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Genome-wide Mapping of the Coactivator Ada2p Yields Insight into the Functional Roles of SAGA/ADA Complex in *Candida albicans*

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The SAGA/ADA coactivator complex, which regulates numerous cellular processes by coordinating histone acetylation, is widely conserved throughout eukaryotes, and analysis of the *Candida albicans* genome identifies the components of this complex in the fungal pathogen. We investigated the multiple functions of SAGA/ADA in *C. albicans* by determining the genome-wide occupancy of Ada2p using chromatin immunoprecipitation (ChIP). Ada2p is recruited to 200 promoters upstream of genes involved in different stress-response functions and metabolic processes. Phenotypic and transcriptomic analysis of *ada2* mutant showed that Ada2p is required for the responses to oxidative stress, as well as to treatments with tunicamycin and fluconazole. Ada2p recruitment to the promoters of oxidative resistance genes is mediated by the transcription factor Cap1p, and coactivator function were also established for Gal4p, which recruits Ada2p to the promoters of glycolysis and pyruvate metabolism genes. Cooccupancy of Ada2p and the drug resistance regulator Mrr1p on the promoters of core resistance genes characterizing drug resistance in clinical strains was also demonstrated. Ada2p recruitment to the promoters of these genes were shown to be completely dependent on Mrr1p. Furthermore, *ADA2* deletion causes a decrease in H3K9 acetylation levels of target genes, thus illustrating its importance for histone acetyl transferase activity.

INTRODUCTION

Candida albicans is a major cause of morbidity and mortality in bloodstream infections. This pathogen can also colonize various biomaterials and readily forms dense biofilms that are resistant to most antifungal agents. Because of the challenges of drug resistance (Kontoyiannis and Lewis, 2002), extensive efforts are underway to identify new drug targets for therapeutic intervention. Despite the large number of studies undertaken on the genetic determinism of *C. albicans* pathogenesis, transcriptional regulation involving chromatin remodeling in this potentially virulent commensal remains largely unknown.

In eukaryotic cells, remodeling of chromatin structure is a critical factor in the control of gene expression because nucleosomes create an inherent physical obstacle for the binding of transacting factors, such as transcription factors (TFs) and RNA polymerases. Histone proteins function as building blocks to package eukaryotic DNA into repeating nucleosomal units that can be organized into highly condensed chromatin fibers (Kornberg and Lorch, 1999). Histone tails protruding beyond the nucleosome core are subjected to many posttranslational modifications, such as acetylation, methylation, phosphorylation, and ubiquitination, all of which can affect chromatin structure and thus regulation of gene expression (Grant, 2001). The Spt-Ada-Gcn5-acetyltransferase (SAGA) coactivator complex regulates numerous cellular processes through coordination of histone posttranslational modifications (Baker and Grant, 2007). Histone acetylation mediated by the SAGA complex through the histone acetyl transferase (HAT) Gcn5p has been widely studied. SAGA is targeted specifically to inducible gene promoters primarily through direct interaction with acidic activator domains of TFs, such as Gal4p and Gcn4p (Baker and Grant, 2007). In the budding yeast *Saccharomyces cerevisiae*, SAGA modulates the expression of ~10% of the measurable genome. The SAGA-dominated genes are strongly enriched in stress-responsive genes involved in challenges such as heat, oxidative agents, acidity, DNA damage, carbon or nitrogen starvation, and unfolded proteins (Daniel and Grant, 2007). Other investigations showed that transcriptional activation of some processes such as amino acid, phosphate, and galactose metabolism were completely dependant on chromatin remodeling mediated by SAGA (Berger *et al.*, 1992). Although best known for its role in regulating transcriptional activation, SAGA is also required for optimal transcription elongation, mRNA export, and DNA repair (Huisinga and Pugh, 2004).

The Ada1-5 proteins (Alteration/deficiency in activation) are components of SAGA and are encoded by genes that,

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Abbreviations used: ChIP-CHIP, chromatin immunoprecipitation–ChIP mircoarray; HAT, histone acetyl transferase; SAGA, Spt-Ada-Gcn5-acetyltransferase; TF, transcription factor.

