. Wild-type cells showed a significant increase in the mitochondrial membrane potential in glycerol compared with glucose media. The zap1Δ mutant showed 50% the level of membrane potential in glycerol compared with wild-type, and etr1Δ showed reduced membrane potential (Fig. 4D). We assessed mitochondrial morphology in vivo by observing the fluorescence of mitochondria specific stains. Cells were grown in synthetic media in the presence of glucose or glycerol until reaching stationary phase; cells were then stained with Mito Tracker CMTMRos and observed under a microscope. In wild-type cells, the mitochondria appeared as tubular structures, a phenotype that was more profound and appeared in a network-type formation when grown in glycerol. In zap1Δ, the mitochondria were either
fragmented or globular. The etr1Δ was used as a control (Fig. 4E). These experiments suggested that Zap1p is involved in maintaining mitochondrial integrity and that deletion of the gene affects the mitochondrial function.

**ETR1 deletion causes an increase in TAG level**

The wild-type and etr1Δ strains were grown in synthetic media in the presence of [14C]acetate until reaching stationary phase; the lipids were then extracted and resolved by TLC. The etr1Δ showed a significant increase in neutral lipids compared with wild-type (Fig. 5A). An increase in the number of lipid droplets was also observed in etr1Δ in comparison to wild-type (Fig. 5B). The expression level of ETR1 was reduced in zap1Δ, which caused a defect in mtFAS and resulted in an accumulation of TAG in zap1Δ. To validate this result, ETR1 was cloned into the low-copy number constitutive expression vector p415 ADH and expressed in zap1Δ and etr1Δ strains. The levels of neutral lipids in [14C]acetate-labelled stationary-phase cells were analyzed by TLC. The levels of TAG in the cells transformed with p415 ADH-ETR1 were significantly reduced compared with cells transformed with the vector control (Fig. 5C). These experiments suggested that the deletion of ETR1 caused an increase in TAG in etr1Δ and that it is responsible for the increase in TAG in zap1Δ.

**Zap1p interacts with the predicted binding site sequence in the ETR1 promoter**

The Zap1p binding sites are known as zinc-responsive elements (ZREs). The consensus sequence for ZRE is ACCTTNAAGGT (Zhao, 1998; Lyons, 2000). The sequence 1000 bp upstream of the ETR1 transcription start site was considered to be the promoter sequence of ETR1 and contains a predicted Zap1p binding site, ACCCGCGGT (Fig. 6A). To examine the binding of Zap1p to the ETR1 promoter, electrophoretic mobility shift assays (EMSA) were performed using the recombinant Zap1p from bacterial cells. The protein was purified by affinity chromatography using glutathione (GSH)-agarose (Fig. 6B). With increasing amounts of protein, an increase in the DNA-protein complex was observed (Fig. 6C, inset). The specificity of the interaction was examined by mutating the binding-site sequence of ETR1 to a non-consensus sequence (tggagaccag). These mutations abolished Zap1p binding to the ETR1 promoter (Fig. 6C, inset).

To determine whether Zap1p activates the transcription of ETR1 in vivo, wild-type and zap1Δ cells expressing lacZ under the control of the native and mutated ETR1 promoter were grown to log phase in complete synthetic medium. The β-galactosidase activity of the zap1Δ strain was substantially lower (9.1 ± 2.5 nmol/min/mg) than that of the wild-type (43.27 ± 3.2 nmol/min/mg) under the control of native ETR1 promoter (Nt). Wild-type cells expressing lacZ under the control of the mutated promoter (Mt) showed a reduction in β-galactosidase activity (approximately five-fold) when compared with wild-type cells expressing the native promoter. However, there was no significant change in the β-galactosidase activity of zap1Δ cells expressing lacZ under the control of the mutated promoter (Fig. 6C). These results suggest that Zap1p positively regulates ETR1 expression.

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Fig. 3. ZAP1 affects the mitochondrial fatty acid biosynthesis genes. Cells were grown in synthetic medium in presence of either 2% glucose or 2% glycerol. Total RNA was isolated and 1 μg of total RNA was used for cDNA synthesis. A 1:20 dilution of cDNA was used for the quantitative expression analysis. Actin was used as an endogenous control.

A. Quantitative expression analysis of the genes involved in the mitochondrial fatty acid pathway in zap1Δ and wild-type strains. Dotted line represents the base value 1.

B. Expression analysis of the genes involved in lipoic acid synthesis and attachment in zap1Δ compared with wild-type. Dotted line represents the base value 1. MtFAS, mitochondrial fatty acid biosynthesis; PDH, pyruvate dehydrogenase complex; α-KGDH, α-ketoglutarate dehydrogenase; GCV3, GlyCine cleavage.

C. Graph represents the lipoic acid content in zap1Δ compared with wild-type. Values are the means ± S.D. of three independent determinations, and each experiment was carried out in triplicate.

