The switch from fermentation to respiration in *Saccharomyces cerevisiae* is regulated by the Ert1 transcriptional activator/repressor

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Running title:
Ert1 is a regulator of gluconeogenesis

Keywords
Gluconeogenesis, transcriptional regulator, *Saccharomyces cerevisiae*, fermentation, zinc cluster proteins

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, fermentation is the major pathway for energy production, even under aerobic conditions. However, when glucose becomes scarce, ethanol produced during fermentation is used as a carbon source, requiring a shift to respiration. This adaptation results in massive reprogramming of gene expression. Increased expression of genes for gluconeogenesis and the glyoxylate cycle is observed upon a shift to ethanol and, conversely, expression of some fermentation genes is reduced. The zinc cluster proteins Cat8, Sip4 and Rds2, as well as Adr1, have been shown to mediate this reprogramming of gene expression. In this study, we have characterized the gene *YBR239C* encoding a putative zinc cluster protein and it was named *ERT1* (Ethanol Regulated Transcription factor 1). ChIP-chip analysis showed that Ert1 binds to a limited number of targets in the presence of glucose. The strongest enrichment was observed at the promoter of *PCK1* encoding an important gluconeogenic enzyme. With ethanol as the carbon source, enrichment was observed with many additional genes involved in gluconeogenesis and mitochondrial function. Use of lacZ reporters and qRT-PCR analyses demonstrated that Ert1 regulates expression of its target genes in a manner that is highly redundant with other regulators of gluconeogenesis. Interestingly, in the presence of ethanol, Ert1 is a repressor of *PDC1* encoding an important enzyme for fermentation. We also show that Ert1 binds directly to the *PCK1* and *PDC1* promoters. In summary, Ert1 is a novel factor involved in the regulation of gluconeogenesis as well as a key fermentation gene.
INTRODUCTION

In the yeast *Saccharomyces cerevisiae*, glucose is the preferred carbon source and fermentation is the major pathway for energy production, even under aerobic conditions. However, when glucose becomes scarce, ethanol produced during fermentation is used as a carbon source, a process requiring a shift to a respiration mode. Other non-fermentable carbon sources, such as lactate, acetate or glycerol, can also be used by yeast (Turcotte et al. 2011). The shift from fermentative to non-fermentative metabolism results in massive reprogramming of gene expression for the use of ethanol (DeRisi et al. 1997; Roberts and Hudson 2006). For example, increased expression of genes for gluconeogenesis and the glyoxylate cycle is observed upon a shift to ethanol and, conversely, expression of some fermentation genes is reduced under these conditions. An important player in the regulation of this process is the Snf1 kinase (Broach 2012; Hedbacker and Carlson 2008; Zaman et al. 2008; Zhang et al. 2010). Snf1 becomes activated under low glucose conditions resulting in the phosphorylation of a number of substrates that include DNA binding proteins such as Mig1, Cat8, Sip4 and Rds2 (for reviews see Schüller 2003; Turcotte et al. 2011).

Mig1 and Adr1 belong to the family of zinc finger proteins of the Cys2His2 type. Mig1 is a transcriptional repressor that, following phosphorylation by Snf1, undergoes nucleocytoplasmic shuffling allowing derepression of target genes such as *CAT8*. Adr1 is a transcriptional activator of over one hundred genes involved in the utilization of ethanol, glycerol and fatty acids as well as genes involved in peroxisome biogenesis (Young et al. 2003). For example, Adr1 is a positive regulator of *ADH2* encoding alcohol dehydrogenase, an enzyme involved in the conversion of ethanol to acetaldehyde. Cat8, Sip4 and Rds2 are members of
the family of zinc cluster proteins (MacPherson et al. 2006). These transcriptional regulators are characterized by the presence of a well-conserved motif (CysX_2CysX_6CysX_5-12CysX_2CysX_6-8Cys) where the cysteines bind to two zinc atoms that facilitate folding of the zinc cluster domain involved in DNA recognition (MacPherson et al. 2006). Zinc cluster proteins preferentially bind to CGG triplets oriented as direct (CGGNxC GG), inverted (CGGNxCCG), or everted repeats (CCGNxCCG) (MacPherson et al. 2006). These factors have been shown to bind DNA as monomers, homodimers or heterodimers (MacPherson et al. 2006). Expression of CAT8 is increased under low glucose conditions and Cat8 regulates the expression of SIP4 (Hedges et al. 1995; Lesage et al. 1996). These factors control expression of various genes involved in the utilization of non-fermentable carbon sources. For example, expression of PCK1 encoding phosphoenolpyruvate carboxykinase (an essential enzyme for gluconeogenesis) is controlled by Cat8, Sip4 and Rds2. These factors mediate their effects by binding to carbon source response elements (CSREs) found in the promoters of target genes (Schüller 2003; Turcotte et al. 2011).

In this study, we focused on the gene YBR239C encoding a putative zinc cluster protein that we named ERT1 (Ethanol Regulated Transcription factor 1). Ert1 shows high homology to Rds2 and data from a large-scale two-hybrid analysis suggests that the two proteins interact with each other (Ito et al. 2001). Importantly, AcuK, a homologue of Ert1 in Aspergillus nidulans, regulates expression of gluconeogenic genes (Suzuki et al. 2012). To unravel the role of Ert1, we have performed genome-wide location analysis (ChIP-chip). Results show that Ert1 binds to a limited number of promoters (e.g. PCK1) when cells were grown in the presence of glucose. However, enrichment of Ert1 with many genes involved in gluconeogenesis and other aspects of
carbon metabolism was observed when ethanol was used as a carbon source. Finally, our results show that Ert1 acts redundantly with other regulators of gluconeogenesis, behaving as an activator of gluconeogenesis genes as well as a repressor of \textit{PDC1}, a key fermentation gene. Taken together, our data identified Ert1 as a novel transcriptional regulator of the switch from fermentation to respiration.

**MATERIALS AND METHODS**

**Strains and media**

Yeast strains used in this study are listed in Table 1. Wild-type strain is BY4741 (BRACHMANN \textit{et al.} 1998). The open reading frame (ORF) of \textit{ERT1} was N-terminally tagged with a triple HA epitope using the approach of Schneider \textit{et al.} (SCHNEIDER \textit{et al.} 1995). Briefly, strain BY4741 was transformed with a PCR product generated using oligonucleotides PETYBR239-1 and PETYBR239-2 (Table S2) and, as a template, plasmid pMPY-3XHA. Colonies were selected on plates lacking uracil. Transformants were grown in the absence of selection to allow loss of the \textit{URA3} marker by internal recombination and Ura⁻ cells were obtained using the 5-fluoroorotic acid selection (SCHNEIDER \textit{et al.} 1995). Integrity of the DNA sequences encompassing the tag was verified by amplifying that region with oligonucleotides ERT1-Acheck and D2 and sequencing of the PCR product (Table S2). To generate the double deletion strains SC112A, SC113 and SC114 (Table 1), genomic DNA from \textit{Candida glabrata} (strain ATCC 66032) was isolated and used as a template to amplify the \textit{HIS3} gene from -696 to +240 relative to the ORF using oligonucleotides ERT1-CgHIS3-1 and ERT1-CgHIS3-2 (Table S2). The PCR product containing sequences homologous to the 5’ and 3’ regions of the \textit{ERT1} ORF was transformed in single-deletion strains SC102A, SC103 and SC104 or wild-type W303-
1A (Table 1) and transformants were selected on plates lacking histidine. Media were prepared according to Adams (Adams et al. 1997). Plasmid pPDC1(-850)-lacZ and mutants were linearized with StuI (site located in the URA3 gene) and integrated at the ura3-52 locus of strain YPH499 by selection on minimal plates lacking uracil and histidine. Quantitative PCR showed that two copies of the plasmids were integrated for selected clones.

