

# The Switch from Fermentation to Respiration in *Saccharomyces cerevisiae* Is Regulated by the Ert1 Transcriptional Activator/Repressor

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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 quantitative RT-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.

**I**N 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 nonfermentable carbon sources, such as lactate, acetate, or glycerol, can also be used by yeast (Turcotte *et al.* 2011). The shift from fermentative to nonfermentative 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 (Hedbacker and Carlson 2008; Zaman *et al.* 2008; Zhang *et al.* 2010; Broach 2012). 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 Cys<sub>2</sub>His<sub>2</sub> 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 >100 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

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**Table 1** Strains used in this study

| Strain  | Parent  | Genotype  | Reference                      |
|---------|---------|---|--------------------------------|
| BY4741  | N/A     | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>  | Brachmann <i>et al.</i> (1998) |
| SC12    | BY4741  | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> [ERT1 tagged with three HA epitopes at the N-terminus] | This study                     |
| SC102A  | BY4741  | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cat8Δ::KanMX</i>   | Winzeler <i>et al.</i> (1999)  |
| SC103   | BY4741  | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sip4Δ::KanMX</i>   | Winzeler <i>et al.</i> (1999)  |
| SC104   | BY4741  | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rds2Δ::KanMX</i>   | Winzeler <i>et al.</i> (1999)  |
| SC105A  | BY4741  | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ert1Δ::KanMX</i>   | Winzeler <i>et al.</i> (1999)  |
| SC173   | BY4741  | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1Δ::KanMX</i>   | Winzeler <i>et al.</i> (1999)  |
| SC111A  | SC102A  | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cat8Δ::KanMX ert1Δ::CgHIS3</i>                             | This study                     |
| SC113   | SC103   | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sip4Δ::KanMX ert1Δ::CgHIS3</i>                             | This study                     |
| SC114   | SC104   | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rds2Δ::KanMX ert1Δ::CgHIS3</i>                             | This study                     |
| W303-1A | N/A     | <i>MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</i>                                 | Rothstein <i>et al.</i> (1977) |
| SC14    | W303-1A | <i>MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ert1Δ::CgHIS3</i>                   | This study                     |
| YPH499  | N/A     | <i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>                                | Sikorski and Hieter (1989)     |

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<sub>2</sub>CysX<sub>6</sub>CysX<sub>5-12</sub>CysX<sub>2</sub>CysX<sub>6-8</sub>Cys) 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 (CGGNxCGG), inverted (CGGNxCCG), or everted repeats (CCGNxCGG) (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 nonfermentable 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 homolog 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 repres-

or of *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. The wild-type strain is BY4741 (Brachmann *et al.* 1998). The open reading frame (ORF) of *ERT1* was N-terminally tagged with a triple HA epitope using the approach of Schneider *et al.* (1995). Briefly, strain BY4741 was transformed with a PCR product generated using oligonucleotides PETYBR239-1 and PETYBR239-2 (Supporting Information, 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 *URA3* marker by internal recombination, and Ura<sup>-</sup> cells were obtained using the 5-fluoroorotic acid selection (Schneider *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 SC111A, SC113, and SC114 (Table 1), genomic DNA from *Candida glabrata* (strain ATCC 66032) was isolated and used as a template to amplify the *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 *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 *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.

**Table 2 Selected Ert1 target genes**

| Gene         | Systematic name  | Function   | Enrichment (glucose) | Enrichment (ethanol) | Binding of Adr1, Cat8, or Rds2 |
|--------------|------------------|--|----------------------|----------------------|--------------------------------|
| <i>PCK1</i>  | <i>YKR097W</i>   | Phosphoenolpyruvate carboxykinase, key enzyme in the gluconeogenesis pathway   | 64                   | 47                   | Adr1, Cat8, Rds2               |
| <i>MAE1</i>  | <i>YKL029C</i>   | Mitochondrial malic enzyme, catalyzes the oxidative decarboxylation of malate to pyruvate  | 11                   | 35                   | Rds2                           |
| <i>PDC1</i>  | <i>YLR044C</i>   | Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation   | 4.8                  | 26                   | Rds2                           |
| <i>GAT1</i>  | <i>YFL021W</i>   | Transcriptional activator of genes involved in nitrogen catabolite repression  | NS                   | 17                   | —                              |
| <i>AQR1</i>  | <i>YNL065W</i>   | Plasma membrane transporter of the major facilitator superfamily   | 6.1                  | 15                   | Rds2                           |
| <i>ICY1</i>  | <i>YMR195W</i>   | Protein of unknown function, required for viability in rich media of cells lacking mitochondrial DNA   | 2.7                  | 14                   | Adr1, Cat8, Rds2               |
| <i>LSC2</i>  | <i>YGR244C</i>   | $\beta$ -Subunit of succinyl-CoA ligase  | 3.0                  | 14                   | Adr1, Rds2                     |
| <i>VID24</i> | <i>YBR105C</i>   | GID complex regulatory subunit; regulates fructose-1,6-bisphosphatase targeting to the vacuole   | 4.4                  | 9.4                  | Rds2                           |
| <i>PET9</i>  | <i>YBL30C</i>    | Major ADP/ATP carrier of the mitochondrial inner membrane; exchanges cytosolic ADP for mitochondrially synthesized ATP; also imports heme and ATP  | 2.2                  | 7.1                  | Rds2                           |
| <i>OPI1</i>  | <i>YHL020C</i>   | Negative regulator of phospholipid biosynthetic genes  | 1.9                  | 7.0                  | Rds2                           |
| <i>FBP1</i>  | <i>YLR377C</i>   | Fructose-1,6-bisphosphatase, key regulatory enzyme in the gluconeogenesis pathway  | 2.7                  | NS                   | Adr1, Cat8, Rds2               |
| <i>FMP48</i> | <i>YGR052W</i>   | Putative protein of unknown function; the authentic, nontagged protein is detected in highly purified mitochondria in high-throughput studies  | NS                   | 4.7                  | Adr1, Rds2                     |
| —            | <i>YGR050C</i>   | Unknown function   |                      |                      |                                |
| <i>RPM2</i>  | <i>YML091C</i>   | Protein subunit of mitochondrial RNase P   | NS                   | 4.6                  | Rds2                           |
| <i>YPC1</i>  | <i>YBR183W</i>   | Alkaline ceramidase/   | NS                   | 4.5                  | —                              |
| —            | <i>YBR182C-A</i> | Putative protein of unknown function   |                      |                      |                                |
| —            | <i>YEL020C-B</i> | Dubious open reading frame   | NS                   | 4.4                  | —                              |
| <i>PTR2</i>  | <i>YKR093W</i>   | Integral membrane peptide transporter, mediates transport of di- and tri-peptides  | NS                   | 4.2                  | Adr1, Cat8, Rds2               |
| <i>QDR3</i>  | <i>YBR043C</i>   | Multidrug transporter of the major facilitator superfamily   | NS                   | 4.2                  | Rds2                           |
| <i>PHD1</i>  | <i>YLR043W</i>   | Transcriptional activator that enhances pseudohyphal growth  | NS                   | 3.3                  | —                              |
| —            | <i>YLR044W</i>   | Protein of unknown function  |                      |                      |                                |
| <i>HXT8</i>  | <i>YJL214W</i>   | 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   | NS                   | 3.3                  | Rds2                           |
| <i>IMA5</i>  | <i>YJL216C</i>   | Alpha-glucosidase  |                      |                      |                                |
| <i>PMA1</i>  | <i>YGL008C</i>   | Plasma membrane H <sup>+</sup> -ATPase   | NS                   | 3.2                  | —                              |
| <i>TKL1</i>  | <i>YPR074C</i>   | Transketolase of the pentose phosphate pathway   | NS                   | 2.9                  | Rds2                           |
| <i>DIP5</i>  | <i>YPL265W</i>   | Dicarboxylic amino acid permease; mediates high-affinity and high-capacity transport of L-glutamate and L-aspartate  | NS                   | 2.7                  | Adr1                           |
| <i>ATO2</i>  | <i>YNR002C</i>   | Putative transmembrane protein involved in export of ammonia   | NS                   | 2.6                  | Adr1, Rds2                     |
| <i>HAP4</i>  | <i>YKL109W</i>   | Limiting subunit of the heme-activated, glucose-repressed Hap2/3/4/5 complex involved in regulation of respiration genes   | NS                   | 2.6                  | Adr1, Rds2                     |
| <i>GID8</i>  | <i>YMR135C</i>   | Subunit of the GID complex involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase   | NS                   | 2.3                  | Adr1, Rds2                     |
| <i>FMP43</i> | <i>YGR243W</i>   | 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 | NS                   | 2.1                  | Adr1                           |
| —            | <i>YPR036W-A</i> | Protein of unknown function; transcription is regulated by Pdr1  | NS                   | 2.1                  | Adr1, Cat8                     |
| <i>QCR6</i>  | <i>YFR033C</i>   | Subunit 6 of the ubiquinol cytochrome-c reductase complex  | NS                   | 2.1                  | —                              |
| <i>ADR1</i>  | <i>YDR216W</i>   | Carbon source-responsive zinc-finger transcription factor, required for transcription of peroxisomal protein genes, and of genes required for ethanol, glycerol, and fatty acid utilization  | NS                   | 1.9                  | Adr1, Cat8                     |
| <i>PYC1</i>  | <i>YGL062W</i>   | Pyruvate carboxylase isoform involved in the conversion of pyruvate to oxaloacetate  | NS                   | 1.9                  | —                              |
| <i>HXT2</i>  | <i>YMR011W</i>   | High-affinity glucose transporter of the major facilitator superfamily; expression is induced by low levels of glucose and repressed by high levels of glucose   | NS                   | 1.8                  | Adr1                           |

(continued)

**Table 2, continued**

| Gene        | Systematic name | Function  | Enrichment (glucose) | Enrichment (ethanol) | Binding of Adr1, Cat8, or Rds2 |
|-------------|-----------------|---|----------------------|----------------------|--------------------------------|
| <i>SFP1</i> | <i>YLR403W</i>  | Regulates transcription of ribosomal protein and biogenesis genes; regulates response to nutrients and stress | NS                   | 1.8                  | —                              |
| <i>SFC1</i> | <i>YJR095W</i>  | Mitochondrial succinate-fumarate transporter; required for ethanol and acetate utilization                    | NS                   | 1.8                  | Adr1, Cat8, Rds2               |

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>). 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 Soontornngun *et al.* (2007). For a complete list of targets, see Supporting Information, Table S1.