D. Immunoblot for lipoic acid content. Equal amounts of cell-free lysate (100 μg) were separated by 12% SDS-PAGE. Anti-lipoic acid antibodies were used to check the level of LA and anti-Pgk1 was used as a loading control. Lipoic acid is covalently attached to E3 subunit of pyruvate dehydrogenase complex that corresponds to molecular weight of 54.0 kDa and dihydrolipoyl transsuccinylase of alpha ketoglutarate dehydrogenase complex that corresponds to molecular weight of 50 kDa as shown in the Figure.
Fig. 4. Effect of different carbon sources on mitochondrial function.
A. Serial 10-fold dilutions of wild-type (WT), zap1Δ and etr1Δ strains were spotted on synthetic medium in the presence of 2% glucose or glycerol for observation of growth patterns. Plates were incubated at 30 °C.
B. Oxygen consumption rate of zap1Δ mutant strain. Cells were grown in synthetic medium in the presence of 2% glucose or glycerol.
C. Cytochrome c oxidase activity measurement. Cells were grown in complete synthetic medium in the presence of 2% galactose. Total mitochondrial proteins were used for the assay. Graph represents the percentage of oxidase activity in the mutants compared with the wild-type.
D. Mitochondrial membrane potential (red/green fluorescence) of zap1Δ and etr1Δ strains. Graph represents the percentage of fluorescence ratio compared with wild-type control.
E. Confocal microscopy of wild-type, zap1Δ and etr1Δ strains stained with Mito Tracker Orange CMTMRos. Scale bar = 2 μm. Data represent the means ± S.D. (*P < 0.05) of triplicates from three independent experiments.
Fig. 5. Effect of ETR1 deletion on TAG metabolism. Cells were grown in synthetic medium in 2% glucose to stationary phase. A. [\textsuperscript{14}C]Acetate labelling of neutral lipids in wild-type and etr\textsuperscript{1}Δ cells. Equal amounts (\textit{A}\textsubscript{600} = 20) of cells were taken for lipid extraction and resolved by TLC. The graph represents the incorporation of radiolabel into various lipids. Radiolabel was quantified using a liquid scintillation counter. B. Confocal microscopy of wild-type and etr\textsuperscript{1}Δ lipid droplets. Lipid droplets were stained with BODIPY 493/503 and viewed under confocal microscope. C. Neutral lipid profiles of zap1Δ and etr1Δ cells expressing vector p415 ADH or p415 ADH-ETR1. Transformed cells were grown and labelled with [\textsuperscript{14}C]acetate. Lipids were extracted from the cells (\textit{A}\textsubscript{600} = 20) and analyzed by TLC. Lane 1, zap1Δ strain transformed with p415 ADH vector control; lane 2, zap1Δ cells expressing ETR1; lane 3, etr1Δ transformed with p415 ADH vector control; lane 4, etr1Δ expressing ETR1. WT, wild-type; SE, steryl ester; TAG, triacylglycerol; FFA, free fatty acids; STE, sterol; DAG, diacylglycerol; Scale bar = 2 μm. Values are the means ± SD of three independent determinations, and each experiment was carried out in triplicate.
Expression of native YEpl357-ETR1 in etr1Δ mutant restores TAG levels and mitochondrial function

To further strengthen our finding that Zap1p regulates ETR1 at the transcriptional level, the native (pRN3) and mutated (pRN4) ETR1 promoters along with gene (2143 bp) was cloned into the YEpl357 vector. These constructs were transformed into etr1Δ and zap1Δ strains along with the YEpl357 vector control. Expression analysis showed that the mRNA abundance in etr1Δ cells expressing pRN3 was 1.7-fold higher than the cells expressing pRN4 (Supporting Information Fig. S4A). Zap1p expressed at high levels under zinc-limiting conditions. Therefore, the transformants were grown under both high and zinc-limiting conditions; a reduced level of TAG in the etr1Δ strain expressing the native promoter was found and the effect was more profound under zinc-limiting conditions. There was no significant reduction in TAG levels in the etr1Δ strain expressing mutated promoter (Fig. 7A). The zap1Δ strain expressing the native and mutated promoter showed no change in TAG levels (Supporting Information Fig. S4B). These results were further confirmed by the staining of lipid droplets. The etr1Δ strain expressing the native and mutated promoter were grown to stationary phase, stained with BODIPY 493/503 and viewed under microscope. A reduction in the number of lipid droplets was observed in cells expressing the native promoter in comparison to the vector control, whereas there was no change in the number of lipid droplets in cells expressing the mutated promoter (Fig. 7B).

The mitochondrial morphology and mitochondrial membrane potential were investigated in the etr1Δ strain expressing native and mutated promoters. When Etr1p was expressed under the control of the native promoter, cells showed restoration of mitochondrial integrity in comparison to the etr1Δ vector control, whereas no change was observed under expression by the mutated promoter (Fig. 7C). We found a ~12-fold increase in the membrane potential of cells expressing the native promoter in glycerol medium. However, there was minimal change in cells expressing the mutated promoter (Fig. 7D). These experiments suggest that Zap1p positively
Fig. 7. Mutation of the ZAP1 binding-site in the ETR1 promoter and effects on neutral lipid levels and mitochondrial function.

A. The etr1Δ strain was transformed with the YEp357 vector control, ETR1 with the native (Nt) and mutated promoters (Mt). The transformants were grown in synthetic medium (2% glucose) in the presence of high (+Zn) and low zinc (-Zn) to stationary phase along with [14C]acetate (0.2 µCi mL⁻¹). Equal amounts (A600 = 20) of cells were harvested, followed by lipid extraction. Lipids were separated by silica-TLC using petroleum ether: diethyl ether: acetic acid (70:30:1, v/v) as the solvent system. The graph represents the incorporation of radiolabel into various lipids. These lipids were quantified by scraping the lipid spots from the TLC plate and counting the radioactivity using toluene-based scintillation fluid in a liquid scintillation counter. SE, steryl ester; TAG, triacylglycerol; FFA, free fatty acids; STE, sterol; DAG, diacylglycerol.

B. Confocal microscopy was used to visualize lipid droplets in cells harboring the YEp357 vector control, ETR1 with the native (Nt) and mutated promoters. The transformants were grown in synthetic medium (2% glucose) to stationary phase and then were stained with BODIPY (493/503). Vec, YEp357 vector; Nt, native promoter; Mt, mutated promoter; Scale bar = 2 µm. Data represent the means ± S.D. (*P < 0.05) of triplicates from three independent experiments.