Plasmids

An integrative PDC1-lacZ reporter was constructed in multiple steps. Plasmid pRS303 (Sikorski and Hieter 1989) was cut with PvuII and religated to remove the multi cloning sites yielding pRS303∆PvuII. Oligos LacZ-integr-1 and LacZ-integr-2 were used to amplify by PCR a region of plasmid YEP356 (Myers et al. 1986) containing the lacZ and the URA3 genes. The PCR product was cut with BglII and NheI and subcloned into pRS303∆PvuII cut with the same enzymes to yield pLacZ-integr. The PDC1 promoter (-850 to +52 bp relative to the ATG) was amplified with oligonucleotides PDC1-lacZ-3 and PDC1-lacZ-4 using genomic DNA isolated from strain BY4741. The PCR product was cut with EcoRI and SphI and subcloned into pLacZ-integr cut with the same enzymes to yield pPDC1(-850)-lacZ. Mutations (CGG to CTG, see Fig. 8A) were introduced in pPDC1(-850)-lacZ by site-directed mutagenesis using oligonucleotides PDC1-CGG to CTG-1 and PDC1-CGG to CTG-2 (mutation at position -569) as well as PDC1-CGG to CTG-3 and PDC1-CGG to CTG-4 (mutation at position -587).

A GST-Ert1 (a.a.1-152) bacterial expression vector was constructed by amplifying DNA sequences encoding the DNA binding domain of Ert1 by PCR using oligonucleotides D1 and D2 (Table S2) and genomic DNA isolated from strain BY4741 as a template. The PCR product was
cut with BamHI and EcoRI and subcloned into pGEX-f (HELLAUER et al. 1996) cut with the same enzymes to yield plasmid pGST-Ert1(1-152).

**Chromatin immunoprecipitation (ChIP)**

ChIP-chip assays were performed as described elsewhere (LAROCHELLE et al. 2006). Cells from the wild-type (BY4741) and HA-ERT1 strains were grown in YPD to an approximate \( \text{OD}_{600} \) of 0.7, washed twice in water, and transferred to YPD or YEP media containing 2% of ethanol as a sole carbon source and grown for 3 h. Microarrays used for ChIP-chip (4X 44K) were obtained from Agilent. Significantly enriched regions from the ChIP-chip data were identified as described previously (SOONTORNGUN et al. 2012) and tRNA genes excluded from the analysis. Final assignments were then manually curated. Standard ChIP assays were performed as described (SYLVAIN et al. 2011).

**Quantitative-RT PCR analysis (qRT-PCR)**

For qRT-PCR, wild-type (BY4741 or YPH499) and deletion strains were grown as described above. RNAs were isolated using the hot-phenol extraction method and purified with an RNA clean up kit (Qiagen). The cDNA was synthesized from 1 \( \mu \text{g} \) of total RNA according to the manufacturer's protocol for the Maxima H minus Reverse Transcriptase (Thermo Scientific). The qRT-PCR was performed with an Applied Biosystems 7300 using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific). Quality control of the primers, listed in Table S2, was verified by efficiency and dimerization tests. The PCR parameters were 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s. The relative quantification of each transcript was calculated by the \( 2^{-\Delta\Delta CT} \) method (LIVAK and SCHMITTGEN
2001) using the ACT1 gene (actin) as an internal control. Three independent cDNA preparations obtained from two independent cultures were used for analysis.

**β-galactosidase assays**

Reporters used in this study are pJJ11b (FBP1-lacZ) (Vincent and Gancedo 1995), pPEPCK-lacZ (PCK1-lacZ) (Proft et al. 1995) and pPDC1(-850)-lacZ (this study). Following transformation and selection of the reporters, colonies were grown overnight in rich medium (YPD). Cell were then diluted into selective medium containing 0.67% yeast nitrogen base supplemented with 0.004% leucine, 0.004% histidine, 0.004% methionine, 0.2% glucose and 2% ethanol and grown for approximately 16 h. β-galactosidase assays were performed with permeabilized cells (Guarente 1983).

**Electrophoretic mobility shift assay (EMSA)**

Expression and purification of Ert1(a.a.1-152), probe labeling and the EMSA were performed as described (Hellauer et al. 1996; Larochele et al. 2006). The binding reaction was performed in 4 mM Tris-HCl pH 8, 40 mM NaCl, 3 pmole/ml labeled probe and 25 ug/ml sonicated salmon sperm DNA.

**RESULTS**

**Ert1 binds to the promoters of gene involved in the utilization of ethanol as a carbon source**

Ert1 (Ybr239c) is a putative zinc cluster protein of unknown function and is a homologue of Rds2. In order to determine its function, ERT1 was tagged at its natural chromosomal location with a triple HA epitope and ChIP-chip experiments were performed using tiling microarrays.
covering the entire yeast genome with probes located, on average, at every 290 bp. The initial ChIP-chip analysis was performed with cells grown in rich medium and with glucose as a carbon source. Under these conditions, Ert1 was bound to a limited number of genes (total of 11, see Table 2 and Table S1 for a complete list). The strongest enrichment (64 fold) was observed at the promoter of \textit{PCK1}, a gene encoding phosphoenolpyruvate carboxykinase, an essential enzyme for gluconeogenesis. Ert1 was also detected at the promoter of \textit{FBP1} encoding fructose-1,6-bisphosphatase, another essential gluconeogenic enzyme. Other Ert1 targets include \textit{MAE1}, encoding an additional gluconeogenic enzyme involved in the conversion of malate to pyruvate as well as \textit{PDC1}, encoding a key enzyme for fermentation.

These results suggested that \textit{ERT1} encodes a factor involved in the utilization of non-fermentable carbon sources. To test this hypothesis, ChIP-chip assays were then performed with cells grown in the presence of ethanol as a sole carbon source. Results showed that Ert1 binds to the promoters of 70 different genes (p-value <0.05 with an enrichment >1.8). GO term analysis of Ert1 targets showed a clear enrichment for processes related to glucose metabolism (Table 3). In agreement with this analysis, inspection of Ert1 targets revealed enrichment of Ert1 at genes involved in gluconeogenesis and related processes (Table 2). Strikingly, genes bound by Ert1 under glucose conditions showed increased enrichment with cells grown in the presence of ethanol (e.g. \textit{MAE1, AQR1, VID24}). A noticeable exception is \textit{FBP1} were no significant binding of Ert1 was observed with ethanol-grown cells as opposed to a 2.7-fold enrichment in glucose-grown cells. Additional targets of Ert1 include genes encoding transcription factors such as \textit{ADR1, HAP4} (an activator of respiration genes) and \textit{GAT1} (an activator of genes involved in the utilization of poor nitrogen sources). Interestingly, Ert1 was also detected at the promoter of
PDC1 under both ethanol and glucose conditions. This gene encodes a major pyruvate carboxylase involved in fermentation via conversion of pyruvate to acetaldehyde. Standard ChIP assays for PCK1, PDC1 and FBP1 gave enrichment values highly similar to those obtained by ChIP-chip (Fig. 1).