Table 1. *Candida albicans* strains used in the study

Strain	Genotype	Reference
SC5314	Clinical isolate	Gillum et al. (1984)
BWP17	ura3D::limm434/ura3D::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG	Wilson et al. (1999)
DAY286	ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG-ARG4-URA3/ arg4::hisG	Davis et al. (2002)
VIC1057[a]	ada2Δ::ARG4/ada2Δ::URA3 his1::hisG/his1::hisG	Bruno et al. (2006)
VIC1145[a]	ada2Δ::ARG4/ada2Δ::URA3 pHIS1::his1::hisG/his1::hisG	Bruno et al. (2006)
VIC1197[a]	ada2Δ::ARG4/ada2Δ::URA3 pADA2::HIS1::his1::hisG/his1::hisG	Bruno et al. (2006)
CJD20[b]	ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cap1::hisG-URA3-hisG/cap1::hisG	Alarco and Raymond (1999)
CMM3[a]	gal4Δ::ARG4/gal4Δ::HIS1 his1::hisG/his1::hisG	Martchenko et al. (2007)
AS-20[a]	ADA2/ADA2-TAP-URA3	
AS-21[a]	gal4Δ::ARG4/gal4Δ::HIS1 his1::hisG/his1::hisG ADA2/ADA2-TAP-URA3	This study
AS-22[b]	cap1::hisG-URA3-hisG/cap1::hisG ADA2/ADA2-TAP-URA3	This study
AS-23[a]	MRR1/MRR1-TAP-URA3	This study
MRR1M4B[c]	mrr1Δ::FRT/mrr1Δ::FRT	Morschhauser et al. (2007)
AS-24[c]	mrr1Δ::FRT/mrr1Δ::FRTADA2/ADA2-TAP-SAT1	This study

[a] Strains derived from BWP17 and have the genotype *ura3Δ::λimm434/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG.*
[b] Strain derived from CAI4 and have the genotype *ura3::imm434/ura3::imm434.*
[c] Strain derived from the clinical strain SC5314.

when inactivated, alleviate the toxicity of the chimeric activator GAL4-VPS16 in *S. cerevisiae* (Barrios *et al.*, 2007). Furthermore, it was demonstrated (Marcus *et al.*, 1994) that the Ada2/Ada3/Gcn5 complex is sufficient for robust histone and nucleosomal HAT activity. Gcn5p interacts with Ada2p in vivo and in vitro, thus establishing a physical and genetic link between these transcriptional components (Marcus *et al.*, 1994). Moreover, biochemical studies have shown that Ada2p interacts directly with the activation domains of Gcn4p and Gal4p, and indirectly with the TATA-binding protein (TBP), arguing that Ada2p may mediate interactions between the acidic activator domains of TFs and the basal transcriptional machinery component TFIID (Barlev *et al.*, 1995; Bhaumik and Green, 2001; Larschan and Winston, 2001).

The first evidence in *C. albicans* of a role for chromatin remodeling was the report (Klar *et al.*, 2001) that treating cells with the deacetylase inhibitor trichostatin A or deletion of the deacetylase-encoding genes *HDA1* or *RPD3* caused increases in the frequency of white-opaque switching. A recent study undertaken in *C. albicans* has also shown that Ada2p is required for the cell wall damage response, most probably by acting in combination with a variety of other regulators (Bruno *et al.*, 2006). However, up to now there has been little information about the biological processes controlled by the *C. albicans* SAGA/ADA coactivator complex.

In this study we have begun to elucidate the multiple functions of SAGA/ADA coactivator complex in *C. albicans*. Using chromatin immunoprecipitation (ChIP) coupled with microarray analysis (ChIP–CHIP), we have established the promoter occupancy of the SAGA/ADA component Ada2p. Our results yield insight into the role of Ada2p in drug, oxidative stress, and unfolded protein responses (UPRs) as well as virulence. We also investigated the importance of Ada2p recruitment by the transcription factors Cap1p and Gal4p to the promoters of oxidative stress responsive and glycolysis genes, respectively. Interestingly, we showed that Ada2p was involved in fluconazole tolerance, and its cooccupancy with the drug resistance regulator Mrr1p on the promoters of core resistance genes characterizing drug resistance in *MDR1*-overexpressing clinical strains was demonstrated. Likewise, we have shown that an *ada2* deletion causes a clear decrease of histone acetylation in vivo. These data demonstrate the role of Ada2p in chromatin remodeling through histone acetylation in addition to its function as a specific transcriptional coactivator recruited by TFs to their target promoters.