C. Mitochondrial morphology of etr1Δ cells harboring the YEp357 vector control, ETR1 with the native and mutated promoters. Cells were grown in the presence of glucose or glycerol in complete synthetic medium to stationary phase and then were stained with Mito Tracker CMTMRos Scale bar = 2 µm.

D. Mitochondrial membrane potential. Cells were stained with JC-1 stain. Graph represents the ratio of red/green fluorescence. Wild-type vector was used as the control to determine the percentage change in membrane potential in etr1Δ transformed cells. Vec, YEp357 vector; Nt, native promoter; Mt, mutated promoter; Scale bar = 2 µm. Data represent the means ± SD (*P < 0.05) of triplicates from three independent experiments.

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regulates the expression of ETR1 at the transcriptional level. In the zap1Δ mutant strain, expression of ETR1 was downregulated, which resulted in lower mtFAS, leading to altered mitochondrial function and TAG accumulation.

Flux of carbon and accumulation of TAG in zap1Δ

In zap1Δ, downregulation of ETR1 expression led to a loss of mitochondrial function. To understand the accumulation of TAG in zap1Δ, we analyzed the expression of genes involved in the fate of pyruvate produced through glycolysis. We observed the expression of MPC (MPC 1, 2 and 3), which code for the mitochondrial pyruvate carrier protein and together facilitate the entry of pyruvate into mitochondria for energy production. Expression of MPC was normal in zap1Δ, whereas the expression of PDC1, the first enzyme of the alcoholic fermentation pathway, which competes with MPC for pyruvate, was significantly increased (2.14-fold) in zap1Δ compared with wild-type, increasing the conversion of pyruvate into acetaldehyde. We observed reduced expression of all ADH genes in zap1Δ, which led to a reduction in fermentation in the mutant. ALD6, which converts acetaldehyde into acetate, showed normal expression in the mutant. ACS1 and ACS2, which are involved in the conversion of acetate to acetyl-CoA, were expressed at levels 1.5-fold higher in zap1Δ compared with wild-type (Fig. 8). The genes involved in TAG biosynthesis were expressed at high levels, whereas the expression of TAG hydrolytic genes was significantly downregulated in zap1Δ compared with wild-type. The expression analysis of these genes together with the other results show that the reduction in LA content due to compromised mtFAS may lead to reduction in the mitochondrial pyruvate utilization; as a result, pyruvate is channeled toward cytosolic decarboxylation and further to lipid biosynthesis, which results in accumulation of TAG in zap1Δ.

In eukaryotes fatty acids synthesis takes place in two subcellular compartments: in the cytoplasm (FAS type I) and in mitochondria (FAS type II). Earlier experimental results showed altered mtFAS in zap1Δ. To know the expression level of genes involved in cytosolic fatty acid synthesis, we checked their expression level and we observed that expression of ACC1 was 1.5 fold high whereas FAS1 and FAS2 showed normal expression level.

Fig. 8. TAG accumulation in zap1Δ. Graph represents the expression levels of genes involved in carbon flux from pyruvate produced through glycolysis in different pathways and lipid metabolism in zap1Δ compared with wild-type. Actin was used as an endogenous control. Each data point represents the average of triplicate results from three independent experiments ± S.D. LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol.
Discussion

The activities of critical enzymes involved in lipid metabolism are tightly controlled by different nutritional, environmental and intracellular conditions. Yeast has the ability to adapt to a variety of stressful conditions by regulating the expression of genes, including those involved in phospholipid metabolism. The effect of zinc depletion on the regulation of phospholipid metabolism in *S. cerevisiae* is well established; the process is mediated by the Zap1p transcription factor. These genes contain UASZRE in their promoters and are regulated by Zap1p at the transcriptional level. Other genes involved in lipid metabolism that are regulated by Zap1p include *DPP1* (Han et al., 2001) and *PAH1* (Soto-Cardalda et al., 2012).