**Ert1 is redundant with other factors involved in the utilization of ethanol as a carbon source**

We were then interested to determine if Ert1 regulates the expression of the target genes identified by ChIP-chip analysis. We performed expression profiling experiments comparing mRNA levels of wild-type and Δert1 strains grown in the presence of glucose or ethanol as a carbon source. Minimal differences were observed between the wild-type and the Δert1 strains (data not shown). A control experiment showed that a shift from glucose to ethanol resulted in a massive effect on gene expression (data not shown). The lack of correlation between ChIP-chip data and expression profiling suggested that Ert1 is redundant with other factors involved in utilization of non-fermentable carbon sources, namely ethanol. In order to test this hypothesis, we generated strains carrying double deletions of zinc cluster genes involved in utilization of non-fermentable carbon sources. Strains were grown in rich medium, washed in water and diluted before spotting on plates containing glucose or ethanol as a carbon source (Fig. 2). With plates containing glucose, growth was similar for all strains tested with the exception of Δsnfl where slightly reduced growth was observed (Fig. 2, left panel). With ethanol as a sole carbon source, no growth was observed with a strain carrying a deletion of SNFl or CAT8, either alone or in combination with other mutants (Fig. 2, right panel), a result expected from previous observations (CELENZA and CARLSON 1984; HEDGES et al. 1995). While single deletions of
ERT1, RDS2 or SIP4 had no effect on growth with ethanol as a carbon source, a strain lacking ERT1 and SIP4 showed greatly impaired growth on ethanol plates. Interestingly, no growth defect was observed with a strain carrying deletions of both ERT1 and RDS2. Collectively, these results strongly suggest that ERT1 is redundant with SIP4.

**Ert1 controls the promoter activity of the gluconeogenic genes PCK1 and FBP1**

Given these results, we were interested to determine if Ert1 controls the promoter activity of key gluconeogenic genes such as PCK1 and FBP1. Since regulation of these genes occurs at both the transcriptional and mRNA stability levels (Mercado et al. 1994), we assayed the activity of the PCK1 and FBP1 promoters using lacZ reporters transformed in the strains described above. For both reporters, background activity was measured when the wild-type strain was grown in the presence of glucose (data not shown). As expected (Proft et al. 1995), high activity of the PCK1-lacZ reporter was observed under inducing conditions (0.2% glucose and 2% ethanol) (Fig. 3, top panel). Deletion of CAT8 reduced activity by 14-fold while deletion of ERT1 or SIP4 reduced the activity only by 2-fold. In contrast, deletion of RDS2 had no significant effect on the activity of the PCK1 promoter. Similarly, with the double deletion strain ∆ert1∆sip4, no major change in activity was observed as compared to single deletion strains while deletion of Rds2 did revert the downregulation observed in the ∆ert1 strain. Finally, deletion of both CAT8 and ERT1 resulted in background activity.

With the FBP1-lacZ reporter, deletion of CAT8 strongly reduced β-galactosidase activity while a slight increase was observed for a strain lacking SIP4 (Fig. 3, bottom panel), in agreement with previous observations (Zaragoza et al. 2001). Deleting ERT1 gave β-
galactosidase activity at levels comparable to a wild-type strain. Interestingly, the double mutant \( \Delta e rt1 \Delta s ip4 \) showed a ten-fold reduction in activity while deletion of both \( ERT1 \) and \( CAT8 \) resulted in background activity. Thus, the marked reduction in activity of the \( FBP1-lacZ \) reporter in strains \( \Delta c at8, \Delta e rt1 \Delta c at8, \) and \( \Delta e rt1 \Delta s ip4 \) correlates with their impaired growth, as assayed with ethanol as the sole carbon source. In summary, our results show that Ert1 controls the activity of the \( PCK1 \) and \( FBP1 \) promoters through a complex interplay with other zinc cluster transcription factors.

**Ert1 acts as an activator and a repressor**

We then focused on additional targets of Ert1 in order to determine if these genes are also regulated by this factor using quantitative RT-PCR (qRT-PCR). Control experiments showed that no mRNA for \( ERT1, CAT8, RDS2, \) and \( SIP4 \) could be detected in the corresponding deletion strains (data not shown). We focused on 5 different genes: \( FBP1, SFC1 \) (encoding a mitochondrial transporter for succinate and fumarate), \( PDC1, ADR1, \) and \( GAT1 \). We determined changes in mRNA levels in a wild-type strain following a shift from glucose to ethanol (Fig. 4A). As expected, the expression of \( FBP1 \) and \( SFC1 \) was greatly increased (\( FBP1: \) 380 fold; \( SFC1: \) 182 fold) while the expression of the fermentation gene \( PDC1 \) was strongly repressed (33 fold) upon a shift from glucose to ethanol (BOJUNGA et al. 1998; SCHMITT et al. 1983). The expression of \( GAT1 \) and \( ADR1 \) was increased 3.2 and 5.9 fold, respectively. We then performed qRT-PCR with RNA prepared from wild-type and deletion strains that were grown under inducing conditions (0.2% glucose and 2% ethanol) (Fig. 4B). Overall, results for \( FBP1 \) correlate with those obtained with a \( FBP1-lacZ \) reporter (Fig. 3). For instance, reduced \( \beta \)-galactosidase activity and \( FBP1 \) mRNA levels were observed for the \( \Delta c at8 \) and the \( \Delta e rt1 \Delta s ip4 \) strains.
However, deletion of CAT8 reduced promoter activity by 8-fold while no FBP1 mRNA could be detected in that strain. This difference could be due to changes in FBP1 mRNA stability (MERCADO et al. 1994). As expected (BOJUNGA et al. 1998), deletion of CAT8 greatly diminished SFC1 mRNA levels while a similar effect was observed upon deletion of SIP4 (Fig. 4B). Single deletions of ERT1 or RDS2 had minor effects on expression of SFC1 but a ∆ert1∆rds2 strain showed reduction of SFC1 mRNA levels by almost 2-fold. For ADR1, single deletion strains had minor effects on expression of this gene and the strongest effect on mRNA levels was observed with the strain ∆ert1∆rds2 (approximately 2-fold reduction). Similarly for GAT1, the strongest effect (2-fold reduction) was observed with the double deletion strain ∆ert1∆sip4.

As stated above, expression of PDC1 is repressed under non-fermentative conditions (Fig. 4A). This gene encodes pyruvate decarboxylase, a key enzyme acting at the pyruvate branch-point (PRONK et al. 1996). Pyruvate, the end product of glycolysis, can either enter the TCA cycle or be converted to acetaldehyde by pyruvate decarboxylase followed by conversion to ethanol. Thus, repression of PDC1 is one mechanism allowing cells to switch from fermentative to non-fermentative metabolism. Interestingly, deletion of ERT1 increased PDC1 mRNA levels by almost 3-fold (Fig. 4B). These results suggest that Ert1 is not only involved in activation of genes for non-fermentative metabolism but is also involved, in part, in shutting down the fermentative process. Deletion of RDS2 slightly increased PDC1 mRNA levels but no additive effect was observed with the double deletion ∆ert1∆rds2. Deletion of CAT8 or SIP4 had minor effects on PDC1 mRNA levels. However, strains ∆ert1∆cat8 and ∆ert1∆sip4 had reduced mRNA levels for PDC1. We also tested whether the effect of Ert1 on PDC1 expression is
specific for the strain BT4741 or not. To this end, we generated a Δert1 strain in the W303-1A background. Deleting ERT1 resulted in increased (2.8 fold) PDC1 mRNA levels (Fig. 4C), as observed in the BY4741 background. In summary, deletion of ERT1 minimally alters the expression of gluconeogenic genes but a more pronounced effect was observed in strains lacking ERT1 in combination with CAT8, SIP4 or RDS2. These results strongly suggest redundancy of function among these transcriptional regulators.