### 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 multicloning sites yielding pRS303 $\Delta$ *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 *BglIII* and *NheI* and subcloned into pRS303 $\Delta$ *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 Figure 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 (aa 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

Chromatin immunoprecipitation (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 OD<sub>600</sub> of 0.7, washed twice in water, and transferred to YPD or YEP media containing 2% ethanol as a sole carbon source and grown for 3 hr. Microarrays used for ChIP-chip (4× 44K) were obtained from Agilent. Significantly enriched regions from the ChIP-chip data were identified as described previously (Soontornngun *et al.* 2012) and tRNA genes were 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

For quantitative RT-PCR (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 μg 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 (2×) (Thermo Scientific). Quality control of the primers, listed in Table S2, was verified by efficiency and dimerization tests. The PCR parameters were 95° for 10 min, followed by 40 cycles at 95° for 10 sec, 60° for 15 sec, and 72° for 30 sec. The relative quantification of each transcript was calculated by the 2<sup>−ΔΔCT</sup> 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.

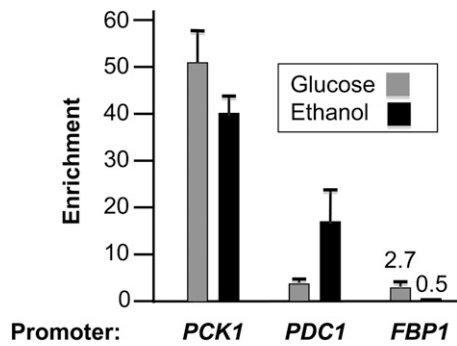
### β-Galactosidase assays

Reporters used in this study are pJJ11b (*FBP1-lacZ*) (Vincent and Gancedo 1995), pPEPCK-*lacZ* (*PCK1-lacZ*) (Proft *et al.*

**Table 3 Gene ontology enrichment of the Ert1 target genes identified by ChIP-chip analysis**

| GO term (process)           | Fraction of the query | Fraction of the query/fraction of the genome (enrichment) | <i>P</i> value         |
|-----------------------------|-----------------------|---|------------------------|
| Glucose metabolism          | 8 of 75 genes         | 9.7   | 4.8 × 10 <sup>−4</sup> |
| Hexose metabolism           | 8 of 75 genes         | 8.2   | 1.6 × 10 <sup>−3</sup> |
| Monosaccharide metabolism   | 8 of 75 genes         | 7.6   | 2.7 × 10 <sup>−3</sup> |
| Gluconeogenesis             | 5 of 75 genes         | 16.8  | 4.0 × 10 <sup>−3</sup> |
| Hexose biosynthetic process | 5 of 75 genes         | 16.8  | 4.0 × 10 <sup>−3</sup> |

GO, Gene Ontology.



**Figure 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 *ARN1* as an internal control.

1995), and p*PDC1*(–850)-*lacZ* (this study). Following transformation and selection of the reporters, colonies were grown overnight in rich medium (YPD). Cells 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 ~16 hr.  $\beta$ -Galactosidase assays were performed with permeabilized cells (Guarente 1983).

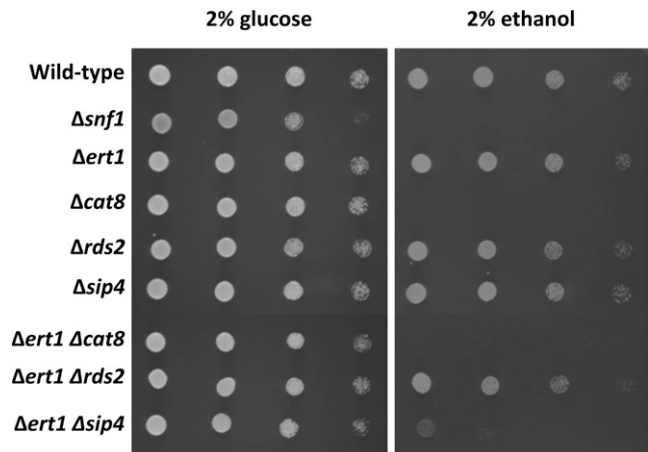
#### Electrophoretic mobility shift assay

Expression and purification of Ert1(aa 1–152), probe labeling and the electrophoretic mobility shift assay (EMSA) were performed as described (Hellauer *et al.* 1996; Larochelle *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  $\mu$ g/ml sonicated salmon sperm DNA.

## Results

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

Ert1 (Ybr239c) is a putative zinc cluster protein of unknown function and is a homolog of Rds2. 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 *PCK1*, a gene encoding phosphoenolpyruvate carboxykinase, an essential enzyme for gluconeogenesis. Ert1 was also detected at the promoter of *FBP1* encoding fructose-1,6-bisphosphatase, another essential gluconeogenic enzyme. Other Ert1 targets include *MAE1*, encoding an additional gluconeogenic enzyme involved in the conversion of malate to pyruvate as well as *PDC1*, encoding a key enzyme for fermentation.



**Figure 2** Redundancy among transcriptional regulators of gluconeogenesis. Wild-type (BY4741) and deletion strains (Table 1) were grown overnight in YPD and washed twice in H<sub>2</sub>O. Cells were then serially diluted (from left to right: 25-, 100-, 400-, and 2000-fold). Ten microliters 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 at the top. Glucose and ethanol plates were incubated at 30° for 1 and 5 days, respectively.

These results suggested that *ERT1* encodes a factor involved in the utilization of nonfermentable 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). Gene Ontology (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., *MAE1*, *AQR1*, and *VID24*). A noticeable exception is *FBP1*, where 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 *ADR1*, *HAP4* (an activator of respiration genes), and *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 (Figure 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

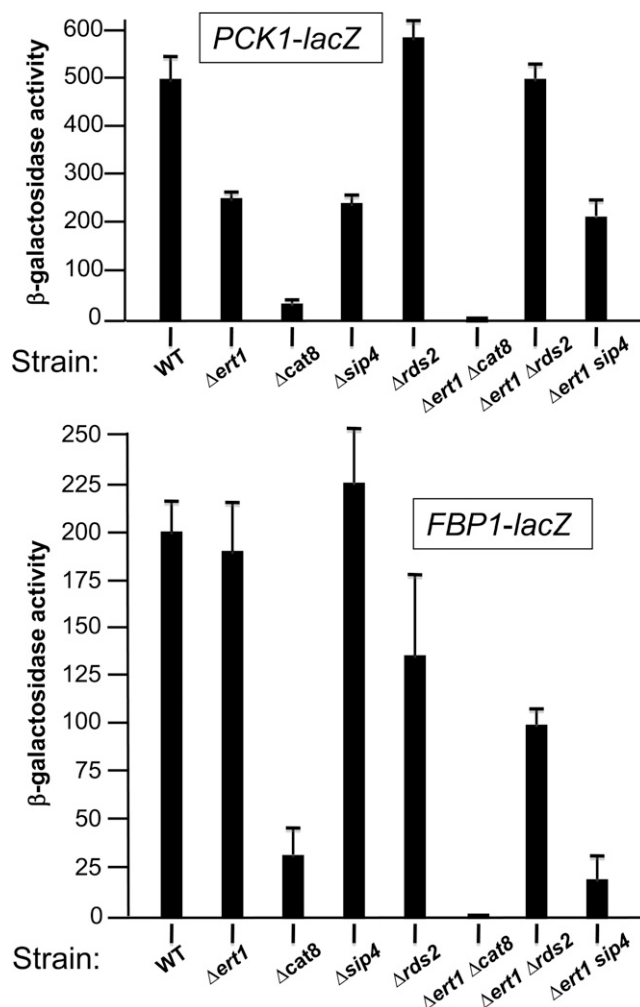


analysis. We performed expression profiling experiments comparing mRNA levels of wild-type and  $\Delta$ ert1 strains grown in the presence of glucose or ethanol as a carbon source. Minimal differences were observed between the wild-type and the  $\Delta$ ert1 strains (data not shown). A control experiment 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 the utilization of nonfermentable carbon sources, namely ethanol. To test this hypothesis, we generated strains carrying double deletions of zinc cluster genes involved in utilization of nonfermentable 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 (Figure 2). With plates containing glucose, growth was similar for all strains tested with the exception of  $\Delta$ snf1, where slightly reduced growth was observed (Figure 2, left panel). With ethanol as a sole carbon source, no growth was observed with a strain carrying a deletion of *SNF1* or *CAT8*, either alone or in combination with other mutants (Figure 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 in determining 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) (Figure 3, top panel). Deletion of *CAT8* reduced activity by 14-fold, while deletion of *ERT1* or *SIP4* reduced the activity by only 2-fold. In contrast, deletion of *RDS2* had no significant effect on the activity of the *PCK1* promoter. Similarly, with the double deletion strain  $\Delta$ ert1 $\Delta$ 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  $\Delta$ ert1 strain. Finally, deletion of both *CAT8* and *ERT1* resulted in background activity.