The role of Zap1p has been well explored with respect to zinc homeostasis and phospholipids, but less information is available on the regulation of triacylglycerol metabolism in yeast. The accumulation of neutral lipids is observed in conditions of inositol deficiency (Paltauf and Johnston, 1970, Hayashi et al., 1976), sporulation (Hunter and Rose, 1972) and low temperatures (Illingworth et al., 1973). In the present study, we aimed to understand the effect of zinc-regulated genes on TAG metabolism. Among these genes, deletion of *ZAP1* caused a significant increase in TAG without causing a change in PE and PI levels, which suggested that Zap1p regulates expression of Kennedy pathway genes under zinc-limiting conditions and that CDP-DAG pathway remains operational. We found that the expression of *ETR1*, an enzyme involved in mtFAS, was significantly reduced in *zap1Δ*, and the expression of this gene was modulated in a *ZAP1*-dependent manner. MtFAS is a conserved pathway throughout eukaryotes and is catalyzed by a discrete set of enzymes. In eukaryotes, fatty acid synthesis takes place in two subcellular organelles: the cytoplasm and the mitochondria. MtFAS has been implicated as the only source of octadecanoids (oxidative decarboxylation of pyruvate), and may result in reduced mitochondrial pyruvate utilization and causes pyruvate to be metabolized to acetyl-CoA was not as efficient as in wild-type cells. The reduced level of LA, which serves as a cofactor for the pyruvate dehydrogenase complex slows down the activity of enzyme (oxidative decarboxylation of pyruvate), and may result in reduced mitochondrial pyruvate utilization and causes pyruvate to be metabolized to acetaldehyde by pyruvate carboxylase (*PDC1*) in the cytosol. The product, acetaldheyde can be either metabolized through fermentation into ethanol or acetate by aldehyde dehydrogenase (*ALD6*), which can be directed for lipid biosynthesis. *ZAP1* is one of the essential genes required for fermentation, and deletion of the gene arrests or delays the fermentation process (Walker et al., 2014). Our expression results also showed the reduced expression of genes involved in fermentation. Therefore, in *zap1Δ*, pyruvate is metabolized to acetate, which results in TAG accumulation (Fig. 9B). Deletion of *ETR1* causes an increase in neutral lipids and the complementation of this gene restored the TAG level in *etr1Δ*. Other studies have shown that supplementation of LA reduces TAG accumulation in rats by increasing energy expenditure (Kim et al., 2004). A significant reduction in LA content was observed in the *zap1Δ* mutant compared with wild-type. Expression of the *ETR1* gene in *zap1Δ* suppresses the
TAG level, as the gene is involved in mtFAS and produces octanoic acid required for LA production which is essential for mitochondrial enzymes involved in aerobic respiration. The ETR1 promoter does not contain a consensus binding sequence for Zap1p. However, a putative binding site was identified, and the interaction of Zap1p with the ETR1 promoter was confirmed by EMSA. Deviations from the consensus sequence are known to reduce the interaction of Zap1p; as a result, the induction of ETR1 under zinc-limiting conditions was not as strong as that of identified Zap1p targets (Zhao and Eide, 1996a; Zhao and Eide, 1996b). Moreover, mutation of the Zap1p-binding site abolished the interaction of Zap1p with the ETR1 promoter. Further validation that Zap1p mediates the ETR1 regulation at the transcriptional level was provided by the evidence that the expression of ETR1 and the TAG level did not change in the zap1Δ strain that was transformed with plasmids pRN3 and pRN4 due to the absence of the transcription factor; however, when the etr1Δ strain was transformed with plasmids pRN3 and pRN4, the TAG level and mitochondrial function were restored. Our results demonstrate that ETR1 is positively regulated by Zap1p at transcriptional level. ZAP1 affects the overall mtFAS pathway and the lipoic acid content in yeast, which possibly leads to accumulation of TAG in the zap1Δ strain.

### Experimental procedures

#### Materials

Yeast deletion strains were procured from Euroscarf. All chemicals were reagent-grade. Yeast nitrogenous base was obtained from Difco. Restriction endonucleases and Phusion high-fidelity DNA polymerase were purchased from New England Biolabs. The yeast transformation kit was obtained from Clontech. Silica-TLC plates and nitrocellulose membrane were obtained from Merck. The vector pVT100U-mtGFP for mitochondrial studies was procured from Addgene (Westermann and Neupert, 2000). The yeast expression vectors and fluorescence-based electrophoretic mobility shift assay kit were purchased from Life Technologies. The cDNA synthesis kit and Power SYBR Green PCR Master Mix were purchased from Applied Biosystems. Glass beads, Folin’s reagent, DNA purification kits, cytochrome c oxidase assay kit, yeast synthetic drop-out medium, amino acids, GST beads, antibiotics, bovine serum albumin, ortho-nitrophenyl β-D-galactopyranoside,
Table 1. Strains used in this study.

| Strain | Genotype | Source |
|--------|----------|--------|
| E. coli | F' Δ80lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hisD177 (his m') phoA supE44 thi-1 gyrA96 relA1 | Invitrogen |
| BL21(DE3)pLysS | F' ompT hsdSB (rB mB ) gal dcm (DE3) pLysS | Invitrogen |

| **S. cerevisiae** | **BY4741** | MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 | Euroscarf |
| **zrg1Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR284c::kanMX4 | Euroscarf |
| **zrg5Δ/Δadh4Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGL256w::kanMX4 | Euroscarf |
| **zrg6Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPL171c::kanMX4 | Euroscarf |
| **zrg7Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNL014w::kanMX4 | Euroscarf |
| **zrg8Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YER033c::kanMX4 | Euroscarf |
| **zrg10Δ/zap1Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJL056c::kanMX4 | Euroscarf |
| **zrg11Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YL046w::kanMX4 | Euroscarf |
| **zrg12Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOR030w::kanMX4 | Euroscarf |
| **zrg13Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNL139c::kanMX4 | Euroscarf |
| **zrg14Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YML028w::kanMX4 | Euroscarf |
| **zrg15Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLR443w::kanMX4 | Euroscarf |
| **zrg17Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNR039c::kanMX4 | Euroscarf |
| **etr1Δ** | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YBR026c::kanMX4 | Euroscarf |

SDS-PAGE reagents, oligonucleotides and anti-poly-His monoclonal antibody were obtained from Sigma-Aldrich. Anti-PgK1 and anti-lipoic acid antibodies were obtained from Abcam. The QuikChange Lightning Site-directed Mutagenesis kit purchased from Agilent Technologies.

**Strains, media and culture conditions**

The strains used in this study are listed in Table 1. Yeast cells were grown in YEPD medium (1% yeast extract, 2% peptone and 2% glucose) or in synthetic defined (SD) medium containing 2% glucose and kanamycin (50 μg/mL) at 30 °C. For the selection of plasmid-harboring yeast cells, the appropriate amino acids were omitted from the synthetic medium. Low-zinc medium consisted of yeast nitrogen base with low concentration of zinc. For low-zinc conditions, cells were first grown in YEPD for 24 h and then cells were harvested, washed with water and subcultured in synthetic medium for 24 h in the absence of zinc to deplete the internal stores of zinc (Iwanyshyn, 2004). The zinc-depleted cells were grown in synthetic medium in the presence of 50 nM zinc (low zinc) and 2 μM zinc (high zinc) for further studies. For the growth of yeast and bacteria on solid media, agar plates were prepared with supplementation of 2% agar to the respective broth.