**Ert1 binds directly to the PCK1 and PDC1 promoters.**

We were interested to determine if Ert1 directly interacts with DNA and, if so, what is the DNA recognition site? The DNA binding domain of Ert1 (a.a. 1 to 152) was expressed in bacteria as a GST fusion protein, purified and the GST moiety removed by thrombin cleavage. The purified DNA binding domain of Ert1 was used in an electrophoretic mobility shift assay (EMSA). We initially focused on the PCK1 promoter. A series of DNA probes spanning the regulatory region of PCK1 (UAS1, UAS2, URS) was tested for Ert1(1-152) binding (Fig. 5A). Binding was detected only when using probe X (Fig. 5B, left panel). No Ert1(1-152) binding was observed when using probes located between probes Z2 and Z3 (data not shown). Thus, Ert1(1-152) binds to a region located upstream of UAS1 (a regulatory site recognized by the factors Cat8 and Sip4). With probe X, two complexes were observed. We compared the relative mobility of the Ert1(1-152)-DNA complex to zinc cluster proteins that bind DNA as homodimers (e.g. Gal4). Results suggest that the fast migrating complex is a monomer while the slow migrating complex would composed of two monomers bound to separate sites on probe X (data not shown). Use of the smaller probes Z4 and Z5 resulted in the formation of a single Ert1(1-152)-DNA complex (Fig. 5B, middle panel). In addition, Ert1(1-152) bound to two smaller
probes (Z6, Z7) that do not overlap. Binding at Z7 was weaker when compared to site Z6. Thus, Ert1 binds to two distinct sites in the PCK1 promoter. As stated above, zinc cluster proteins preferentially bind to CGG triplets (MacPherson et al. 2006). As shown in Fig. 5C, the Z6 probe contains two CGG triplets. Mutating CGG #2 to CTG had no effect on Ert1(1-152) binding. In contrast, a mutation in CGG #1 almost completely abolished binding of Ert1(1-152). Three CGG triplets are found in probe Z7; strikingly, only a mutation in CGG #2 reduced binding of Ert1(1-152).

We used the same approach described above to identify Ert1 binding site(s) in the PDC1 promoter (Fig. 6). Since ChIP-chip experiments suggested that Ert1 binds to a site located at approximately 500 bp upstream of the ATG, we designed 5 probes (A to E) covering this region, including the UAS (Fig. 6A). Binding of Ert1(1-152) was observed only with probe B (Fig. 6B). In analogy to PCK1, two Ert1(1-152)-DNA complexes were observed with that probe. With smaller probes (F, G, H), only a single complex of fast mobility was observed (Fig. 6, right panel). Binding of Ert1(1-152) was also observed with probe I that does not overlap with probes G or H (see below). Thus, the PDC1 promoter contains two binding sites for Ert1. We then performed mutational analysis of these two sites. Probe H contains three CGG triplets (Fig. 7A). Slightly reduced binding of Ert1(1-152) was observed when mutating CGG #1 or #3. In contrast, mutation in the second CGG triplet completely abolished binding of Ert1(1-152) (Fig. 7A top, panel). We tested additional mutations in the region encompassing CGG triplet #2 using transversions. No reduced binding of Ert1(1-152) was observed when mutating nucleotides labeled a, b, i, j, k, and l (Fig. 7A). However, diminished binding was observed with mutations at positions c, d, e, f, and h. A similar scan was performed with probe I (Fig. 7B). EMSA analysis
showed that nucleotides located at positions c, d, e, f, g and, to a lesser extent, nucleotide at position b are critical for Ert1. Taken together, results show that the binding site of Ert1 at PDC1 is CGGAAY (where Y is C or T).

**Mutations that prevent Ert1 binding result in increased activity of a PDC1-lacZ reporter.**

We were interested to determine the effect of mutations that prevent binding of Ert1 on the PDC1 promoter activity. To this end, we constructed a PDC1-lacZ reporter that was integrated at the URA3 locus (Fig. 8A). Three mutant versions were also tested. Mutant #1 has a mutation in the second CGG triplet while Mutant #2 has a mutation in the first CGG triplet. These mutations abolished binding of Ert1(1-152) in vitro (Fig. 7). A double mutant was also tested (Fig. 8A). As expected with a wild-type PDC1 promoter (Butler and McConnell 1988), high β-galactosidase activity was observed with cells grown in the presence of glucose while growth with ethanol as a carbon source gave background activity (Fig. 8B). With glucose, Mutants #1 and #2 gave β-galactosidase activity similar to the wild-type reporter. Interestingly, increased activity was observed for both mutants when assayed with ethanol. Finally, Mutant #3 (carrying mutations in both CGG triplets) showed slightly increased β-galactosidase activity with glucose as compared to wild-type PDC1 while the activity was also slightly increased in the presence of ethanol when compared to Mutants #1 and #2. In a Δert1 strain grown in the presence of ethanol, PDC1 mRNA levels are at approximately 10% of the levels measured in a wild-type strain grown in the presence of glucose (Fig. 4A and B). Strikingly, a similar ratio (12%) was observed with lacZ reporters. Taken together, our results strongly suggest that Ert1 represses PDC1 promoter activity by binding directly to sites adjacent to the UAS.
DISCUSSION

In this study, we have characterized the ORF YBR239C encoding a putative transcriptional regulator of the family of zinc cluster proteins and we named this gene ERT1 (Ethanol Regulated Transcription factor 1). Fig. 10 provides a schematic view of the pathways of fermentative and non-fermentative metabolism as well as some target genes of Ert1. ChIP-chip analysis showed that Ert1 is bound to a limited number of genes (total of 11), as assayed in rich medium supplemented with glucose. The strongest enrichment (64-fold) was observed at the promoter of the gluconeogenic gene PCK1 (Table 2). Given this result, we hypothesized that Ert1 is involved in regulating gluconeogenesis. When the ChIP-chip assay was repeated in the presence of ethanol as a carbon source, binding of Ert1 was detected at 70 promoters including the one of PYC1, another gluconeogenic gene. A similar phenomenon was observed for Rds2 where an increased number of targets was observed with non-fermentable carbon sources as compared to glucose (SOONTORNGUN et al. 2012; SOONTORNGUN et al. 2007). A number of Ert1 target genes encode mitochondrial components. For example, SFC1 (ACR1) encodes a fumarate/succinate transporter while the FMP43 gene product is a subunit of the pyruvate transporter (BRICKER et al. 2012; HERZIG et al. 2012; PALMIERI et al. 1997). Some other Ert1 targets (PET9, FMP48, TAM41, RMP2, QCR6, and YHR080C) also encode mitochondrial proteins.