With the *FBP1-lacZ* reporter, deletion of *CAT8* strongly reduced  $\beta$ -galactosidase activity, while a slight increase was observed for a strain lacking *SIP4* (Figure 3, bottom

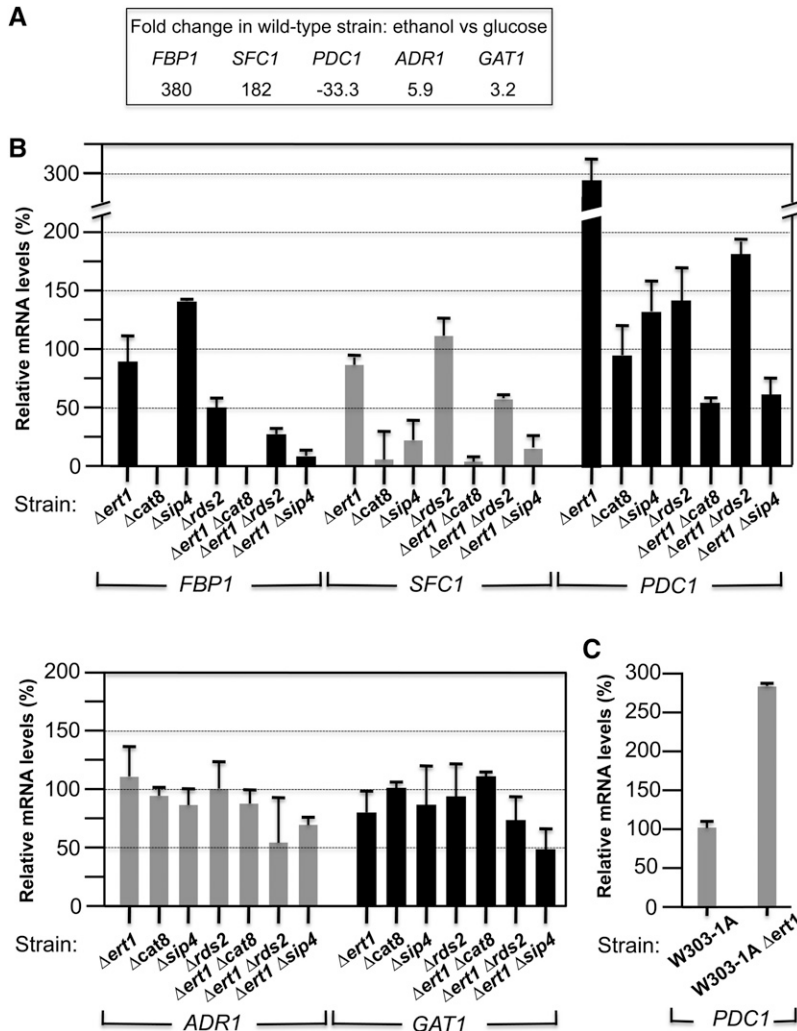


**Figure 3** Ert1 controls the activity of the *PCK1* and *FBP1* promoters. Wild-type strain BY4741 and strains carrying deletion of genes encoding various zinc cluster proteins (as indicated at the bottom) were transformed with the *lacZ* reporters for *PCK1* or *FBP1*. Cells were grown in the presence of 0.2% glucose and 2% ethanol to induce reporter activity (see *Materials and Methods* for more details).

panel), in agreement with previous observations (Zaragoza *et al.* 2001). Deleting *ERT1* gave  $\beta$ -galactosidase activity at levels comparable to a wild-type strain. Interestingly, the double mutant  $\Delta$ ert1 $\Delta$ sip4 showed a 10-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  $\Delta$ cat8,  $\Delta$ ert1 $\Delta$ cat8, and  $\Delta$ ert1 $\Delta$ sip4 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 to determine if these genes are also regulated by this factor using qRT-PCR.

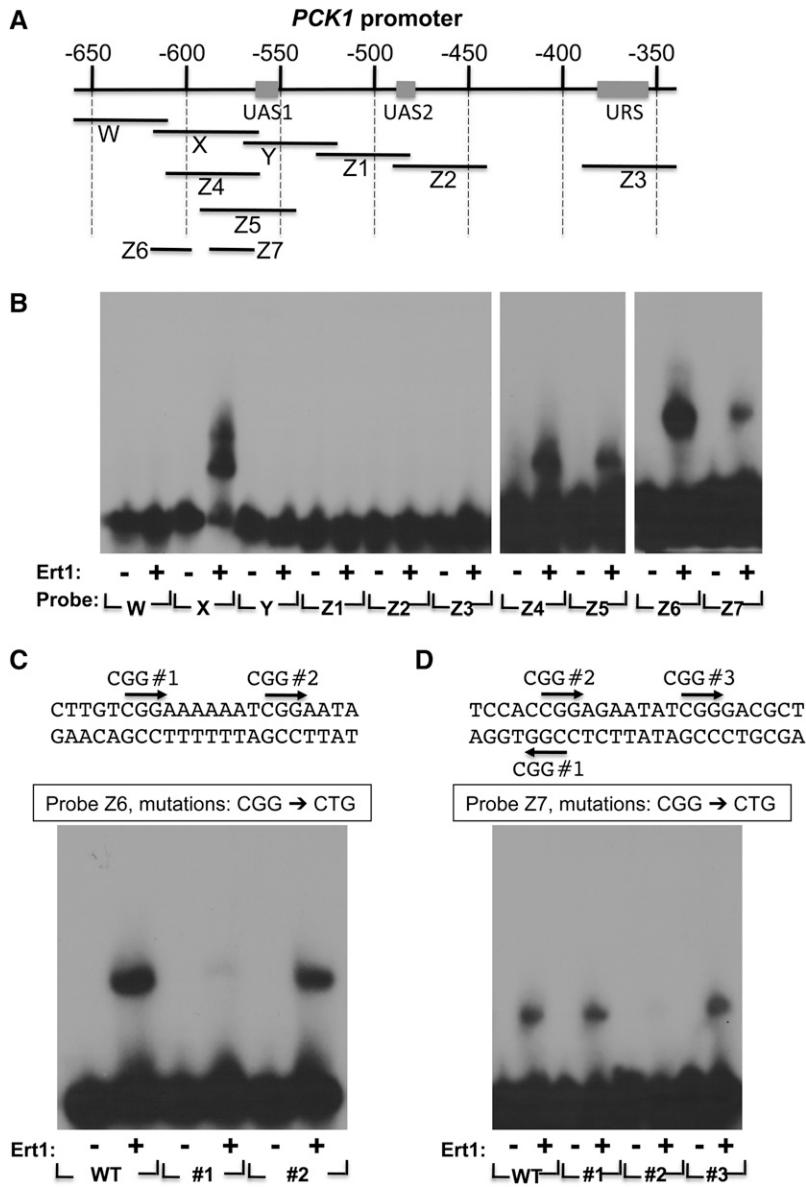


**Figure 4** Expression of *Ert1* target genes in strains lacking various transcriptional regulators. (A) Wild-type strain BY4741 was grown in the presence of 2% glucose or 2% ethanol and RNA isolated for qRT-PCR analysis. Fold changes are given as ratios between mRNA levels measured in ethanol- and glucose-grown cells. (B) Wild-type strain BY4741 and deletion strains (Table 1) were grown in the presence of 2% ethanol and RNA was 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.

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 five 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 (Figure 4A). As expected, the expression of *FBP1* and *SCF1* 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 (Schmitt *et al.* 1983; Bojunga *et al.* 1998). 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) (Figure 4B). Overall, results for *FBP1* correlate with those obtained with a *FBP1-lacZ* reporter (Figure 3). For instance, reduced  $\beta$ -galactosidase activity and *FBP1* mRNA levels were observed for the  $\Delta cat8$  and the  $\Delta ert1\Delta sip4$  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* (Figure 4B). Single deletions of *ERT1* or *RDS2* had minor effects on expression of *SFC1* but a  $\Delta ert1\Delta rds2$  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  $\Delta ert1\Delta rds2$  (~2-fold reduction). Similarly for *GAT1*, the strongest effect (2-fold reduction) was observed with the double deletion strain  $\Delta ert1\Delta sip4$ .

As stated above, expression of *PDC1* is repressed under nonfermentative conditions (Figure 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 tricarboxylic acid 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 nonfermentative metabolism. Interestingly, deletion of *ERT1* increased *PDC1* mRNA levels by almost



**Figure 5** Ert1 binds to the *PCK1* promoter. (A) Schematic view of the *PCK1* promoter. Coordinates (in base pairs) are given relative to the ATG (+1 bp). Upstream activating sequences (UAS1 and UAS2) and an upstream repressing sequence (URS) (Proft *et al.* 1995) are shown as rectangles. Probes used for EMSA are shown as black lines. (B) Ert1 (aa 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 at the top as well as the two CGG triplets (CGG no. 1 and no. 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 at the top as well as the three CGG triplets (labeled CGG nos. 1, 2, and 3). The effect of mutating either CGG triplets to CTG on Ert1 binding was tested by EMSA.

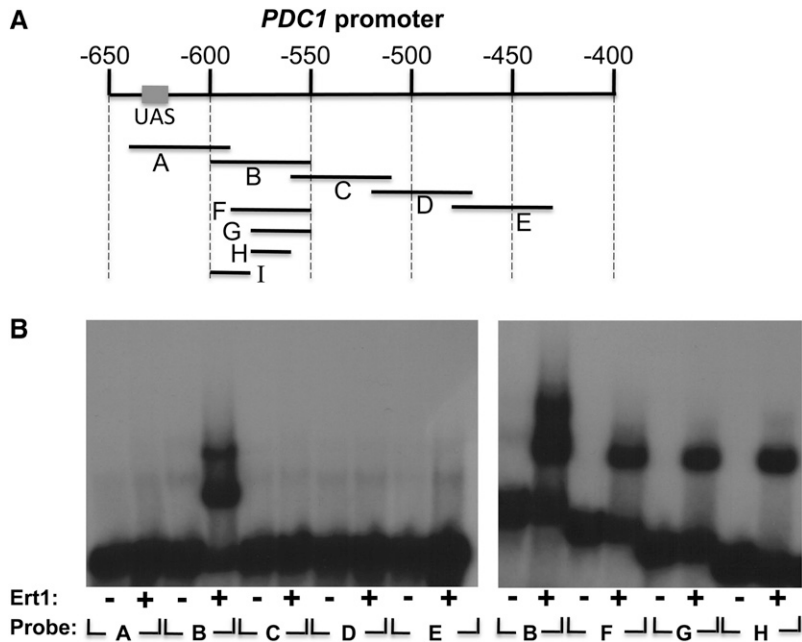
three-fold (Figure 4B). These results suggest that Ert1 is not only involved in activation of genes for nonfermentative metabolism but is also involved, in part, in shutting down the fermentative process. Deletion of *RDS2* slightly increased *PDC1* mRNA levels but no additive effect was observed with the double deletion  $\Delta$ *ert1* $\Delta$ *rds2*. Deletion of *CAT8* or *SIP4* had minor effects on *PDC1* mRNA levels. However, strains  $\Delta$ *ert1* $\Delta$ *cat8* and  $\Delta$ *ert1* $\Delta$ *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  $\Delta$ *ert1* strain in the W303-1A background. Deleting *ERT1* resulted in increased (2.8-fold) *PDC1* mRNA levels (Figure 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 in determining if Ert1 directly interacts with DNA and, if so, what the DNA recognition site is. The DNA binding domain of Ert1 (aa 1–152) 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 EMSA. We initially focused on the *PCK1* promoter. A series of DNA probes spanning the regulatory region of *PCK1* (upstream activating sequences 1 and 2 (UAS1 and UAS2) and upstream repressing sequence (URS) was tested for Ert1(1–152) binding (Figure 5A). Binding was detected only when using probe X (Figure 5B, left panel). No Ert1(1–152) binding was observed when using probes located between probes Z2 and





**Figure 6** Ert1 binds to the *PDC1* promoter. (A) Schematic view of the *PDC1* promoter. Coordinates (in base pairs) are given relative to the ATG (+1 bp). The upstream activating sequence (UAS) (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 Figure 7.