**Spotting assay/growth on solid media**

Wild-type and deletion strains were grown in SD medium until the cultures reached logarithmic phase. Cells were then harvested and the cell density was adjusted to A600 of 1.0. A serial dilution series of undiluted, 1:10, 1:100, 1:1000 and 1:10000 was made and 2 μl of each dilution were spotted on the SD media agar plate. For the growth study of ZRGs in zinc conditions, cells were spotted on plates and grown in low (50 nM) and high zinc (2 μM) at 30 °C for two or three days, respectively. To assess cell growth on different carbon sources, cells were spotted on agar plates containing either 2% glucose or 2% glycerol as a carbon source and grown at 30 °C for two or five days, respectively.

**Atomic absorption spectroscopy**

The total cellular zinc was measured by atomic absorption spectroscopy (AAS). Cells were harvested by centrifugation and washed twice with distilled water. Equal amounts of cells were placed in a silica crucible and dried in an oven. The crucible was kept in a programmable furnace at 450 °C for at least 10 h. Ash was washed with 2 mL water and water was evaporated. The crucible was again kept in the furnace and re-ashed. Ash was digested with HNO3. Zinc content was measured using a Thermo Scientific iCE 3000 atomic absorption spectrometer.

**Construction of plasmids, expression and purification of recombinant proteins**

The plasmids used in this work are listed in Table 2. Yeast genomic DNA was prepared as previously described (Sambrook et al., 1989). The ZAP1 (YJC056C) gene was amplified from yeast genomic DNA using gene-specific primers. The gene was cloned into the bacterial and yeast expression vectors pGEX-6P-1 (NotI and XhoI) and pYES2-NT/C (KpnI and XhoI) respectively. For promoter cloning, fragments that included the 1000 base pairs upstream of the ETR1 gene sequence along with 24 nucleotides from the start site were amplified and cloned into the YEPL357 vector using the KpnI and XbaI sites (pPR1). Plasmid pPR2 was derived from plasmid pPR1 by mutating Zap1p-binding site. ETR1 along with its promoter were cloned into the YEPL37.
vector using the KpnI and PstI sites and named as pRN3. Plasmid pRN4 was derivative of pRN3 in which binding site was mutated in ETR1 promoter. This plasmid was used as a template to create mutations in the ZAP1-binding site of the ETR1 promoter by PCR-mediated site-directed mutagenesis. The ETR1 (YBR026C) gene was cloned into the yeast expression vector p415ADH using the Xhol and PstI restriction sites. Bacterially overexpressed recombinant Zap1p was purified using GST beads, and the protein concentrations were determined using Lowry’s reagents. The purified proteins were resolved on a 8% SDS-PAGE followed by Coomassie brilliant blue staining, and the presence of the recombinant protein was confirmed by immunoblot analysis using the GST antibody. All of the primers used in cloning are listed in Table 3. The constructs used in the study were confirmed by DNA sequencing.

**Table 2.** Plasmids used in this study.

| Plasmids                  | Relevant characteristics                                      | Source                      |
|---------------------------|----------------------------------------------------------------|-----------------------------|
| pGEX-6P-1                 | E. coli expression vector with GST tag fusion                 | Invitrogen                  |
| pYES2NT-C                 | Yeast expression vector with N-terminal His6 tag fusion        | Invitrogen                  |
| YEplac22                   | Yeast episomal plasmids with lacZ reporter gene                | ATCC                        |
| p415 ADH                  | Yeast expression vector with leucine selection                 | ATCC                        |
| pVT100U-mtGFP              | Yeast expression vector with GFP tag                           | (Westermann et al., 2000)   |
| pGEX-6P-1-ZAP1            | ZAP1 coding sequence inserted into pGEX-6P-1                   | This study                  |
| pYES2-NT-C-ZAP1           | ZAP1 coding sequence inserted into pYES2-NT/C                  | This study                  |
| p415 ADH-ETR1             | ETR1 coding sequence inserted into p415 ADH                    | This study                  |
| pRN1                      | PZAP1lacZ reporter gene containing the ETR1 promoter into EYe357| This study                  |
| pRN2                      | PZAP1lacZ reporter gene derived from pRN1 in which Zap1p binding site mutated | This study                  |
| pRN3                      | The ETR1 promoter along with ETR1 (2143bp) into YEp357         | This study                  |
| pRN4                      | Derived from pRN3 in which Zap1p binding site mutated          | This study                  |

**In vivo labelling and lipid extraction from yeast cells**

Yeast cells were grown in synthetic media (2% glucose) until reaching stationary phase in the presence of [14C]acetate (0.2 μCi/mL) for incorporation of radiolabel into cellular lipids. Cells (A600 = 20) were harvested at different time intervals and washed with cold water. Cells were lysed with acid-washed glass beads and 2% orthophosphoric acid. Total lipids were extracted using a chloroform:methanol mixture (1:1, v/v) as a solvent system. The identities of the labelled lipids on the TLC plates were confirmed by comparison with standards after exposure to iodine vapor. Radiolabelled lipids were visualized under a phosphorimager (Typhoon FLA9500) and quantified in a liquid scintillation counter using a toluene-based scintillation cocktail (Perkin Elmer Micro Beta2 2450 Microplate counter).