Ert1 is a DNA binding protein

EMSA analysis showed that Ert1(1-152) binds to specific DNA sequences in the PCK1 and the PDC1 promoters. Mutational analysis of the Ert1 binding sites at PDC1 suggests that the DNA recognition sequence of this factor is CGGAAY (where Y is C or T) (Fig. 7). Strikingly,
this matches exactly the consensus sequence obtained by protein binding microarray analysis (BADIS et al. 2008). One site identified at PCK1 has the sequence CGGAAA suggesting that the last position can tolerate some changes. In agreement with this observation, mutating the sequence CGGAAC found in PDC1 to CGGAAA reduced, but did not abolish, binding of Ert1(1-152) (Fig. 7B). A similar affect was observed with the mutation CGGAAT to CGGAAG (Fig. 7A). Finally, Ert1 bound weakly to probe Z7 located in the PCK1 promoter (Fig. 6D), an observation that could be explained by the fact that this site (CGGAGAA) does not fit the consensus sequence. Our EMSA analysis suggests that Ert1(1-152) binds as a monomer. Although binding of a zinc cluster proteins to DNA as monomer has been reported (CAHUZAC et al. 2001), it is quite possible that Ert1 binds to DNA as a homo- or a heterodimer. For example, the DNA binding domain used in our studies may lack a dimerization domain. Alternatively, Ert1 may bind with a partner that remains to be identified. AcuM and AcuK are two Aspergillus nidulans homologues of Ert1/Rds2. AcuM and AcuK also are involved in the regulation of gluconeogenic genes and, interestingly, bind to DNA in an interdependent manner (SUZUKI et al. 2012).

**Redundancy of Ert1 with other transcriptional regulators**

Our ChIP-chip analysis suggests that Ert1 is involved in controlling the expression of genes involved in the utilization of non-fermentable carbon sources such as ethanol. However, expression profiling using wild-type and Δert1 strains did not reveal any significant changes in expression of the Ert1 targets that were identified by ChIP-chip analysis (data not shown). These results suggest redundancy among transcriptional regulators. In agreement with these results, deletion of ERT1 did not impair growth on plates containing ethanol as a carbon source (Fig. 2).
However, a strain carrying deletions of both $ERT1$ and $SIP4$ showed greatly reduced growth when assayed with ethanol as a carbon source. These observations correlate with the minimal effect of a deletion of $ERT1$ on the activity of a $FBP1$-lacZ reporter. This contrasts with the marked reduction of promoter activity observed in a $\Deltaert1\Delta sip4$ strain (Fig. 3). These results were confirmed by qRT-PCR analysis (Fig. 4B). Unexpectedly, Ert1 was detected at the $FBP1$ promoter under glucose conditions but not in the presence of ethanol as determined by ChIP-chip and standard ChIP analysis (Table 2, Fig. 1). This lack of correlation between elevated expression of $FBP1$ and binding of Ert1 may be due to the possibility that, upon a shift to ethanol, binding of additional factors at the $FBP1$ promoter masks the epitope of HA-Ert1. Alternatively, the effect of Ert1 on expression of $FBP1$ may be indirect. For instance, in the presence of ethanol, Ert1 may increase the expression of a gene encoding positive regulator of $FBP1$.

**Ert1 is a repressor of the fermentation gene $PDC1$**

As stated above, Pdc1 is a key enzyme for fermentation and its pyruvate decarboxylase activity is elevated under glucose conditions promoting fueling of the glycolytic product pyruvate towards ethanol production. In contrast, low levels of pyruvate decarboxylase activity are observed with cells shifted to non-fermentative metabolism (Promk et al. 1996). A major level of this regulation occurs via control of $PDC1$ transcription (Butler and McConnell 1988). With glucose as a carbon source, the transcription factor Pdc2 allows high levels of $PDC1$ expression (Hohmann 1993). However, it was not known how repression of $PDC1$ in the presence of ethanol is exerted. Our results show that Ert1 is responsible in part for this repression since a 3-fold increase of $PDC1$ mRNA levels is observed in cells lacking Ert1, as
assayed in ethanol (Fig. 4B). In agreement with these results, mutations that prevent binding of Ert1(1-152) in vitro result in increased activity of a PDC1-lacZ reporter (Fig. 8). These results suggest that Ert1 can act both as a transcriptional repressor and an activator, as observed for Rds2 (SOONTORNGUN et al. 2007). Rds2 also binds to the PDC1 promoter (SOONTORNGUN et al. 2007) and cells lacking RDS2 show a slight increase in PDC1 mRNA levels. However, a double mutant (Δert1Δrds2) had PDC1 mRNA at lower levels as compared to a Δert1 strain. The effect of these two factors may be more apparent when assayed during the transition from fermentative to non-fermentative metabolism. It is also likely that additional transcriptional regulators control the expression of PDC1.

Transcriptional regulators of gluconeogenesis have common and distinct target genes

In analogy to the role played by PDC1 for fermentative metabolism, PCK1 encodes a key enzyme for committing cells towards gluconeogenesis. Gsm1 (Glucose Starvation Modulator 1) is another zinc cluster protein that may control gluconeogenesis (TURCOTTE et al. 2011). This factor binds to the PCK1 promoter under glucose (VAN BAKEL et al. 2008) and ethanol conditions (N. Gasmi, unpublished results). Remarkably, Ert1, Cat8, Sip4, Rds2, Gsm1, and Adr1 all bind to the PCK1 promoter, as determined by ChIP-chip analysis (HARBISON et al. 2004; SOONTORNGUN et al. 2007; TACHIBANA et al. 2005; VAN BAKEL et al. 2008). Thus, the multiplicity of transcription factors bound to PCK1 may allow integration of multiple signals for a precise regulation of this key gluconeogenic gene. However, it is more common for these factors to have both common and distinct targets. For example, we have shown that approximately one third of the Rds2 targets are bound by Adr1 (SOONTORNGUN et al. 2012). Similarly, Adr1 and Cat8 have common and distinct target genes (TACHIBANA et al. 2005). With
respect to Ert1, our results show that this regulator shares many targets with Rds2 and Adr1 while the overlap with Cat8 is minimal (Fig. 9). These observations point to a complex combinatorial network of transcription factors involved in the utilization of non-fermentable carbon sources. Bmh proteins (also called 14-3-3 proteins) may also play an important role in this process. Adr1 and Cat8 are both required for maximal expression of the ADH2 gene (encoding alcohol dehydrogenase). Interestingly, inactivation of Bmh1 and 2 bypasses the requirement of Cat8 for expression of ADH2 (but not FBP1) (BRAUN et al. 2013). It has been proposed that Bmh1 and 2 are involved in combinatorial regulation of gene expression (BRAUN et al. 2013). It will be interesting to determine if Bmh proteins control the activity of additional transcriptional regulators of non-fermentative metabolism.

Interplay among transcriptional regulators of gluconeogenesis

An additional level of regulation is exerted by the interplay among these transcriptional regulators. Expression of ERT1 and RDS2 does not vary much under fermentative and non-fermentative conditions (SOONTORNGUN et al. 2007 and unpublished results). However, expression of CAT8, SIP4, GSM1, and ADR1 is increased under low glucose levels (Fig. 4B and refs. (HEDGES et al. 1995; LESAGE et al. 1996; ROBERTS and HUDSON 2006)). Similarly, expression of HAP4, encoding the limiting subunit of the Hap2/3/4/5 complex involved in the up-regulation of respiration genes, is increased upon a shift to respiration (DERISI et al. 1997; FORSBURG and GUARENTE 1989; SOONTORNGUN et al. 2007). Cat8 controls the expression of SIP4 (LESAGE et al. 1996) while Ert1 and Rds2 control, in part, the expression of ADR1 (Fig. 4B). Finally, Rds2 is a regulator of the expression of HAP4 (SOONTORNGUN et al. 2007). These observations suggest an elaborate interplay among these factors involving complex dynamics. In
summary, our studies on ERT1 have led to the identification of a novel factor involved in the regulation of gluconeogenesis as well as a key fermentation gene.