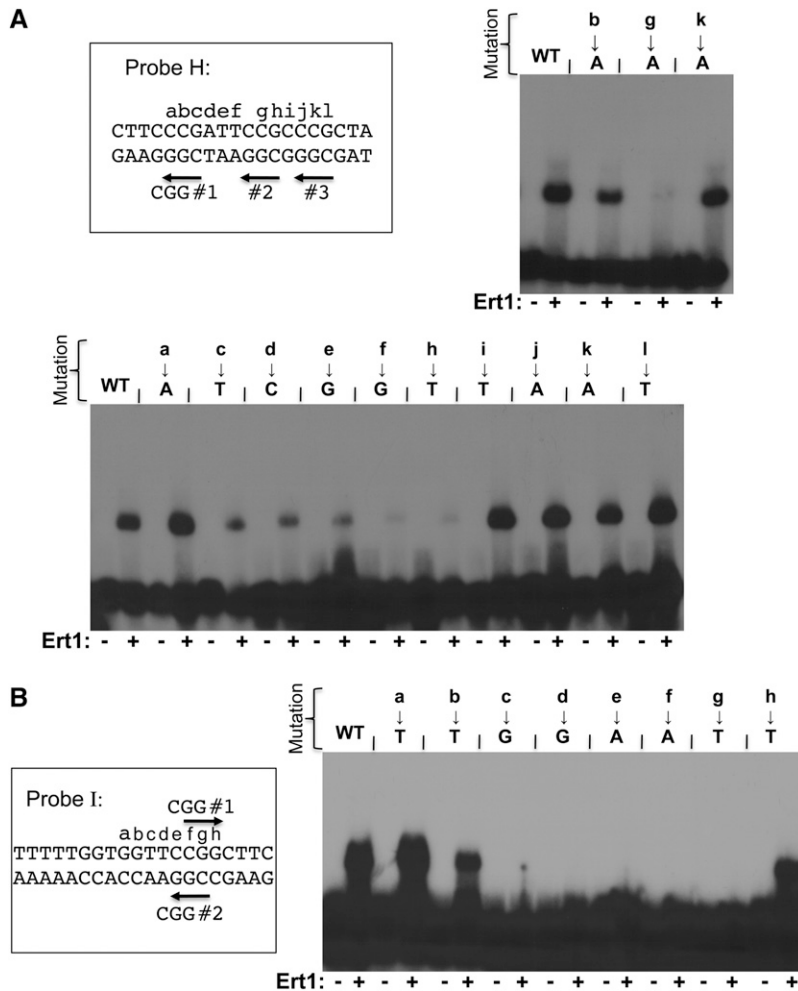
Z3 (data not shown). Thus, Ert1(1–152) binds to a region located upstream of UAS1 (a regulatory site recognized by the factors Cat8 and Sip4). With probe X, two complexes were observed. We compared the relative mobility of the Ert1(1–152)–DNA complex to zinc cluster proteins that bind DNA as homodimers (e.g., Gal4). Results suggest that the fast migrating complex is a monomer, while the slow migrating complex would be 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 (Figure 5B, middle panel). In addition, Ert1(1–152) bound to two smaller probes (Z6 and 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 Figure 5C, the Z6 probe contains two CGG triplets. Mutating CGG no. 2 to CTG had no effect on Ert1(1–152) binding. In contrast, a mutation in CGG no. 1 almost completely abolished binding of Ert1(1–152). Three CGG triplets are found in probe Z7; strikingly, only a mutation in CGG no. 2 reduced binding of Ert1(1–152).

We used the same approach described above to identify Ert1 binding site(s) in the *PDC1* promoter (Figure 6). Since ChIP-chip experiments suggested that Ert1 binds to a site located at ~500 bp upstream of the ATG, we designed five probes (A–E) covering this region, including the UAS (Figure 6A). Binding of Ert1(1–152) was observed only with probe B (Figure 6B). In analogy to *PCK1*, two Ert1(1–152)–DNA complexes were observed with that probe. With smaller probes (F, G, and H), only a single complex of fast mobility was observed (Figure 6, right panel). Binding of Ert1(1–152) was also observed with probe I, which 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 (Figure 7A). Slightly reduced binding of Ert1(1–152) was observed when mutating CGG no. 1 or no. 3. In contrast, mutation in the second CGG triplet completely abolished binding of Ert1(1–152) (Figure 7A, top panel). We tested additional mutations in the region encompassing CGG triplet no. 2 using transversions. No reduced binding of Ert1(1–152) was observed when mutating nucleotides labeled a, b, and i–l (Figure 7A). However, diminished binding was observed with mutations at positions c–f and h. A similar scan was performed with probe I (Figure 7B). EMSA analysis showed that nucleotides located at positions c–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 in determining 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 (Figure 8A). Three mutant versions were also tested. Mutant no. 1 has a mutation in the second CGG triplet while mutant no. 2 has a mutation in the first CGG triplet. These mutations abolished binding of Ert1(1–152) *in vitro* (Figure 7). A double mutant was also tested (Figure 8A). As expected with a wild-type *PDC1* promoter (Butler and McConnell 1988), high  $\beta$ -galactosidase activity was observed with cells grown in the presence of glucose, while growth with ethanol as a carbon source gave background activity (Figure 8B). With glucose, mutants no. 1 and no. 2 gave  $\beta$ -galactosidase activity similar to the wild-type reporter. Interestingly, increased activity was observed



**Figure 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 (Figure 6A) is shown at the top as well as the three CGG triplets (labeled CGG nos. 1, 2, and 3). Letters a–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 (aa 1–152), as shown in the top right panel. Additional nucleotides were also mutated (bottom panel). WT, wild-type. (B) Mutational analysis of the binding site I of Ert1. DNA sequence of probe I (Figure 6A) is shown on the left as well as the two CGG triplets (labeled CGG nos. 1 and 2). Letters a–h correspond to nucleotides that were mutated. Mutants were analyzed by EMSA using the purified DNA binding domain of Ert1 (aa 1–152) (right panel).

for both mutants when assayed with ethanol. Finally, mutant no. 3 (carrying mutations in both CGG triplets) showed slightly increased  $\beta$ -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 no. 1 and no. 2. In a  $\Delta$ *ert1* strain grown in the presence of ethanol, *PDC1* mRNA levels are at ~10% of the levels measured in a wild-type strain grown in the presence of glucose (Figure 4, A 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.

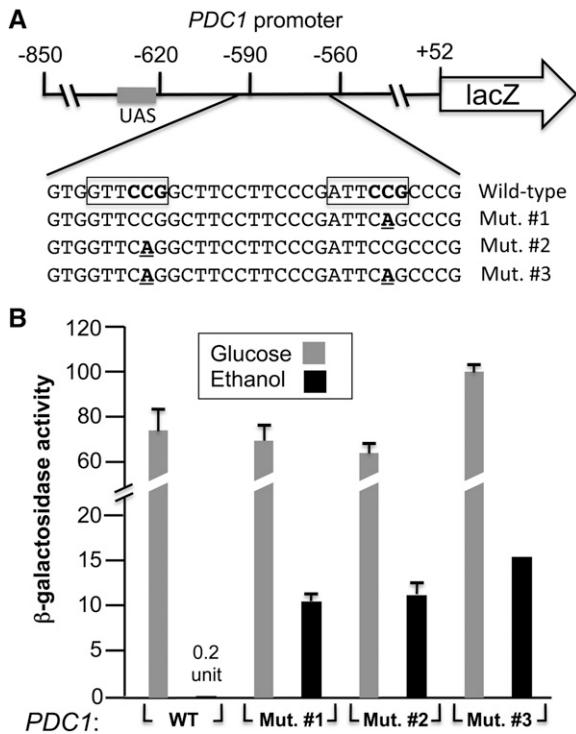
## Discussion

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). Figure 9 provides a schematic view of the pathways of fermentative and nonfermentative 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 *PYCI*, another gluconeogenic gene. A similar phenomenon was observed for Rds2 where an increased number of targets was observed with nonfermentable carbon sources as compared to glucose (Soontorngun *et al.* 2007, 2012). 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 (Palmieri *et al.* 1997; Bricker *et al.* 2012; Herzig *et al.* 2012). 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



**Figure 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 boldface type. Mutations that were introduced in the reporter are underlined and in boldface type. (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  $\beta$ -galactosidase activity was measured (see *Materials 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.