**Confocal microscopy**

The intracellular zinc in wild-type cells was measured by zinc-staining dye, zinquin ethyl ester, which specifically binds to zinc. Cells pre-grown in YPD medium were subcultured at an initial A600 = 0.2 in SD medium without zinc for 24 h. Cells were washed and subcultured in synthetic medium (2% glucose) in the presence of 50 nM (low) and 2 μM zinc (high) for 24 h. Cells were pelleted and washed with 1× PBS. The washed cells were fixed with formaldehyde and after fixation, washed with 1× PBS. Cells were resuspended in 1× PBS containing 25 μM zinquin ethyl ester and incubated for 1 h at RT. Cells were subsequently washed in 1× PBS to remove extracellular zinc (Devirgiliis et al., 2004). Finally, cells were resuspended in 1× PBS, mounted on an agarose-coated slide and viewed under a confocal microscope (ZEISS, LSM 700).

Stationary-phase cells grown in synthetic media were pelleted and washed thrice with 1× PBS. The washed cells were fixed with 3% formaldehyde for 15 min. After fixing, cells were resuspended in 200 μl PBS containing 1 μg of the neutral lipid-specific dye BODIPY™ 493/503. The cells were incubated in the dark for 30 min at room temperature. After incubation, cells were pelleted and washed thrice with 1× PBS. Finally, the cells were resuspended in 20 μl PBS containing 50% glycerol and spotted onto agarose-coated slides and lipid droplets were observed under the microscope.

Cells harboring the plasmid pVT100U-mtGFP were grown in synthetic media without uracil in glucose or glycerol for assessing mitochondrial morphology. The growth of zap1Δ and etr1Δ strains were defective in presence of glycerol as a carbon source therefore we have taken enough number of cells and incubated in SM media in presence of glycerol to induce mitochondrial biogenesis. For staining of yeast mitochondria, cells grown in synthetic media with glucose or glycerol. Cells were pelleted and washed with 1× PBS. After washing, cells were resuspended in 10 mM HEPES buffer (pH 7.4) containing 5% glucose, and Mitotracker CM/Tracker Ros 554/576 (100 nM final concentration) was added to the sample. The cells were incubated at room temperature for 30 min in the dark. After incubation, cells were pelleted and washed with 1× PBS. Cells were resuspended in HEPES buffer and mitochondria were viewed under the microscope.

**Gas chromatography**

The total lipid content was extracted from wild-type and zap1Δ cells grown to stationary phase in synthetic media

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| No. | Gene     | Forward primer            | Reverse primer            |
|-----|----------|---------------------------|---------------------------|
| 1   | ACT1.qRT | GATATGGAAAAGATCTGGCATCATAC | CGCCATTTTGAGAATCTTTG     |
| 2   | ZAP1.qRT | GACGATAGTGGCTCGGCCAACAC  | ATGGCTAGTCCTCGGATGAAA    |
| 3   | ICT1.qRT | CCCCCCAAGCAGGAGACACT    | TCCCTCGCATTGCTGCTAT      |
| 4   | ARE2.qRT | GCAACTCCCAAGCCAAAGTAA   | ATGCCGAGTCCTGGTCTTTG     |
| 5   | DPP1.qRT | TCTCTCTTGCTTTAACACATTG  | TCATCCCCGATCTGCTTTG     |
| 6   | TGL1.qRT | TCTACCAAGCTGATTGCTCA    | TCATACCCGCTTACAGGCTT     |
| 7   | YSR3.qRT | ACAGGACAGATCTGATCTTCTT  | CGTCTGACAGCTGACAGATCT     |
| 8   | HFA1.qRT | ACTAAGACAGCCGGTCCT      | GTGATCTGGATGCTTCTG       |
| 9   | MCT1.qRT | CCCGGGCAATGTGATTTA       | CCGCCTTGTGACAGGCT       |
| 10  | CEM1.qRT | AAGGTGACCCAGGAGACACT    | CAAGGACAGCTGACAGATCT     |
| 11  | OAR1.qRT | AGAGCGCACCGCGATAGATA    | CTGGTGAGGAGATCTGGT       |
| 12  | MDM10.qRT | TAAGGGCATTTTATCAGAGCACC | TGCTGACAGCTGACAGATCT     |
| 13  | MDM33.qRT | TCACGGAAGATACAGGAGAAGAGA | TGGTGAGGAGATCTGGT       |
| 14  | TIM11.qRT | GGAGCAGGCACAGTACGAGG    | TGGTGAGGAGATCTGGT       |
| 15  | TIM12.qRT | AATGATTCTCCGCTGAGG      | AAGGACAGCTGACAGATCT     |
| 16  | TOM22.qRT | AAGACACTCGTCCCCCAGGT    | AAGGACAGCTGACAGATCT     |
| 17  | COX2.qRT | TCTCTCTCTGATTATATCATGGA | TCTCTCTCTGATTATATCATGGA |
| 18  | COX3.qRT | TGTGTTTATTCTGAGCTTATCTA  | TCTCTCTCTGATTATATCATGGA |
| 19  | LIP2.qRT | TGGCAATGAAAGCAGATCTA   | TCTCTCTCTGATTATATCATGGA |
| 20  | LIP3.qRT | GCAAAAGAGGTCCCGATAAG    | TCTCTCTCTGATTATATCATGGA |
| 21  | LIP5.qRT | GGCAACGGCAACAATTATGC   | TCTCTCTCTGATTATATCATGGA |
| 22  | GCV3.qRT | GAGTTGCCAGAAGTGGGCAC    | TCTCTCTCTGATTATATCATGGA |
| 23  | ZAP1.qRT | GCTCGAGATGGATGCGTTGACTCC | TCTCTCTCTGATTATATCATGGA |
| 24  | ZAP1.qRT | TTGGTACCCATGGATGCGTTGACT | TCTCTCTCTGATTATATCATGGA |
| 25  | ETR1.qRT | ATGATCCATGCTCCACTTTCCAAACC | TCTCTCTCTGATTATATCATGGA |
| 26  | HTD2.qRT | AGCGAACAAGAAGGTTATG     | TCTCTCTCTGATTATATCATGGA |
| 27  | PAH1.qRT | CTGTGTTGCGGCGGGATG     | TCTCTCTCTGATTATATCATGGA |
| 28  | DGA1.qRT | TGGTGGACAGCAGCAAGGATG   | TCTCTCTCTGATTATATCATGGA |
| 29  | LPP1.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 30  | LRO1.qRT | CGTACAACCCTGCCGCCGAAAT  | TCTCTCTCTGATTATATCATGGA |
| 31  | TGL2.qRT | CTGGCTCCTGCAGAGAAGAATG | TCTCTCTCTGATTATATCATGGA |
| 32  | TGL4.qRT | CAAACGGGCAAGGTCGATACT  | TCTCTCTCTGATTATATCATGGA |
| 33  | MDC1.qRT | CCCGTGCTGAGACACGCTG     | TCTCTCTCTGATTATATCATGGA |
| 34  | MDC2.qRT | CTTGCTGATTTTGCAGCTG    | TCTCTCTCTGATTATATCATGGA |
| 35  | MDC3.qRT | GGTCTTCGCAGGGCTAATG    | TCTCTCTCTGATTATATCATGGA |
| 36  | MDC4.qRT | CCTGTTGCGGAGGCTAAGT    | TCTCTCTCTGATTATATCATGGA |
| 37  | MDC5.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 38  | MDC6.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 39  | MDC7.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 40  | MDC8.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 41  | MDC9.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 42  | MDC10.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 43  | MDC11.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 44  | MDC12.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 45  | MDC13.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 46  | MDC14.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 47  | MDC15.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 48  | MDC16.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 49  | MDC17.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 50  | MDC18.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 51  | MDC19.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 52  | MDC20.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 53  | MDC21.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 54  | MDC22.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 55  | MDC23.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 56  | MDC24.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 57  | MDC25.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 58  | MDC26.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 59  | MDC27.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 60  | MDC28.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |
| 61  | MDC29.qRT | AGTTGCATTTGGTGCCCTTT   | TCTCTCTCTGATTATATCATGGA |