ACKNOWLEDGMENTS

We thank Drs. Peter Kötter, Karl-Dieter Entian, Juana Maria Gancedo, and Hans-Joachim Schüller for the gift of reporter plasmids. We are grateful to Dr. Nitnipa Soontorngun, Xiao Bei Liang and Piyasuda Thepnok for their input in this project. We thank Dr. Hugh Bennett for critical review of the manuscript. This work was supported by a grant to B.T. from the Natural Sciences and Engineering Research Council of Canada. F.R. was supported by a grant from the Canadian Institutes of Health Research (grant # 82891).

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FIGURE LEGENDS

Fig. 1

Confirmation of ChIP-chip results for selected genes by standard ChIP.

ChIP assays were performed with the PCK1, PDC1 and FBP1 genes using qPCR. Enrichment was calculated by normalization to signals obtained with an untagged strain and ARNI as an internal control.

Fig. 2

Redundancy among transcriptional regulators of gluconeogenesis.

Wild-type (BY4741) and deletion strains (Table 1) were grown overnight in YPD and washed twice in H2O. Cells were then serially diluted (from left to right: 25-, 100-, 400-, and 2000-fold). Ten µl of diluted cells were spotted on minimal plates supplemented with 0.2% casamino acids
and histidine, leucine, methionine, uracil (each at 0.004%) and 2% glucose or 2% ethanol, as indicated on top of the Fig. Glucose and ethanol plates were incubated at 30°C for one and five days, respectively.

**Fig. 3**

**Ert1 controls the activity of the PCK1 and FBP1 promoters.**

Wild-type strain BY4741 (“WT”) and strains carrying deletion of genes encoding various zinc cluster proteins (as indicated at the bottom) were transformed with the lacZ reporters for PCK1 or FBP1. Cells were grown in the presence of 0.2% glucose and 2% ethanol to induce reporter activity (see Material and Methods for more details).

**Fig. 4**

**Expression of Ert1 target genes in strains lacking various transcriptional regulators**

A) Wild-type strain BY4741 was grown in the presence of 2% glucose or 2% ethanol and RNA isolated for qRT-PCR analysis. Fold changes are given as ratios between mRNA levels measured in ethanol- and glucose-grown cells.

B) Wild-type strain BY4741 and deletion strains (Table 1) were grown in the presence of 2% ethanol and RNA isolated for qRT-PCR analysis. mRNA levels were measured for various genes (as indicated at the bottom of each panel) and are given relative to the wild-type strain (100%).

C) Same as in “B” with the exception that strain W303-1A was used instead of BY4741.

**Fig. 5**

**Ert1 binds to the PCK1 promoter**
A) Schematic view of the *PCK1* promoter. Coordinates (in bp) are given relative to the ATG (+1 bp). Upstream activating sequences (UAS1 and UAS2) and an upstream repressing sequence (URS) (ref. (PROFT *et al.* 1995)) are shown as rectangles. Probes used for EMSA are shown as black lines.

B) Ert1 (a.a. 1-152) binds to two sites in the *PCK1* promoter. Various probes were incubated (“+”) or not “-“ with the purified DNA binding domain of Ert1 for EMSA analysis.

C) Mutational analysis of the binding site Z6 of Ert1. DNA sequence of probe Z6 (see “A”) is shown on top as well as the two CGG triplets (labeled “CGG #1” and “2”). The effect of mutating either CGG triplets to CTG on Ert1 binding was tested by EMSA. “WT”, wild-type.

D) Mutational analysis of the binding site Z7 of Ert1. DNA sequence of probe Z7 (see “A”) is shown on top as well as the three CGG triplets (labeled “CGG #1”, “2” and “3”). The effect of mutating either CGG triplets to CTG on Ert1 binding was tested by EMSA. “WT”, wild-type.

**Fig. 6**

**Ert1 binds to the *PDC1* promoter**

A) Schematic view of the *PDC1* promoter. Coordinates (in bp) are given relative to the ATG (+1 bp). The upstream activating sequence (UAS) (ref. (BUTLER and MCCONNELL 1988)) is shown as a rectangle. Probes used for EMSA are shown as black lines.

B) Various probes were incubated (“+”) or not “-“ with the purified DNA binding domain of Ert1 for EMSA analysis. Probe I was tested in Fig. 7.

**Fig. 7**

**Ert1 binds to two sites in the *PDC1* promoter**
A) Mutational analysis of the binding site “H” of PDC1. DNA sequence of probe H (Fig. 6A) is shown on top as well as the three CGG triplets (labeled “CGG #1”, “2” and “3”). Letters (a to l) correspond to nucleotides that were mutated. The effect of mutating either CGG triplets to CTG on Ert1 binding was tested by EMSA using the purified DNA binding domain of Ert1 (a.a. 1-152), as shown on the top right panel. Additional nucleotides were also mutated (lower panel). “WT”, wild-type.

B) Mutational analysis of the binding site “I” of Ert1. DNA sequence of probe I (Fig. 6A) is shown on the left as well as the two CGG triplets (labeled “CGG #1” and “2”). Letters (a to h) correspond to nucleotides that were mutated. Mutants were analyzed by EMSA using the purified DNA binding domain of Ert1 (a.a. 1-152) (right panel). “WT”, wild-type.

Fig. 8

Mutations that prevent binding of Ert1 to the PDC1 promoter result in increased activity of a PDC1-lacZ reporter.

A) Schematic view of the PDC1-lacZ reporter. The grey rectangle corresponds to the UAS (upstream activating sequence) present in the PDC1 promoter. The DNA sequence of the region bound by Ert1 is shown below. The key nucleotides for Ert1 binding are boxed and the CGG triplets (opposite strand) necessary for Ert1 binding are in bold characters. Mutations that were introduced in the reporter are underlined and in bold characters.

B) Strain YPH499 containing a wild-type PDC1-lacZ reporter (or mutants) integrated at the URA3 locus was grown in the presence of 2% glucose or 2% ethanol and β-galactosidase activity measured (see Material and Methods for more details). Results are the average of two experiments performed in duplicate and with two independent strains. In addition to histograms,
one numerical value is given when a low $\beta$-galactosidase activity was measured. “WT”, wild-type.

**Fig. 9**

*Overlap between Ert1 targets and those of Cat8, Rds2 and Adr1.*

Values in the Venn diagram (left panel) refer to the number of genes that are common targets for specified regulators. The number of overlapping genes with Ert1 and a specific regulator is also indicated on the right panel. Forty-one percent ($29/70$) of the Ert1 target genes did not overlap with the Cat8, Rds2 and Adr1 targets. ChIP-chip data for Adr1 and Cat8 were taken from (Tachibana et al. 2005) while data for Rds2 were from (Soontorngun et al. 2007). ChIP-chip data for Cat8 and Adr1 were obtained with cells grown under low glucose conditions while the assay for Rds2 was performed with ethanol as a carbon source.

**Fig. 10**

*Major Ert1 targets in the pathways of fermentative and non-fermentative metabolism.*

Some of the Ert1 target genes are indicated in bold characters. Only the mitochondrial compartment is shown in this Fig. *SFC1* encode a fumarate/succinate transporter and *FMP43* encode a subunit of the pyruvate transporter. *PET9* encodes an ATP/ADP carrier.