(where Y is C or T) (Figure 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) (Figure 7B). A similar effect was observed with the mutation CGGAAT to CGGAAG (Figure 7A). Finally, Ert1 bound weakly to probe Z7 located in the *PCK1* promoter (Figure 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 protein to DNA as monomer has been reported (Cahuzac *et al.* 2001), it is quite possible that Ert1 binds to DNA as a homodimer. For example, the DNA binding domain used in our studies may lack a dimerization domain. Alter-

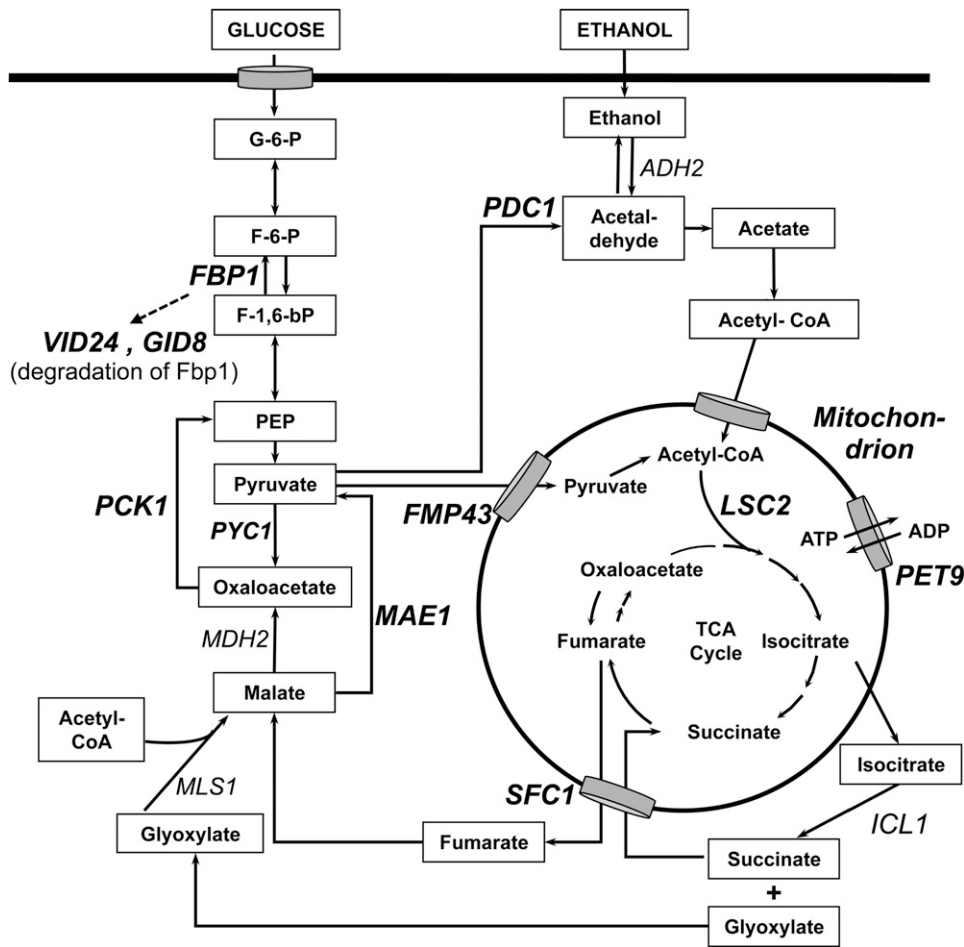
natively, Ert1 may bind with a partner that remains to be identified. AcuM and AcuK are two *A. nidulans* homologs of Ert1/Rds2. AcuM and AcuK also are involved in the regulation of gluconeogenic genes and, interestingly, bind to DNA in an interdependent manner (Suzuki *et al.* 2012).

### 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 nonfermentable carbon sources such as ethanol. However, expression profiling using wild-type and  $\Delta$ *ert1* 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 (Figure 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  $\Delta$ *ert1* $\Delta$ *sip4* strain (Figure 3). These results were confirmed by qRT-PCR analysis (Figure 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, Figure 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 toward ethanol production. In contrast, low levels of pyruvate decarboxylase activity are observed with cells shifted to nonfermentative metabolism (Pronk *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 (Figure 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 (Figure 8). These results suggest that Ert1 can act both as a transcriptional repressor and an activator, as observed for Rds2



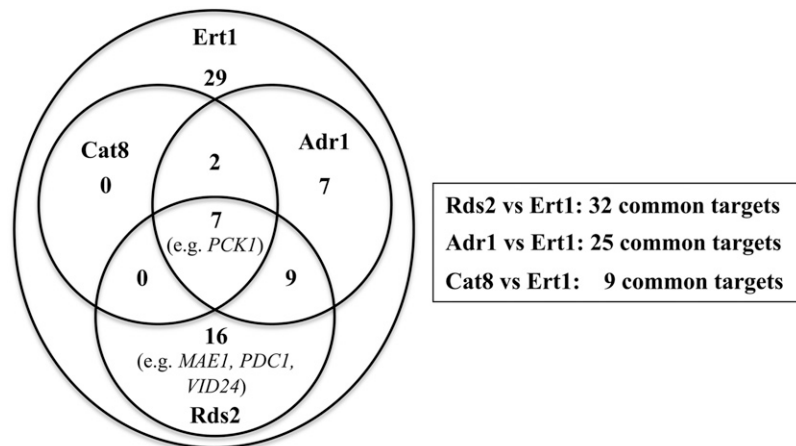
**Figure 9** Major Ert1 targets in the pathways of fermentative and nonfermentative metabolism. Some of the Ert1 target genes are indicated in boldface type. Only the mitochondrial compartment is shown. *SFC1* encodes a fumarate/succinate transporter and *FMP43* encodes a subunit of the pyruvate transporter. *PET9* encodes an ATP/ADP carrier.

(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 ( $\Delta$ *ert1* $\Delta$ *rds2*) had *PDC1* mRNA at lower levels as compared to a  $\Delta$ *ert1* strain. The effect of these two factors may be more apparent when assayed during the transition from fermentative to nonfermentative metab-

olism. 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



toward 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; Tachibana *et al.* 2005; Soontornngun *et al.* 2007; 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 (Soontornngun *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 (Figure 10). These observations point to a complex combinatorial network of transcription factors involved in the utilization of nonfermentable 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 nonfermentative 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 nonfermentative conditions (Soontornngun *et al.* 2007 and unpublished results). However, expression of *CAT8*, *SIP4*, *GSM1*, and *ADR1* is increased under low glucose levels (Figure 4B) (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 (Forsburg and Guarente 1989; Derisi *et al.* 1997; Soontornngun *et al.* 2007). Cat8 controls the expression of *SIP4* (Lesage *et al.* 1996) while Ert1 and Rds2 control, in part, the expression of *ADR1* (Figure 4B). Finally, Rds2 is a regulator of the expression of *HAP4* (Soontornngun *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

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# GENETICS

Supporting Information

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## **The Switch from Fermentation to Respiration in *Saccharomyces cerevisiae* Is Regulated by the Ert1 Transcriptional Activator/Repressor**

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**Table S1****List of Ert1 targets (P <0.05 with enrichment values >1.8).**

Gene functions were taken from the *Saccharomyces cerevisiae* database (<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 as the carbon source) were taken from (SOONTORNGUN *et al.* 2007).

| Gene         | Systematic name | Function  | Enrichment (glucose) | Enrichment (ethanol) | Binding of Adr1, Cat8 or Rds2 |
|--------------|-----------------|---|----------------------|----------------------|-------------------------------|
| <i>PCK1</i>  | <i>YKR097W</i>  | Phosphoenolpyruvate carboxykinase, key enzyme in the gluconeogenesis pathway  | 64                   | 47                   | Adr1, Cat8, Rds2              |
| <i>MAE1</i>  | <i>YKL029C</i>  | Mitochondrial malic enzyme, catalyzes the oxidative decarboxylation of malate to pyruvate   | 11                   | 35                   | Rds2                          |
| <i>PDC1</i>  | <i>YLR044C</i>  | Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation  | 4.8                  | 26                   | Rds2                          |
| <i>GAT1</i>  | <i>YFL021W</i>  | Transcriptional activator of genes involved in nitrogen catabolite repression   | NS                   | 17                   | -                             |
| <i>AQR1</i>  | <i>YNL065W</i>  | Plasma membrane transporter of the major facilitator superfamily  | 6.1                  | 15                   | Rds2                          |
| <i>ICY1</i>  | <i>YMR195W</i>  | Protein of unknown function, required for viability in rich media of cells lacking mitochondrial DNA  | 2.7                  | 14                   | Adr1, Cat8, Rds2              |
| <i>LSC2</i>  | <i>YGR244C</i>  | $\beta$ -subunit of succinyl-CoA ligase   | 3.0                  | 14                   | Adr1, Rds2                    |
| <i>VID24</i> | <i>YBR105C</i>  | GID complex regulatory subunit; regulates fructose-1,6-bisphosphatase targeting to the vacuole  | 4.4                  | 9.4                  | Rds2                          |
| <i>PET9</i>  | <i>YBL30C</i>   | Major ADP/ATP carrier of the mitochondrial inner membrane; exchanges cytosolic ADP for mitochondrially synthesized ATP; also imports heme and ATP | 2.2                  | 7.1                  | Rds2                          |
| <i>OPI1</i>  | <i>YHL020C</i>  | Negative regulator of phospholipid biosynthetic genes   | 1.9                  | 7.0                  | Rds2                          |
| <i>FBP1</i>  | <i>YLR377C</i>  | Fructose-1,6-bisphosphatase, key regulatory enzyme in the gluconeogenesis pathway   | 2.7                  | NS                   | Adr1, Cat8, Rds2              |
| <i>PRP28</i> | <i>YDR243C</i>  | RNA helicase in the DEAD-box family, involved in RNA isomerization at the 5' splice site  | NS                   | 6.2                  | Rds2                          |

|                  |                  |  |    |     |                  |
|------------------|------------------|--|----|-----|------------------|
| <i>PEX5</i>      | <i>YDR244W</i>   | Peroxisomal membrane signal receptor required for peroxisomal matrix protein import  |    |     |                  |
| <i>HSP30</i>     | <i>YCR021C</i>   | Hydrophobic plasma membrane localized, stress-responsive protein that negatively regulates the H(+)-ATPase Pma1p; induced by heat shock, ethanol treatment, weak organic acid, glucose limitation, and entry into stationary phase | NS | 5.6 | -                |
| <i>IXR1</i>      | <i>YKL032C</i>   | Transcription factor that mediates aerobic transcriptional repression of <i>COX5b</i>  | NS | 5.3 | -                |
| <i>FMP4</i><br>8 | <i>YGR052W</i>   | Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies   | NS | 4.7 | Adr1, Rds2       |
| -                | <i>YGR050C</i>   | Unknown function   |    |     |                  |
| <i>RPM2</i>      | <i>YML091C</i>   | Protein subunit of mitochondrial RNase P   | NS | 4.6 | Rds2             |
| <i>YPC1</i>      | <i>YBR183W</i>   | Alkaline ceramidase/   |    |     |                  |
| -                | <i>YBR182C-A</i> | Putative protein of unknown function   | NS | 4.5 | -                |
| -                | <i>YEL020C-B</i> | Dubious open reading frame   | NS | 4.4 | -                |
| <i>PTR2</i>      | <i>YKR093W</i>   | Integral membrane peptide transporter, mediates transport of di- and tri-peptides  | NS | 4.2 | Adr1, Cat8, Rds2 |
| <i>QDR3</i>      | <i>YBR043C</i>   | Multidrug transporter of the major facilitator superfamily   | NS | 4.2 | Rds2             |
| <i>HMS1</i>      | <i>YOR032C</i>   | Basic helix-loop-helix (bHLH) protein with similarity to myc-family transcription factors  | NS | 3.7 | -                |
| <i>PHD1</i>      | <i>YKL043W</i>   | Transcriptional activator that enhances pseudohyphal growth  | NS | 3.3 | -                |
| -                | <i>YKL044W</i>   | Protein of unknown function  |    |     |                  |
| <i>HXT8</i>      | <i>YJL214W</i>   | 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   | NS | 3.3 | Rds2             |
| <i>IMA5</i>      | <i>YJL216C</i>   | Alpha-glucosidase  |    |     |                  |
| <i>PMA1</i>      | <i>YGL008C</i>   | Plasma membrane H <sup>+</sup> -ATPase   | NS | 3.2 | -                |
| <i>TKL1</i>      | <i>YPR074C</i>   | Transketolase of the pentose phosphate pathway   | NS | 2.9 | Rds2             |
| <i>DSF1</i>      | <i>YEL070W</i>   | Putative mannitol dehydrogenase  | NS | 2.8 | -                |
| <i>DIP5</i>      | <i>YPL265W</i>   | Dicarboxylic amino acid permease; mediates high-affinity and high-capacity transport of L-glutamate and L-aspartate  | NS | 2.7 | Adr1             |
| -                | <i>YGR161W-C</i> | Putative protein of unknown function   | NS | 2.7 | -                |