MATERIALS AND METHODS

C. albicans Strain Construction, Plasmids, and Media

Strains used in this study are listed in Table 1. Cell growth, transformation, and DNA preparation were carried out using standard yeast procedures. Cells were grown at 30°C in YPD media (1% yeast extract, 2% peptone, 2% dextrose). *ADA2* and *MRR1* were tandem affinity purification (TAP)-tagged in vivo with a TAP-*URA3* PCR product containing 100-bp homology up- and downstream of each open reading frame (ORF) as described by Lavoie *et al.* (2008). Transformants were selected on YPD −ura plates and correct integration of the TAP-tag was checked by PCR and sequencing. For Ada2-TAP ChIP in *mrr1* mutant, the TAP-tagging was performed using pFA-TAP-SAT1 plasmid to generate a cassette that contains a dominant nourseothricin resistance marker (SAT1). pFA-TAP-SAT1 was generated as follows: the *SAT1* gene was amplified by PCR from the previously published plasmid (Reuss *et al.*, 2004) using primers SAT1F and SAT1R. Subcloning of the *C. albicans SAT1* marker was done by ligation of AscI-PmeI PCR fragments in AscI-PmeI–digested pFA-TAP-URA3.

ADA2-TAP expressed in BWP17 strain was fully functional based on complementation of the oxidative stress sensitivity phenotype. Deleting the nontagged allele in the ADA2-TAP strain revealed that this strain has a comparable sensitivity to the single knockout strain and even to the parental strain. The same procedure was used to demonstrate the functionality of MRR1-TAP using the fluconazole sensitivity phenotype.

Drug Susceptibility Tests

Stock solutions were prepared using ethanol as the solvent for menadione (200 mM) and DMSO for fluconazole (100 mg/ml). Growth inhibition by menadione was assessed using a serial dilution inhibition test as described by Bruno *et al.* (2006). Fluconazole susceptibility was quantified using a microtiter plate liquid assay. The data are presented as the percent of relative growth of the cells in fluconazole-containing medium compared with the growth of the same strain in fluconazole-free medium. The values represent the means ± SDs of three independent experiments performed in triplicate.

Whole-Genome Location Profiling by ChIP-CHIP and ChIP-Real Time Quantitative PCR

ChIP experiments were performed as described previously with some modifications (Guillemette *et al.*, 2005). Briefly, cells were grown to an optical density at 600 nm of 2 in 40 ml of YPD. We followed the ChIP protocol

available at http://www.ircm.qc.ca/microsites/francoisrobert/en/317.html with the following modifications: chromatin was sonicated to an average 300 bp, and 700 μl of whole-cell extract (WCE) was incubated with IgG Sepharose beads (Amersham, Piscataway, NJ). Immunoprecipitated DNA was used for either whole-genome location profiling or gene-specific real time quantitative PCR analysis. For whole-genome location profiling, tagged ChIPs were labeled with Cy5 dye, and untagged (mock) ChIPs were labeled with Cy3 dye. Microarrays containing 11,817 70-mer oligonucleotide probes were cohybridized with tagged immunoprecipitated (Cy5-labeled) and mock-immunoprecipitated (untagged strain; Cy3-labeled) DNA samples. Microarray hybridization, washing, and scanning were performed as described (Nantel *et al.*, 2006). Prehybridization and hybridization solutions consisted of DIG Easy Hyb solution (Roche Diagnostics, Mannheim, Germany) with 0.45% salmon sperm DNA and 0.45% yeast tRNA. Slides were washed once in 1.0% SSC, 0.2% SDS at 42°C for 10 min; twice in 0.1% SSC, 0.2% SDS at 42°C for 10 min; and once in 0.1% SCC at 24°C for 5 min, followed by four rinses in 0.1% SSC. ChIPs were air dried before being scanned using a ScanArray Lite microarray scanner (PerkinElmer, Waltham, MA). QuantArray was used to quantify fluorescence intensities. Data handling and analysis were carried out using Genespring v.7.3 (Agilent Technologies, Palo Alto, CA). The significance cutoff was determined using the distribution of log-ratios for each factor. It was set at 2 SDs from the mean of log-transformed fold enrichments. Values shown are an average of two biological replicates derived from independently isolated transformants of tagged and mock constructs.