**Table 1**

*Strains used in this study.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>N/A</td>
<td><em>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</em></td>
<td>(Brachmann et al. 1998)</td>
</tr>
<tr>
<td>SC12</td>
<td>BY4741</td>
<td><em>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [ERT1 tagged]</em></td>
<td>This study</td>
</tr>
</tbody>
</table>
with three HA epitopes at the N-terminus]

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Genotypes</th>
<th>Mutations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC102A</td>
<td>BY4741</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cat8Δ::KanMX</td>
<td>(WINZELER et al. 1999)</td>
<td></td>
</tr>
<tr>
<td>SC103</td>
<td>BY4741</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sip4Δ::KanMX</td>
<td>(WINZELER et al. 1999)</td>
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</tr>
<tr>
<td>SC104</td>
<td>BY4741</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rds2Δ::KanMX</td>
<td>(WINZELER et al. 1999)</td>
<td></td>
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<tr>
<td>SC105A</td>
<td>BY4741</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ert1Δ::KanMX</td>
<td>(WINZELER et al. 1999)</td>
<td></td>
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<tr>
<td>SC173</td>
<td>BY4741</td>
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<td>(WINZELER et al. 1999)</td>
<td></td>
</tr>
<tr>
<td>SC111A</td>
<td>SC102A</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cat8Δ::KanMX ert1Δ::CgHIS3</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>SC113</td>
<td>SC103</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sip4Δ::KanMX ert1Δ::CgHIS3</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>SC114</td>
<td>SC104</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rds2Δ::KanMX ert1Δ::CgHIS3</td>
<td>This study</td>
<td></td>
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<tr>
<td>W303-1A</td>
<td>N/A</td>
<td>MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</td>
<td>(ROTHSTEIN et al. 1977)</td>
<td></td>
</tr>
<tr>
<td>SC14</td>
<td>W303-1A</td>
<td>MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ert1Δ::CgHIS3</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>YPH499</td>
<td>N/A</td>
<td>MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</td>
<td>(SIKORSKI and HIETER 1989)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2

Selected Ert1 target genes.

Major targets of Ert1 are listed (P <0.05 and with enrichment values >1.8). Gene functions were taken from the *Saccharomyces cerevisiae* database ([http://www.yeastgenome.org/](http://www.yeastgenome.org/)). Enrichment at a particular promoter is given for cells grown in glucose and ethanol; NS: not significant. ChIP-chip data for Adr1 and Cat8 (obtained under low glucose conditions) were taken from ([Tachibana et al. 2005](#)) while data for Rds2 (obtained with ethanol) were taken from ([Soontorngun et al. 2007](#)). For a complete list of targets, see Supplementary Table S1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Systematic name</th>
<th>Function</th>
<th>Enrichment (glucose)</th>
<th>Enrichment (ethanol)</th>
<th>Binding of Adr1, Cat8 or Rds2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PCK1</em></td>
<td>YKR097W</td>
<td>Phosphoenolpyruvate carboxykinase, key enzyme in the gluconeogenesis pathway</td>
<td>64</td>
<td>47</td>
<td>Adr1, Cat8, Rds2</td>
</tr>
<tr>
<td><em>MAE1</em></td>
<td>YKL029C</td>
<td>Mitochondrial malic enzyme, catalyzes the oxidative decarboxylation of malate to pyruvate</td>
<td>11</td>
<td>35</td>
<td>Rds2</td>
</tr>
<tr>
<td><em>PDC1</em></td>
<td>YLR044C</td>
<td>Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation</td>
<td>4.8</td>
<td>26</td>
<td>Rds2</td>
</tr>
<tr>
<td><em>GAT1</em></td>
<td>YFL021W</td>
<td>Transcriptional activator of genes involved in nitrogen catabolite repression</td>
<td>NS</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td><em>AQR1</em></td>
<td>YNL065W</td>
<td>Plasma membrane transporter of the major facilitator superfamily</td>
<td>6.1</td>
<td>15</td>
<td>Rds2</td>
</tr>
<tr>
<td><em>ICY1</em></td>
<td>YMR195W</td>
<td>Protein of unknown function, required for viability in rich media of cells lacking mitochondrial DNA</td>
<td>2.7</td>
<td>14</td>
<td>Adr1, Cat8, Rds2</td>
</tr>
<tr>
<td><em>LSC2</em></td>
<td>YGR244C</td>
<td>β-subunit of succinyl-CoA ligase</td>
<td>3.0</td>
<td>14</td>
<td>Adr1, Rds2</td>
</tr>
<tr>
<td><em>VID24</em></td>
<td>YBR105C</td>
<td>GID complex regulatory subunit; regulates fructose-1,6-bisphosphatase targeting to the vacuole</td>
<td>4.4</td>
<td>9.4</td>
<td>Rds2</td>
</tr>
<tr>
<td><em>PET9</em></td>
<td>YBL30C</td>
<td>Major ADP/ATP carrier of the mitochondrial inner membrane; exchanges cytosolic ADP for mitochondrially synthesized ATP; also imports heme and ATP</td>
<td>2.2</td>
<td>7.1</td>
<td>Rds2</td>
</tr>
<tr>
<td><em>OP11</em></td>
<td>YHL020C</td>
<td>Negative regulator of phospholipid biosynthetic genes</td>
<td>1.9</td>
<td>7.0</td>
<td>Rds2</td>
</tr>
<tr>
<td><em>FBP1</em></td>
<td>YLR377C</td>
<td>Fructose-1,6-bisphosphatase, key regulatory enzyme in the gluconeogenesis pathway</td>
<td>2.7</td>
<td>NS</td>
<td>Adr1, Cat8, Rds2</td>
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<td><em>FMP48</em></td>
<td>YGR052W</td>
<td>Putative protein of unknown function;</td>
<td>NS</td>
<td>4.7</td>
<td>Adr1, Rds2</td>
</tr>
<tr>
<td>Gene</td>
<td>Method</td>
<td>Function</td>
<td>Expression Level</td>
<td>Regulators</td>
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<td>------------</td>
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<tr>
<td>YGR050C</td>
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<td>the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies</td>
<td>Unknown function</td>
<td></td>
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<td>RPM2</td>
<td>YML091C</td>
<td>Protein subunit of mitochondrial RNase P</td>
<td>NS</td>
<td>4.6 Rds2</td>
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<td>YPC1</td>
<td>YBR183W</td>
<td>Alkaline ceramidase</td>
<td>NS</td>
<td>4.5 -</td>
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<td>-</td>
<td>YBR182C-A</td>
<td>Putative protein of unknown function</td>
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<tr>
<td>-</td>
<td>YEL020C-B</td>
<td>Dubious open reading frame</td>
<td>NS</td>
<td>4.4 -</td>
<td></td>
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<td>PTR2</td>
<td>YKR093W</td>
<td>Integral membrane peptide transporter, mediates transport of di- and tri-peptides</td>
<td>NS</td>
<td>4.2 Adr1, Cat8, Rds2</td>
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<tr>
<td>QDR3</td>
<td>YBR043C</td>
<td>Multidrug transporter of the major facilitator superfamily</td>
<td>NS</td>
<td>4.2 Rds2</td>
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<td>PHD1</td>
<td>YKL044W</td>
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<td>NS</td>
<td>3.3 -</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>YKL043W</td>
<td>Transcriptional activator that enhances pseudohyphal growth</td>
<td></td>
<td></td>
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<tr>
<td>HXT8</td>
<td>YJL214W</td>
<td>Protein of unknown function with similarity to hexose transporter family members, expression is induced by low levels of glucose and repressed by high levels of glucose</td>
<td>NS</td>
<td>3.3 Rds2</td>
<td></td>
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<tr>
<td>IMA5</td>
<td>YJL216C</td>
<td>Alpha-glucosidase</td>
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<td>PMA1</td>
<td>YGL008C</td>
<td>Plasma membrane H\textsuperscript+ATPase</td>
<td>NS</td>
<td>3.2 -</td>
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<tr>
<td>TKL1</td>
<td>YPR074C</td>
<td>Transketolase of the pentose phosphate pathway</td>
<td>NS</td>
<td>2.9 Rds2</td>
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<tr>
<td>DIP5</td>
<td>YPL265W</td>
<td>Dicarboxylic amino acid permease; mediates high-affinity and high-capacity transport of L-glutamate and L-aspartate</td>
<td>NS</td>
<td>2.7 Adr1</td>
<td></td>
</tr>
<tr>
<td>ATO2</td>
<td>YNR002C</td>
<td>Putative transmembrane protein involved in export of ammonia</td>
<td>NS</td>
<td>2.6 Adr1, Rds2</td>
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<td>HAP4</td>
<td>YKL109W</td>
<td>Limiting subunit of the heme-activated, glucose-repressed Hap2/3/4/5 complex involved in regulation of respiration genes</td>
<td>NS</td>
<td>2.6 Adr1, Rds2</td>
<td></td>
</tr>
<tr>
<td>GID8</td>
<td>YMR135C</td>
<td>Subunit of the GID complex involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase</td>
<td>NS</td>
<td>2.3 Adr1, Rds2</td>
<td></td>
</tr>
<tr>
<td>FMP43</td>
<td>YGR243W</td>
<td>Highly conserved subunit of the mitochondrial pyruvate carrier; a mitochondrial inner membrane complex comprised of Fmp37p/Mpc1p and either Mpc2p or Fmp43p/Mpc3p mediates mitochondrial pyruvate uptake; more highly expressed in glucose-containing minimal medium than in lactate-containing medium</td>
<td>NS</td>
<td>2.1 Adr1</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>YPR036W-A</td>
<td>Protein of unknown function; transcription is regulated by Pdr1</td>
<td>NS</td>
<td>2.1 Adr1, Cat8</td>
<td></td>
</tr>
<tr>
<td>QCR6</td>
<td>YFR033C</td>
<td>Subunit 6 of the ubiquinol cytochrome-c reductase complex</td>
<td>NS</td>
<td>2.1 -</td>
<td></td>
</tr>
<tr>
<td>ADR1</td>
<td>YDR216W</td>
<td>Carbon source-responsive zinc-finger</td>
<td>NS</td>
<td>1.9 Adr1, Cat8</td>
<td></td>
</tr>
</tbody>
</table>
transcription factor, required for transcription of peroxisomal protein genes, and of genes required for ethanol, glycerol, and fatty acid utilization