qRT, Quantitative real-time PCR; Pro, Promoter; Nt, Native; Mt, Mutated.
(2% glucose). The extracted lipids were methylated using boron trifluoride-methanol and incubated at 65 °C for 15 min (Araujo et al., 2008). The reaction mixture was immediately cooled on ice for 5 min and fatty acid methyl esters were extracted using hexane. The moisture content was removed from the hexane fraction through sodium sulfate and concentrated samples were subjected to gas chromatography. Heptadecanoic acid (100 μg; C17:0) was used as an internal standard for each reaction. Capillary gas chromatography analysis of FAMEs was performed on a 60 m length, 0.25-μm thickness, and 0.25-mm inner diameter DB Wax-23 column (Agilent Technologies). The method used for separation of FAMEs on the GC system was as follows: FAMEs were heated up to 50 °C for 2 min, the temperature was raised to 180 °C at the rate of 5 °C/min and was thereafter raised to 240 °C at the rate of 5 °C/min. The column was further maintained at 240 °C for 5 min, and then the temperature was raised to 300 °C at the rate of 30 °C/min and maintained at 300 °C for 5 min. The total run time was 34 min.

RNA isolation and expression analysis

Total RNA was isolated using the Nucleospin RNA II RNA isolation kit. Complementary DNA (cDNA) was prepared using the high-capacity cDNA reverse transcription kit with 1× RT buffer, 1× random primer, 4 mM dNTP mix, 50 U/μl reverse transcriptase and 1 μg total RNA. The primers were designed using the Primer ExpressR Software 3.0 (Applied Biosystems), and the sequences are listed in Table 1. For the real-time expression analysis, 1 μl of a 1:20-diluted cDNA sample was amplified using an Applied Biosystems machine (SDS 2.1) with the Power SYBR Green PCR Master Mix. The samples were analyzed in triplicate, and the results were analyzed using relative quantification test.

Preparation of cell-free lysate

Cell-free lysate was prepared by lysing the cells with glass beads in lysis buffer. Cells were vortexed for 15 cycles of 30 s while keeping the cells on ice for 1 min between cycles. The lysis buffer contained 50 mM Tris-HCl (pH 8.0), 1 mM MgCl2, 10% glycerol and 1 mM phenylmethyleneasulfonfyl fluoride (PMSF). Unbroken cells were removed by centrifugation at 3600 × g for 5 min at 4 °C.

Immunoblotting analysis

Immunoblotting was performed by transferring the proteins onto a nitrocellulose membrane. The blots were incubated for 3 h with anti-lipoic acid primary antibodies (1:1000, v/v) raised in rabbit followed by incubation with an alkaline phosphatase-tagged anti-IgG secondary antibodies (1:5000, v/v) for 1 h. Finally, the blots were developed in the dark using alkaline phosphate substrate.