|              |                  |   |    |     |                  |
|--------------|------------------|---|----|-----|------------------|
| <i>RTS3</i>  | <i>YGR161C</i>   | Putative component of the protein phosphatase type 2A complex   |    |     |                  |
| <i>ATO2</i>  | <i>YNR002C</i>   | Putative transmembrane protein involved in export of ammonia  | NS | 2.6 | Adr1, Rds2       |
| <i>HAP4</i>  | <i>YKL109W</i>   | Limiting subunit of the heme-activated, glucose-repressed Hap2/3/4/5 complex involve in regulation of respiration genes   | NS | 2.6 | Adr1, Rds2       |
| <i>ZEO1</i>  | <i>YOL109W</i>   | Peripheral membrane protein of the plasma membrane; regulates the cell integrity pathway mediated by Pkc1p and Slr2p. The authentic protein is detected in a phosphorylated state in highly purified mitochondria | NS | 2.6 | Adr1, Cat8, Rds2 |
| <i>TAM41</i> | <i>YGR046W</i>   | Mitochondrial protein required for cardiolipin biosynthesis   |    |     |                  |
| <i>RME1</i>  | <i>YGR044C</i>   | Zinc finger protein involved in control of meiosis  | NS | 2.5 | Rds2             |
| <i>AQY1</i>  | <i>YPR192W</i>   | Spore-specific water channel that mediates the transport of water across cell membranes   | NS | 2.5 | Rds2             |
| <i>YCF1</i>  | <i>YDR135C</i>   | Vacuolar glutathione S-conjugate transporter; member of the ATP-binding cassette family   | NS | 2.4 | -                |
| <i>TPO1</i>  | <i>YLL028W</i>   | Polyamine transporter of the major facilitator superfamily  | NS | 2.4 | -                |
| <i>RPS3</i>  | <i>YNL179C</i>   | Protein component of the small (40S) ribosomal subunit  | NS | 2.3 | Adr1, Rds2       |
|              | <i>YNL180C</i>   |   |    |     |                  |
| <i>GID8</i>  | <i>YMR135C</i>   | Subunit of the GID complex involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase  | NS | 2.3 | Adr1, Rds2       |
| <i>ERG11</i> | <i>YHR007C</i>   | Lanosterol 14- $\alpha$ -demethylase  | NS | 2.3 | -                |
| <i>SNA2</i>  | <i>YDR525W-A</i> | Protein of unknown function, has similarity to Pmp3p, which is involved in cation transport   | NS | 2.3 | Adr1, Rds2       |
| <i>HOR7</i>  | <i>YMR251W-A</i> | Protein of unknown function; overexpression suppresses Ca <sup>2+</sup> sensitivity of mutants lacking inositol phosphorylceramide mannosyltransferases   | NS | 2.3 | Adr1, Rds2       |
| <i>MCM1</i>  | <i>YMR043W</i>   | Transcription factor involved in cell-type-specific transcription and pheromone response  | NS | 2.3 | Rds2             |
| <i>RSC3</i>  | <i>YDR303C</i>   | Component of the RSC chromatin remodeling complex; required for maintenance of proper ploidy and regulation of ribosomal protein genes and the cell wall/stress response  | NS | 2.2 | Rds2             |
| <i>MLP1</i>  | <i>YKR095W</i>   | Myosin-like protein associated with the nuclear envelope, connects the nuclear  | NS | 2.2 | -                |

|                  |                  |  |     |     |                  |
|------------------|------------------|--|-----|-----|------------------|
|                  |                  | pore complex with the nuclear interior   |     |     |                  |
| <i>MGAI</i>      | <i>YGR249W</i>   | Protein similar to heat shock transcription factor   | NS  | 2.2 | -                |
| <i>SFK1</i>      | <i>YKL051W</i>   | Plasma membrane protein that may act together with or upstream of Stt4p to generate normal levels of the essential phospholipid PI4P   | NS  | 2.2 | Rds2             |
| <i>HSP12</i>     | <i>YFL014W</i>   | Plasma membrane protein involved in maintaining membrane organization in stress conditions; induced by heat shock, oxidative stress, osmotic stress, stationary phase, glucose depletion, oleate and alcohol   | NS  | 2.2 | Adr1             |
| <i>ROX1</i>      | <i>YPR065W</i>   | Heme-dependent repressor of hypoxic genes  | NS  | 2.2 | -                |
| <i>SKS1</i>      | <i>YPL026C</i>   | Putative serine/threonine protein kinase; involved in the adaptation to low concentrations of glucose independent of the SNF3 regulated pathway  | NS  | 2.1 | -                |
| <i>FMP4</i><br>3 | <i>YGR243W</i>   | 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 | NS  | 2.1 | Adr1             |
| -                | <i>YPR036W-A</i> | Protein of unknown function; transcription is regulated by Pdr1  | NS  | 2.1 | Adr1, Cat8       |
| <i>QCR6</i>      | <i>YFR033C</i>   | Subunit 6 of the ubiquinol cytochrome-c reductase complex  | NS  | 2.1 | -                |
| <i>RGI2</i>      | <i>YIL057C</i>   | Protein of unknown function involved in energy metabolism under respiratory conditions; expression induced under carbon limitation and repressed under high glucose  | NS  | 2.1 | Adr1, Cat8, Rds2 |
| <i>VHR1</i>      | <i>YIL056W</i>   | Transcriptional activator; required for the vitamin H-responsive element (VHRE) mediated induction of VHT1 (Vitamin H transporter)   |     |     |                  |
| <i>BASI</i>      | <i>YKR099W</i>   | Myb-related transcription factor involved in regulating basal and induced expression of genes of the purine and histidine biosynthesis pathways  | 2,5 | 2.1 | -                |
| <i>TIP1</i>      | <i>YBR067C</i>   | Major cell wall mannoprotein with possible lipase activity   | NS  | 2.0 | Adr1, Rds2       |
| <i>MTL1</i>      | <i>YGR023W</i>   | Putative plasma membrane sensor, involved in cell integrity signaling and stress response during glucose starvation and oxidative stress   | NS  | 2.0 | Adr1             |
| -                | <i>YKL027W</i>   | Protein of unknown function, localized   | NS  | 1.9 | -                |

|              |                |   |    |     |                  |
|--------------|----------------|---|----|-----|------------------|
|              |                | to the mitochondrial outer membrane   |    |     |                  |
| <i>PEX1</i>  | <i>YKL197C</i> | AAA-peroxin that heterodimerizes with AAA-peroxin Pex6p and participates in the recycling of peroxisomal signal receptor Pex5p from the peroxisomal membrane to the cytosol                 | NS | 1.9 | -                |
| <i>ADR1</i>  | <i>YDR216W</i> | Carbon source-responsive zinc-finger transcription factor, required for transcription of peroxisomal protein genes, and of genes required for ethanol, glycerol, and fatty acid utilization | NS | 1.9 | Adr1, Cat8       |
| <i>MET13</i> | <i>YGL125W</i> | Major isozyme of methylenetetrahydrofolate reductase  | NS | 1.9 | -                |
| <i>TMA10</i> | <i>YLR327C</i> | Protein of unknown function that associates with ribosomes  | NS | 1.9 | Adr1             |
| <i>CYC8</i>  | <i>YBR112C</i> | General transcriptional co-repressor  | NS | 1.9 | -                |
| <i>PYC1</i>  | <i>YGL062W</i> | Pyruvate carboxylase isoform involved in the conversion of pyruvate to oxaloacetate   | NS | 1.9 | -                |
| <i>NAF1</i>  | <i>YNL124W</i> | RNA-binding protein required for the assembly of box H/ACA snoRNPs and thus for pre-rRNA processing   | NS | 1.8 | -                |
| -            | <i>YHR080C</i> | Protein of unknown function; non-tagged protein is detected in highly purified mitochondria in high-throughput studies  | NS | 1.8 | -                |
| <i>HXT2</i>  | <i>YMR011W</i> | High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose                              | NS | 1.8 | Adr1             |
| <i>MIP6</i>  | <i>YHR015W</i> | Putative RNA-binding protein  | NS | 1.8 | -                |
| <i>SFP1</i>  | <i>YLR403W</i> | Regulates transcription of ribosomal protein and biogenesis genes; regulates response to nutrients and stress   | NS | 1.8 | -                |
| <i>IGD1</i>  | <i>YFR017C</i> | Cytoplasmic protein that inhibits Gdb1 glycogen debranching activity  | NS | 1.8 | -                |
| <i>FAT3</i>  | <i>YKL187C</i> | Putative protein of unknown function; the authentic, non-tagged protein is detected in a phosphorylated state in highly purified mitochondria in high-throughput studies                    | NS | 1.8 | Adr1             |
| <i>SFC1</i>  | <i>YJR095W</i> | Mitochondrial succinate-fumarate transporter; required for ethanol and acetate utilization  | NS | 1.8 | Adr1, Cat8, Rds2 |