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Fraction of the query</th>
<th>Fraction of the query/fraction of the genome (enrichment)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYC1 YGL062W</td>
<td>Pyruvate carboxylase isoform involved in the conversion of pyruvate to oxaloacetate</td>
<td>NS</td>
<td>1.9</td>
<td>-</td>
</tr>
<tr>
<td>HXT2 YMR011W</td>
<td>High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose</td>
<td>NS</td>
<td>1.8</td>
<td>Adr1</td>
</tr>
<tr>
<td>SFP1 YLR403W</td>
<td>Regulates transcription of ribosomal protein and biogenesis genes; regulates response to nutrients and stress</td>
<td>NS</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>SFC1 YJR095W</td>
<td>Mitochondrial succinate-fumarate transporter; required for ethanol and acetate utilization</td>
<td>NS</td>
<td>1.8</td>
<td>Adr1, Cat8, Rds2</td>
</tr>
</tbody>
</table>

Table 3

Gene ontology (GO) enrichment of the Ert1 target genes identified by ChIP-chip analysis.

<table>
<thead>
<tr>
<th>GO term (process)</th>
<th>Fraction of the query</th>
<th>Fraction of the query/fraction of the genome (enrichment)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose metabolism</td>
<td>8 out of 75 genes</td>
<td>9.7</td>
<td>4.8 X10^{-4}</td>
</tr>
<tr>
<td>Hexose metabolism</td>
<td>8 out of 75 genes</td>
<td>8.2</td>
<td>1.6 X10^{-3}</td>
</tr>
<tr>
<td>Monosaccharide metabolism</td>
<td>8 out of 75 genes</td>
<td>7.6</td>
<td>2.7 X10^{-3}</td>
</tr>
<tr>
<td>Gluconeogenesis</td>
<td>5 out of 75 genes</td>
<td>16.8</td>
<td>4.0 X10^{-3}</td>
</tr>
<tr>
<td>Hexose biosynthetic process</td>
<td>5 out of 75 genes</td>
<td>16.8</td>
<td>4.0 X10^{-3}</td>
</tr>
</tbody>
</table>
A) Fold change in wild-type strain: ethanol vs glucose

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBP1</td>
<td>380</td>
</tr>
<tr>
<td>SFC1</td>
<td>182</td>
</tr>
<tr>
<td>PDC1</td>
<td>-33.3</td>
</tr>
<tr>
<td>ADR1</td>
<td>5.9</td>
</tr>
<tr>
<td>GAT1</td>
<td>3.2</td>
</tr>
</tbody>
</table>

B) Relative mRNA levels (%)

C) Relative mRNA levels (%)

Strain: Δert1 Δcat8 Δsip4 Δrds2 Δert1 Δcat8 Δrds2 Δert1 Δrds2 Δert1 Δsip4

Strain: W303-1A Δert1

Strain: W303-1A Δert1
**A)**

Diagram of the PCK1 promoter showing various elements labeled from -650 to -350 with UAS1, UAS2, and URS, among others.

**B)**

A series of autoradiograms showing the effect of Ert1 with and without the presence of probes labeled as W to Z7.

**C)**

sequences for CGG#1 and CGG#2:

- CGG#1: CTTGTCGGAAAAATCGGAATA
- CGG#2: GAACAGCCTTTTTTAGCCTTAT

**D)**

sequences for CGG#2 and CGG#3:

- CGG#2: TCCACCAGAAATATCGGGACGCT
- CGG#3: AGGTGGCCTCTTATAGCCTGCGA

Probes Z6 and Z7 with mutations: CGG → CTG.
A)  

PDC1 promoter

-850 -620 -590 -560 +52

UAS

lacZ

Wild-type
GTGGTTCCGGCTTCCCCTCCCGATTCCGGCCCG

Mut. #1
GTGGTTCCGGCTTCCCCTCCCGATTCA GCCCG

Mut. #2
GTGGTTCAA GGCTTCCCCTCCCGATTCCGGCCCG

Mut. #3
GTGGTTCAA GGCTTCCCCTCCCGATTCA GCCCG

B)  

β-galactosidase activity

Glucose  Ethanol

PDC1:

L  WT  L  Mut. #1  L  Mut. #2  L  Mut. #3

0.2 unit

0  5  10  15  20  25  30  35  40  45  50  60  70  80  90  100  110  120
Rds2 vs Ert1: 32 common targets
Adr1 vs Ert1: 25 common targets
Cat8 vs Ert1: 9 common targets