Quantitative real-time PCR (qPCR) was performed using the Corbett Rotor-Gene RG-3000A (Corbett Research, Sydney, Australia) with SYBR Green fluorescence (Qiagen, Chatsworth, CA). qPCR was performed using 1 ng of TAP-ChIPed DNA or total genomic DNA extracted from WCE. Cycling was for 15 min at 95°C, followed by 45 cycles of 95°C for 10 s, 58°C for 15 s, and 72°C for 15 s. All samples were tested in triplicate and means were used for further calculations. Fold-enrichments of tested promoter sequences were estimated by using the coding sequence of the *C. albicans ACT1* ORF as a reference. *ACT1* was chosen as a reference since no IP enrichment was detected for *ACT1* ORF in the ADA2-TAP strain relative to the control strain BWP17. Fold-enrichment of the tested promoter sequences was estimated using the comparative ΔΔCt method as described by Guillemette *et al.* (2005). Primer sequences used for this analysis are summarized in Supplemental Table S1.

Histone acetylation was assessed by ChIP-qPCR as described above using anti-acetyl-Histone H3 (Lys9; 06-942, Upstate Biotechnology, Lake Placid, NY) and anti-acetyl-Histone H4 (06-598, Upstate Biotechnology) antibodies.

Expression Analysis by qPCR

For fluconazole, tunicamycin, and menadione treatments, cultures were inoculated from a fresh colony and grown overnight in YPD at 30°C. Cultures were then diluted to an OD600 of 0.1 in 100 ml of fresh YPD and grown at the same initial temperature until an OD600 of 0.8. The culture was divided in two volumes of 50 ml; one sample was maintained as the control, and the other treated with compounds cited above. *Candida* cells were exposed to 10 μg/ml fluconazole and 4.73 μM tuncamycin for 1 h and to 0.2 mM menadione for 30 min. Cells were then centrifuged 2 min at 3500 rpm, the supernatants were removed, and the samples were quick-frozen and stored at −80°C.

cDNA was synthesized from 2 μg of total RNA using the reverse-transcription system (50 mm Tris-HCl, 75 mm KCl, 10 mm dithiothreitol (DTT), 3 mm $MgCl_2$, 400 nm oligo$(dT)_{15}$, 1 μm random octamers, 0.5 mm dNTPs, and 200 U Superscript III reverse transcriptase; Invitrogen, Carlsbad, CA). The total volume was adjusted to 20 μl, and the mixture was then incubated for 60 min at 42°C. Aliquots of the resulting first-strand cDNA were used for real-time PCR amplification experiments. Real-time PCRs were performed as described for ChIP-qPCR.

The *HAC1* splicing rate in tunicamycin-challenged cells was evaluated by quantifying specifically the *HAC1* spliced mRNA (sHAC1) by qPCR using the primer pair HAC1F1 and HAC1R1. The primer HAC1R1 was designed to overlap the contiguous exons generated after the excision of the unconventional intron (Wimalasena *et al.*, 2008). The results were normalized using Ct values obtained for *ACT1*. The splicing rate in the *ada2* mutant was determined by comparing sHAC1 levels using the wild type (wt) as a reference.

Virulence Studies

Virulence testing of *C. albicans* was done as previously described (Mullick *et al.*, 2004). Briefly, 8- to 12-wk old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were inoculated via the tail vein with 200 μl of a suspension containing 3×10^5 *C. albicans* in PBS. Five male mice were used for each experimental group. Mice were closely monitored over a period of 21 d for clinical signs of disease such as lethargy, ruffled fur, or hunched back. Mice showing extreme lethargy were considered moribund and were killed. All experimental procedures involving animals were approved by the Biotechnology Research Institute Animal Care Committee, which operated under the guidelines of the Canadian Council of Animal Care.

RESULTS

Ada2p Binds 200 Gene Promoters Associated with Specific Functional Categories

BlastP analysis revealed that, in *C. albicans*, the same SAGA/ADA core components were present as in *S. cerevisiae* (Table 2). Functional domain conservation of the core components GCN5/ADA2/ADA3 was also verified and showed a high degree of similarity (data not shown). In the present study we focused on a core component of the SAGA/ADA complex: Ada2p, which was shown to be crucial for histone acetylation in *S. cerevisiae* (Marcus *et al.*, 1994; Barrios *et al.*, 2007).