**Table S2****Oligonucleotides used in this study.**

| <b>Oligonucleotide</b>              | <b>Sequence (5' → 3')</b>                         | <b>Description</b>   |
|-------------------------------------|---|--|
| D1                                  | CGGGATCCAT <b>GT</b> GCACCTCCAGATGAAAA            | GST-Ert1 expression vector   |
| D2                                  | GGAATTCAGATGATATTCGACAG                           | GST-Ert1 expression vector; verification <i>ERT1</i> tag                             |
| LacZ-integr-1                       | ATCATT <b>GCTAGCT</b> GGCCGATTCATTCCCG            | Construction of pLacZ-integr<br>NheI site in bold characters                         |
| LacZ-integr-2                       | TTTCAGT <b>AGATCT</b> CCAGGAACCGAAATACATACATTG    | Construction of pLacZ-integr<br>BglII site in bold characters                        |
| <i>PDC1-lacZ-3</i>                  | CG <b>GAATTC</b> AAGTAACCTATTCAAAGTAATATCTCATACAT | Construction of pPDC1(-850)- <i>lacZ</i><br>EcoRI site in bold characters            |
| <i>PDC1-lacZ-4</i>                  | TAGGACCG <b>CATGCT</b> GACTTGCTTTAATCTTTTGAACA    | Construction of pPDC1(-850)- <i>lacZ</i><br>NheI site in bold characters             |
| <i>PDC1</i> -CGG to CTG-1 (-569)    | CTTCCCGATTC <b>AGCCCGCT</b> AAAC                  | Site-directed mutagenesis of pPDC1(-850)- <i>lacZ</i><br>Mutation in bold characters |
| <i>PDC1</i> -CGG to CTG-2 (-569)    | GTTTAGCGGGCT <b>GAATCGGGAAG</b>                   | Site-directed mutagenesis of pPDC1(-850)- <i>lacZ</i><br>Mutation in bold characters |
| <i>PDC1</i> -CGG to CTG-3 (-587 bp) | TTTTTGGTGGTTCAGGCTT <b>CTTCCCG</b>                | Site-directed mutagenesis of pPDC1(-850)- <i>lacZ</i><br>Mutation in bold characters |
| <i>PDC1</i> -CGG to CTG-4 (-587 bp) | CGGGAAGGAAGCCTGAACCACCAAAAA                       | Site-directed mutagenesis of pPDC1(-850)- <i>lacZ</i><br>Mutation in bold characters |

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|---------------|--|------------------------------------|
| PETYBR239-1   | ACACGAAGGTATAAAAATAAAATACATTGATGAATCATCGTGATG<br><b>AGGGAACAAAAGCTGGAG</b>   | Tagging <i>ERT1</i>                |
| PETYBR239-2   | ATCTGGGTCAGTTGATGTTTTGTAATCGTTTTTCATCTGGAGTGCAT<br><b>AGGGCGAATTGGGTACC</b>  | Tagging <i>ERT1</i>                |
| ERT1-Acheck   | GCCAAAAGCTTTGCTTGGAT   | Verification <i>ERT1</i> tag       |
| ERT1-CgHIS3-1 | GACACACGAAGGTATAAAAATAAAATACATTGATGAATCATCGTG<br><b>TCTCACACGGGGTGGGAATT</b> | Deletion of <i>ERT1</i>            |
| ERT1-CgHIS3-2 | TATAATACGAGGTAGTAAAATGAGAAAAAAGAAAAAAAAGTC<br><b>AGCTCATCGCCAGAAGTTTT</b>    | Deletion of <i>ERT1</i>            |
| PDC1CHIP STDF | TTGTTGCCTGGTGGCATTG  | Standard ChIP                      |
| PDC1CHIP STDR | TTCCACGAGCCTCATCACAC   | Standard ChIP                      |
| PCK1CHIP STDF | AGAGCACTTGGGCAGAGC   | Standard ChIP                      |
| PCK1CHIP STDR | CGTGTGGGATATTCCGATTT   | Standard ChIP                      |
| FBP1CHIP STDS | CCCATCAAAGTGCATGGTC  | Standard ChIP                      |
| FBP1CHIP STDR | AATTCAAAGCGGCGATTC   | Standard ChIP                      |
| ARN1-1        | TGCACCCATAAAAGCAGGTGT  | Internal control for standard ChIP |
| ARN1-2        | GAGAGCTATCGAATGTTTCCTC   | Internal control for standard ChIP |
| ACT1F         | GCCTTCTACGTTTCC  | qRT-PCR                            |
| ACT1R         | GGCCAAATCGATTCT  | qRT-PCR                            |
| ADR1F         | GAGTGTCATGGACTCTAATCCAAA   | qRT-PCR                            |
| ADR1R         | CACTCACAGCTGGCATTAAACA   | qRT-PCR                            |
| FBP1-RTR      | GTTGTCTGCTTGGGGTTGTT   | qRT-PCR                            |
| FBP1-RTS      | ACTGGGCGAATTCATCTTG  | qRT-PCR                            |
| GAT1-RTS      | CCATCGTCGTCTCTCAATCC   | qRT-PCR                            |

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| GAT1-RTR | GCTGCCACACTTGCTGTATAGT | qRT-PCR |
| PDC1-RTF | GAAGGTATGAGATGGGCTGGT  | qRT-PCR |
| PDC1-RTR | ACACCGAAGGTGGTGATGAT   | qRT-PCR |
| SFC1-RTR | ACCGAAGCTGTTCTTGTCGT   | qRT-PCR |
| SFC1-RTS | ACTGGGCGAATTCATCTTG    | qRT-PCR |

**Probes for EMSA**

The sequence TCGA used for labeling of the oligonucleotides (in bold) is not part of the promoters. Mutations are in bold characters

|                 |  |          |
|-----------------|--|----------|
| <i>PCK1</i> -W1 | <b>TCG</b> AAATTCTGACCAGAGCACTTGGGCAGAGCCCCACCCAGGGC<br>CTTGTCGGA        | Probe W  |
| <i>PCK1</i> -W2 | <b>TCG</b> ATCCGACAAGGCCCTGGGTGGGGGCTCTGCCCAAGTGCTCTG<br>GTCAGAATT       | Probe W  |
| <i>PCK1</i> -X7 | <b>TCG</b> ACTTGTCGGAAAAAATCGGAATATCCCACACGATCCACCGGA<br>GAATATCGGGACGCT | Probe X  |
| <i>PCK1</i> -X8 | <b>TCG</b> AAGCGTCCCGATATTCTCCGGTGGATCGTGTGGGATATTCCGA<br>TTTTTCCGACAAG  | Probe X  |
| <i>PCK1</i> -Y1 | <b>TCG</b> AGGGACGCTGACGGGTGAATGGAGATCTGGATTCCTAATTT<br>GGATACATC        | Probe Y  |
| <i>PCK1</i> -Y2 | <b>TCG</b> AGATGTATCCAAATTAGGAAATCCAGATCTCCATTCACCCGTC<br>AGGGTCCC       | Probe Y  |
| <i>PCK1</i> -Z1 | <b>TCG</b> ATGGATACATCTCTTTTCTTTTTTTGACTCACAATAGGAAAAA<br>ACCGAGCT       | Probe Z1 |
| <i>PCK1</i> -Z2 | <b>TCG</b> AAGCTCGGTTTTTTCCTATTGTGAGTCAAAAAAAGAAAAGAG<br>ATGTATCCA       | Probe Z1 |
| <i>PCK1</i> -Z3 | <b>TCG</b> AAAACCGAGCTTCCTTTCATCCGGCGCGGCTGTGTTCTACATA<br>TCACTGAA       | Probe Z2 |
| <i>PCK1</i> -Z4 | <b>TCG</b> ATTCAGTGATATGTAGAACACAGCCGCGCCGGATGAAAGGAA<br>GCTCGGTTT       | Probe Z2 |
| <i>PCK1</i> -Z5 | <b>TCG</b> ATTTTAGGCTGCTTAACATTATGGATAGGCGGATAAAGGGCC<br>CAAACAGGA       | Probe Z3 |
| <i>PCK1</i> -Z6 | <b>TCG</b> ATCCTGTTTGGGCCCTTTATCCGCCTATCCATAATGTTAAGCA<br>GCCTAAAA       | Probe Z3 |
| <i>PCK1</i> -X1 | <b>TCG</b> AAAAAATCGGAATATCCCACACGATCCACCGGAGAATATCG<br>GGACGCT          | Probe Z4 |
| <i>PCK1</i> -X2 | <b>TCG</b> AATCAGCGTCCCGATATTCTCCGGTGGATCGTGTGGGATATTC<br>CGATTTT        | Probe Z4 |

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| <i>PCK1-X5</i>  | <b>TCGAACACGATCCACCGGAGAATATCGGGACGCTGACGGGTGAATGGAGATCTGG</b> | Probe Z5                             |
| <i>PCK1-X6</i>  | <b>TCGACCAGATCTCCATTCACCCGTCAGCGTCCCGATATTCTCCGGTGGATCGTGT</b> | Probe Z5                             |
| <i>PCK1-X17</i> | <b>TCGACTTGTCGGAAAAAATCGGAATA</b>                              | Probe Z6                             |
| <i>PCK1-X18</i> | <b>TCGATATTCCGATTTTTTCCGACAAG</b>                              | Probe Z6                             |
| <i>PCK1-X11</i> | <b>TCGATCCAACGGAGAATATCGGGACGCT</b>                            | Probe Z6 with mutation in first CGG  |
| <i>PCK1-X12</i> | <b>TCGAAGCGTCCCGATATTCTCCGTTGGA</b>                            | Probe Z6 with mutation in first CGG  |
| <i>PCK1-X13</i> | <b>TCGATCCACCTGAGAATATCGGGACGCT</b>                            | Probe Z6 with mutation in second CGG |
| <i>PCK1-X14</i> | <b>TCGAAGCGTCCCGATATTCTCAGGTGGA</b>                            | Probe Z6 with mutation in second CGG |
| <i>PCK1-X9</i>  | <b>TCGATCCACCGGAGAATATCGGGACGCT</b>                            | Probe Z7                             |
| <i>PCK1-X10</i> | <b>TCGAAGCGTCCCGATATTCTCCGGTGGGA</b>                           | Probe Z7                             |
| <i>PCK1-X11</i> | <b>TCGATCCAACGGAGAATATCGGGACGCT</b>                            | Probe Z7 with mutation in first CGG  |
| <i>PCK1-X12</i> | <b>TCGAAGCGTCCCGATATTCTCCGTTGGA</b>                            | Probe Z7 with mutation in first CGG  |
| <i>PCK1-X13</i> | <b>TCGATCCACCTGAGAATATCGGGACGCT</b>                            | Probe Z7 with mutation in second CGG |
| <i>PCK1-X14</i> | <b>TCGAAGCGTCCCGATATTCTCAGGTGGA</b>                            | Probe Z7 with mutation in second CGG |
| <i>PCK1-X15</i> | <b>TCGATCCACCGGAGAATATCTGGACGCT</b>                            | Probe Z7 with mutation in third CGG  |
| <i>PCK1-X16</i> | <b>TCGAAGCGTCCAGATATTCTCCGGTGGGA</b>                           | Probe Z7 with mutation in third CGG  |
| <i>PDC1-A1</i>  | <b>TCGACACACTGCACCCATACCTTCCTTAAAAACGTAGCTTCCAGTTTTGGTGG</b>   | Probe A                              |
| <i>PDC1-A2</i>  | <b>TCGACCACCAAAAACCTGGAAGCTACGTTTTTAAGGAAGGTATGGGTGCAGTGTG</b> | Probe A                              |