Lipoic acid analysis

Cells were grown in SD medium containing 2% glycerol as a carbon source. Equal amounts of lyophilized samples were taken for lipoic acid (LA) analysis. LA was extracted by base hydrolysis (Kataoka et al., 1993). Sample was dissolved in 0.8 mL of 50 mg/mL BSA in 2.5 M potassium hydroxide in glass tube and volume was made up to 1 mL with water. The mixture was hydrolyzed for 3 h at 110 °C. To the resulting hydrolysate 0.05 mL of 1 mg/mL 2-mercaptoethanol and 0.4 mL of 6 M hydrochloric acid were added. The mixture was extracted twice with 2 mL of methylene chloride, and the pooled methylene chloride extract was evaporated to dryness. The residue was dissolved in 1 mL of 0.01 M sodium hydroxide and used for further analysis. Processed extracts from wild-type, zap1Δ and eTR1Δ cells were used for the mass spectrometric analysis of lipoic acid. For analysis, extracts were diluted in 50% methanol and were injected by direct continuous infusion into an ESI source on a mass spectrometer (QTRAP 6500, AB SCIEX). Detection was achieved in negative ion modes for the lipoic acid standard and the samples.

Electrophoretic mobility shift assays

The YEp357 construct containing the ETR1 promoter was used as a template to amplify the promoter with the primers listed in Table 3. The amplified DNA fragments were gel-purified. The binding assay reactions were carried out in binding buffer (10% glycerol, 12 mM HEPES-NaOH pH 7.9, 4 mM Tris-Cl, 1 mM dithiothreitol, 60 mM KCl, 10 mM MgCl2, 10 μM ZnCl2) and 4 μg DNA with increasing concentrations of Zap1p (200 ng-1000 ng). The reaction mixture was incubated for 1 h at 30 °C and resolved on a 5% non-denaturing polyacrylamide gel. The gel was stained with SYBR Green nucleic acid stain and visualized under a UVP transilluminator at 300 nm. The electrophoresis running buffer (50 mM Tris base, 400 mM glycine, 2 mM EDTA, pH 8.0) was supplemented with 20 μM ZnCl2 to maintain the activity of the Zap1p zinc finger domain (Zhao, 1998).

Site-directed mutagenesis

Mutation of the Zap1p binding site in the ETR1 promoter was introduced by PCR-based amplification of the entire plasmid using the primers listed in the Table 3. The underlined bases represent the mutated nucleotide sequence. Mutations in the plasmids were generated with a Quick-Change Lightning site-directed mutagenesis kit. The binding-site mutations were confirmed by DNA sequencing using specific primers.

β-Galactosidase assay

Wild-type and zap1Δ strains transformed with plasmids (YEp357 vector, pRN1 and pRN2) were grown in synthetic medium without uracil. The saturated cultures were used to inoculate fresh synthetic medium for an A600 of 0.2 and grown until the A600 reached 0.8–1.0. Cells were then
harvested and washed with water, and the cell extracts were prepared as described above. The β-galactosidase activity was measured (Rose and Botstein, 1983) and the specific activity was expressed as nmol/min/mg protein.

**Measurement of oxygen consumption**

Cells were grown in SD medium containing either 2% glucose or glycerol as a carbon source. Equal amounts of cells were used for oxygen measurement. The growth of zap1Δ and etr1Δ strains were defective in presence of glycerol as a carbon source therefore we have taken enough number of cells and incubated in SM media in presence of glycerol to induce mitochondrial biogenesis. The oxygen consumption rate was monitored in a closed chamber equipped with a Clark-type oxygen electrode (Strathkelvin instruments, Model 782) at 30 °C with constant agitation for 5 min. To ensure that the oxygen consumption observed was due to mitochondrial respiration, sodium azide was added to the cultures (final concentration, 0.05%) and the consumption rate was compared with the rate observed without the azide.

**Mitochondria isolation and cytochrome c oxidase assay**

Mitochondria were prepared by sucrose density gradient centrifugation (Zinser et al., 1991). The cytochrome c oxidase activities were measured in the isolated mitochondria using the cytochrome c oxidase assay kit (CYTOCOX1) from Sigma-Aldrich. The assay measures the decrease in absorbance at 550 nm associated with the colorimetric oxidation of ferrocytochrome c to ferricytochrome c by cytochrome c oxidase. Equal concentrations of mitochondrial protein were used for determining the oxidase activity. BSA was used as the standard and protein concentration was determined using Lowry’s reagent.

**Measurement of mitochondrial membrane potential**

Mitochondrial membrane potential was measured using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl benzimidazolyl carbo cyanine iodide) dye obtained from Sigma-Aldrich. JC-1 dye exhibits potential-based accumulation of red fluorescent J-aggregates in the functional mitochondria. A higher red/green fluorescence ratio indicates healthy and active mitochondria, whereas decrease in the red/green fluorescence intensity ratio indicates depolarization. To measure the membrane potential, cells were grown in synthetic media in the presence of fermentable (glucose) or a non-fermentable (glycerol) carbon source. The growth of zap1Δ and etr1Δ strains were defective in presence of glycerol as a carbon source therefore we have taken enough number of cells and incubated in SM media in presence of glycerol to induce mitochondrial biogenesis. Equal amounts of cells were pelleted and washed with 1× PBS. The washed cells were resuspended in synthetic media containing JC-1 stain. The cells were incubated in the dark for 30 min at 30 °C. After incubation, cells were pelleted and washed with 1× PBS. Finally, cells were resuspended in an equal volume of media and samples were aliquoted in a 96-well plate. The red fluorescence (550/600) and green fluorescence (485/535) were measured using the Thermo Scientific Varioskan Flash Multimode fluorescence plate reader. The ratio of red to green fluorescence was determined and the relative mitochondrial membrane potential was expressed as a percentage relative to the control.

**Data analysis**

All quantitative data were analyzed using Student’s t test, and P < 0.05 was considered statistically significant. Each experiment was repeated at least three times. Data are presented as the average ± standard deviation (SD). Statistical analyses, wherever required, were performed using the Sigma Start™ software.

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