We set out to investigate the genomic occupancy of Ada2p using ChIP-CHIP. Cells expressing a TAP-tagged version of Ada2p were lysed and sonicated, and the DNA-Ada2p-TAP was purified. Cross-linking was reversed, and the purified DNA was amplified by ligation-mediated PCR, labeled with Cy5, and hybridized to a microarray containing 5423 intergenic and 6394 intragenic 70-mer oligonucleotide probes. As a control, DNA that was precipitated from BWP17 cells was labeled with the Cy3 fluorochrome. All experiments were repeated twice from samples cross-linked separately. Using a cutoff of two SDs above the mean of log ratios (giving a 1.44-fold enrichment cutoff), Ada2p was found to associate with 200 of the 11,817 probes in our microarray layout (Figure 1A and Supplemental Table S2). To assess the reliability of the ChIP-CHIP method, the immunoprecipitated DNA from two other independent ChIP experiments was quantified using qPCR. A total of 18 promoters were selected and a set of three pairs of PCR primers per promoter were designed to amplify 0 to −200-bp, −200- to −400-bp, and −400- to −600-bp regions upstream the ATG. The result obtained for the 18 selected promoters confirmed binding of Ada2p to the promoters identified by ChIP-CHIP (Figure 1B). The peak intensity of enrichment was most commonly observed in the 0 to −400-bp region.

To gain further insight into the biological function of Ada2p, Gene Ontology (GO) biological process categories were assigned to the 200 genes near Ada2p-occupied loci. For these analyses, all GO categories of genes having an enrichment $p < 0.01$ were selected. Functional categories of Ada2p target genes are summarized in Figure 1C. This analysis revealed a significant enrichment in genes related to metabolic process such as glycolysis ($p = 5.49e-17$), pyruvate metabolism ($p = 1.08e-07$), and protein biosynthesis ($p = 4.08e-05$). Functional categories belonging to stress response were significantly represented in our set. These include genes connected to oxidative stress ($p = 7.69e-05$) and drug response ($p = 1.56e-05$), as well as protein folding ($p = 3.95e-08$). Ada2p was found to occupy cell wall gene promoters ($p = 5.06e-07$) consisting essentially of GPI (glycosylphosphatidylinositol)-anchored proteins. Interestingly, Ada2p was recruited to promoters of proteasome regulatory genes ($p = 3.65e-03$) including the gene encoding the transcription factor Rpn4p. Categories such as lipid metabolism ($p = 2.23e-01$), amino acid metabolism ($p = 5.34e-02$), DNA replication ($p = 8.08$ e-02), early secretion pathway ($p = 8.3e-01$), vacuolar acidification ($p = 1.23e-01$), and chromatin maintenance ($p = 4.01e-01$) did not meet the statistical cutoff criterion in this experiment. Ada2p was also found in the promoter of a large number of hypothetical genes (50 genes).

ADA2 Depletion Affects Histone Acetylation In Vivo

In the budding yeast *S. cerevisiae*, the HAT catalytic subunit of the SAGA coactivator complex, Gcn5p, was shown to preferentially acetylate multiple lysine residues on the N-

Table 2. *Candida albicans* homologs of the *Saccharomyces cerevisiae* SAGA/ADA coactivator complexes