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| <i>PDC1</i> -B1 | <b>TCGATTTTTGGTGGTTCCGGCTTCCTTCCCGATTCCGCCCGCTAAA<br/>CGCATATT</b>   | Probe B                                |
| <i>PDC1</i> -B2 | <b>TCGAAATATGCGTTT TAGCGGGCGGAATCGGGAAGGAAGCCGGAA<br/>CCACCAAAAA</b> | Probe B                                |
| <i>PDC1</i> -C1 | <b>TCGAAACGCATATTTTTGTTGCCTGGTGGCATTGCAAAATGCATA<br/>ACCTATGC</b>    | Probe C                                |
| <i>PDC1</i> -C2 | <b>TCGAGCATAGGTTATGCATTTTGCAAATGCCACCAGGCAACAAAA<br/>ATATGCGTT</b>   | Probe C                                |
| <i>PDC1</i> -D1 | <b>TCGATAACCTATGCATTTAAAAGATTATGTATGCTCTTCTGACTTT<br/>TCGTGTGA</b>   | Probe D                                |
| <i>PDC1</i> -D2 | <b>TCGATCACACGAAAAGTCAGAAGAGCATAACATAATCTTTTAAATG<br/>CATAGGTTA</b>  | Probe D                                |
| <i>PDC1</i> -E1 | <b>TCGATTTTTCGTGTGATGAGGCTCGTGGAAAAAATGAATAATTTATG<br/>AATTTGAG</b>  | Probe E                                |
| <i>PDC1</i> -E2 | <b>TCGACTCAAATTCATAAATTATTCATTTTTCCACGAGCCTCATCA<br/>CACGAAAA</b>    | Probe E                                |
| <i>PDC1</i> -F1 | <b>TCGATTCCGGCTTCCTTCCCGATTCCGCCCGCTAAACGCATATT</b>                  | Probe F                                |
| <i>PDC1</i> -F2 | <b>TCGAAATATGCGTTT TAGCGGGCGGAATCGGGAAGGAAGCCGGAA</b>                | Probe E                                |
| <i>PDC1</i> -G1 | <b>TCGACTTCCCGATTCCGCCCGCTAAACGCATATT</b>                            | Probe G                                |
| <i>PDC1</i> -G2 | <b>TCGAAATATGCGTTT TAGCGGGCGGAATCGGGAAG</b>                          | Probe G                                |
| <i>PDC1</i> -H1 | <b>TCGACTTCCCGATTCCGCCCGCTA</b>                                      | Probe H                                |
| <i>PDC1</i> -H2 | <b>TCGATAGCGGGCGGAATCGGGAAG</b>                                      | Probe H                                |
| <i>PDC1</i> -H3 | <b>TCGACTTCCAGATTCCGCCCGCTA</b>                                      | Probe H with<br>mutation at position b |
| <i>PDC1</i> -H4 | <b>TCGATAGCGGGCGGAATCTGGAAG</b>                                      | Probe H with<br>mutation at position b |
| <i>PDC1</i> -H5 | <b>TCGACTTCCCGATTCCAGCCCGCTA</b>                                     | Probe H with<br>mutation at position g |
| <i>PDC1</i> -H6 | <b>TCGATAGCGGGCTGAATCGGGAAG</b>                                      | Probe H with<br>mutation at position g |
| <i>PDC1</i> -H7 | <b>TCGACTTCCCGATTCCGCCAGCTA</b>                                      | Probe H with<br>mutation at position k |
| <i>PDC1</i> -H8 | <b>TCGATAGCTGGCGGAATCGGGAAG</b>                                      | Probe H with<br>mutation at position k |

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| <i>PDC1</i> -H9  | <b>TCGACTTCACGATTCCGCCCGCTA</b>  | Probe H with mutation at position a |
| <i>PDC1</i> -H10 | <b>TCGATAGCGGGCGGAATCGTGAAG</b>  | Probe H with mutation at position a |
| <i>PDC1</i> -H11 | <b>TCGACTTCCCTATTCCGCCCGCTA</b>  | Probe H with mutation at position c |
| <i>PDC1</i> -H12 | <b>TCGATAGCGGGCGGAATAGGGAAG</b>  | Probe H with mutation at position c |
| <i>PDC1</i> -H13 | <b>TCGACTTCCCGCTTCCGCCCGCTA</b>  | Probe H with mutation at position d |
| <i>PDC1</i> -H14 | <b>TCGATAGCGGGCGGAAGCGGGAAG</b>  | Probe H with mutation at position d |
| <i>PDC1</i> -H15 | <b>TCGACTTCCCGAGTCCGCCCGCTA</b>  | Probe H with mutation at position e |
| <i>PDC1</i> -H16 | <b>TCGATAGCGGGCGGACTCGGGAAG</b>  | Probe H with mutation at position e |
| <i>PDC1</i> -H17 | <b>TCGACTTCCCGATGCCGCCCGCTA</b>  | Probe H with mutation at position f |
| <i>PDC1</i> -H18 | <b>TCGATAGCGGGCGGCATCGGGAAG</b>  | Probe H with mutation at position f |
| <i>PDC1</i> -H19 | <b>TCGACTTCCCGATTCTCCCGCTA</b>   | Probe H with mutation at position h |
| <i>PDC1</i> -H20 | <b>TCGATAGCGGGAGGAATCGGGAAG</b>  | Probe H with mutation at position h |
| <i>PDC1</i> -H21 | <b>TCGACTTCCCGATTCCGCCCGCTA</b>  | Probe H with mutation at position i |
| <i>PDC1</i> -H22 | <b>TCGATAGCGGGCGGAATCGGGAAG</b>  | Probe H with mutation at position i |
| <i>PDC1</i> -H23 | <b>TCGACTTCCCGATTCCGCACGCTA</b>  | Probe H with mutation at position j |
| <i>PDC1</i> -H24 | <b>TCGATAGCGGTGCGGAATCGGGAAG</b> | Probe H with mutation at position j |
| <i>PDC1</i> -H25 | <b>TCGACTTCCCGATTCCGCCAGCTA</b>  | Probe H with mutation at position k |
| <i>PDC1</i> -H26 | <b>TCGATAGCTGGCGGAATCGGGAAG</b>  | Probe H with mutation at position k |
| <i>PDC1</i> -H27 | <b>TCGACTTCCCGATTCCGCCCTCTA</b>  | Probe H with mutation at position l |
| <i>PDC1</i> -H28 | <b>TCGATAGAGGGCGGAATCGGGAAG</b>  | Probe H with mutation at position l |

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| <i>PDC1-i1</i>  | <b>TCGATTTTTGGTGGTTCCGGCTTC</b> | Probe I                             |
| <i>PDC1-i2</i>  | <b>TCGAGAAGCCGGAACCACCAAAAA</b> | Probe I                             |
| <i>PDC1-i3</i>  | <b>TCGATTTTTGGTTGTTCCGGCTTC</b> | Probe I with mutation at position a |
| <i>PDC1-i4</i>  | <b>TCGAGAAGCCGGAACAACCAAAAA</b> | Probe I with mutation at position a |
| <i>PDC1-i5</i>  | <b>TCGATTTTTGGTGTTCGGCTTC</b>   | Probe I with mutation at position b |
| <i>PDC1-i6</i>  | <b>TCGAGAAGCCGGAACACCAAAAA</b>  | Probe I with mutation at position b |
| <i>PDC1-i7</i>  | <b>TCGATTTTTGGTGGTGCCGGCTTC</b> | Probe I with mutation at position d |
| <i>PDC1-i8</i>  | <b>TCGAGAAGCCGGCACCACCAAAAA</b> | Probe I with mutation at position d |
| <i>PDC1-i9</i>  | <b>TCGATTTTTGGTGGTTACGGCTTC</b> | Probe I with mutation at position e |
| <i>PDC1-i10</i> | <b>TCGAGAAGCCGTAACCACCAAAAA</b> | Probe I with mutation at position e |
| <i>PDC1-i11</i> | <b>TCGATTTTTGGTGGTTCAGGCTTC</b> | Probe I with mutation at position f |
| <i>PDC1-i12</i> | <b>TCGAGAAGCCTGAACCACCAAAAA</b> | Probe I with mutation at position f |
| <i>PDC1-i13</i> | <b>TCGATTTTTGGTGGTTCCTGCTTC</b> | Probe I with mutation at position g |
| <i>PDC1-i14</i> | <b>TCGAGAAGCAGGAACCACCAAAAA</b> | Probe I with mutation at position g |
| <i>PDC1-i15</i> | <b>TCGATTTTTGGTGGTTCGTCTTC</b>  | Probe I with mutation at position h |
| <i>PDC1-i16</i> | <b>TCGAGAAGACGGAACCACCAAAAA</b> | Probe I with mutation at position h |
| <i>PDC1-i17</i> | <b>TCGATTTTTGGTGGGTCCGGCTTC</b> | Probe I with mutation at position c |
| <i>PDC1-i18</i> | <b>TCGAGAAGCCGGACCCACCAAAAA</b> | Probe I with mutation at position c |

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