S. cerevisiae protein	Systematic name	*C. albicans* homologs	E value	Function in *S. cerevisiae*
HAT				
Gcn5 (Ada4)	YGR252W	orf19.705	6.1e-153	Histone acetyltransferase
Ada				
Ada1	YPL254W	orf19.307	8.9e-37	Transcription coactivators required for nucleosomal acetylation by Gcn5
Ada2	YDR448W	orf19.2331	7.1e-127	
Ada3	YDR176W	orf19.3023	2.6e-49	
Spt				
Spt3	YDR392W	orf19.7622	6.0e-91	TBP interaction, transcriptional repression
Spt8	YLR055C	orf19.4312	9.1e-87	
Spt7	YBR081C	orf19.7572	9.8e-144	Complex stability and maintenance
Spt20 (Ada5)	YOL148C	orf19.422	1.2e-28	
TAF				
TAF5	YBR198C	orf19.536	4.7e-176	Structural integrity of the complex and interaction with basal transcription machinery
TAF6	YGL112C	orf19.7454	6.8e-106	
TAF9	YMR236W	orf19.1111	3.8e-41	
TAF10	YDR167W	orf19.3242	8.1e-35	
TAF12	YDR145W	orf19.470	1.1e-46	
H2B deubiquitylation				
Ubp8	YMR223W	orf19.1767	4.8e-70	Deubiquitylation of H2BK123
Sgf11	YPL047W	orf19.7360	9.1e-08	Required for association of Ubp8 and Sus1 with SAGA
Sus1	YBR111W-A	orf19.6795	8.8e-10	mRNA export
Chd				
Chd1	YER164W	orf19.3035	0	Recognition of H3K4 methylation and potentiation of histone acetylation by GCN5
Rtg2	YGL252C	—	—	SLIK stability
Other				
Sgf29	YCL010C	orf19.7074	8.8e-58	Unknown
Tra1	YHR099W	orf19.139	0	Interaction with transcriptional activators

terminal tails of histones H3 and H2B (Suka *et al.*, 2001). Furthermore, Ada2p was found to be required for the Gcn5p nucleosomal HAT activity in vivo (Candau *et al.*, 1997). In light of this data, we sought to examine the role of Ada2p in HAT in *C. albicans* by assessing histone acetylation levels and by performing ChIP-qPCR using antibodies directed against acetylated histones H3K9 and H4 in wt and in *ada2* cells.

Acetylation levels of the entire ORF as well as 1 kbp upstream of the *GAL4* and *MDR1* genes were quantified relative to the acetylation level of the *ACT1* coding region (Figure 2). In wt cells, *GAL4* and *MDR1* showed increased H3K9-acetylation compared with the *ACT1* coding region. Notably, the H3K9-acetylation profiles of the two genes were different; although *GAL4* exhibits a peak in the −200-bp promoter region, *MDR1* H3K9-acetylation level peaked at the 3′-extremity of the ORF (Figure 2). In both genes, H3K9-acetylation was significantly decreased but not completely abolished in the *ada2*-deleted strain. For each gene, the wt H4-acetylation profiles were similar to those of H3K9; however, no significant differences were recorded in the *ada2* mutant (data not shown). Thus, these results suggest that Ada2p is required for a specific histone acetylation in vivo.

ada2 Mutant Strains Show Altered Sensitivity to Fluconazole and Oxidative Stress and Display Attenuated Virulence

Genes involved in the oxidative stress and drug responses were significantly overrepresented in Ada2p-bound promoters. This finding prompted us to assess if the inactivation of this regulator affects drug and oxidative stress sensitivity. As shown in Figure 3A, on YPD medium, wt and the *ada2* mutant, as well as the revertant strain, grew similarly. On YPD medium supplemented with 0.2 mM menadione, growth of *ada2* mutant cells was completely abolished, whereas the revertant was slightly inhibited and its sensitivity was found to be comparable to that of the parent strain. The same effect was observed with hydrogen peroxide (data not shown). The sensitivity of an *ada2* mutant strain in the presence of the azole drug fluconazole was evaluated using a liquid microdilution assay. The growth of the *ada2* strain was indeed moderately inhibited in the presence of fluconazole compared with the revertant and parental strains (Figure 3B). This result suggests that in addition to the crucial role in mediating oxidative stress resistance, Ada2p contributes to azole tolerance.

Sensitivity to oxidative stress inducers is often linked to a decrease in virulence. Because *ada2* was hypersensitive to menadione and hydrogen peroxide, we determined if this gene is required for *C. albicans* virulence. The *C. albicans* strains DAY286 (wt), an *ada2* mutant strain, and an *ada2* revertant were tested in a mouse systemic infection model by intravenous tail infection. As shown in Figure 3C, although 100% of the mice infected with the wt strain died within 12 d, only 20% of mice infected with the *ada2* mutant died in this period. The revertant strain showed an intermediate survival rate consistent with the reintroduction of one copy of Ada2p. This finding reveals that the *ada2* deletion causes attenuated virulence during systemic infection.

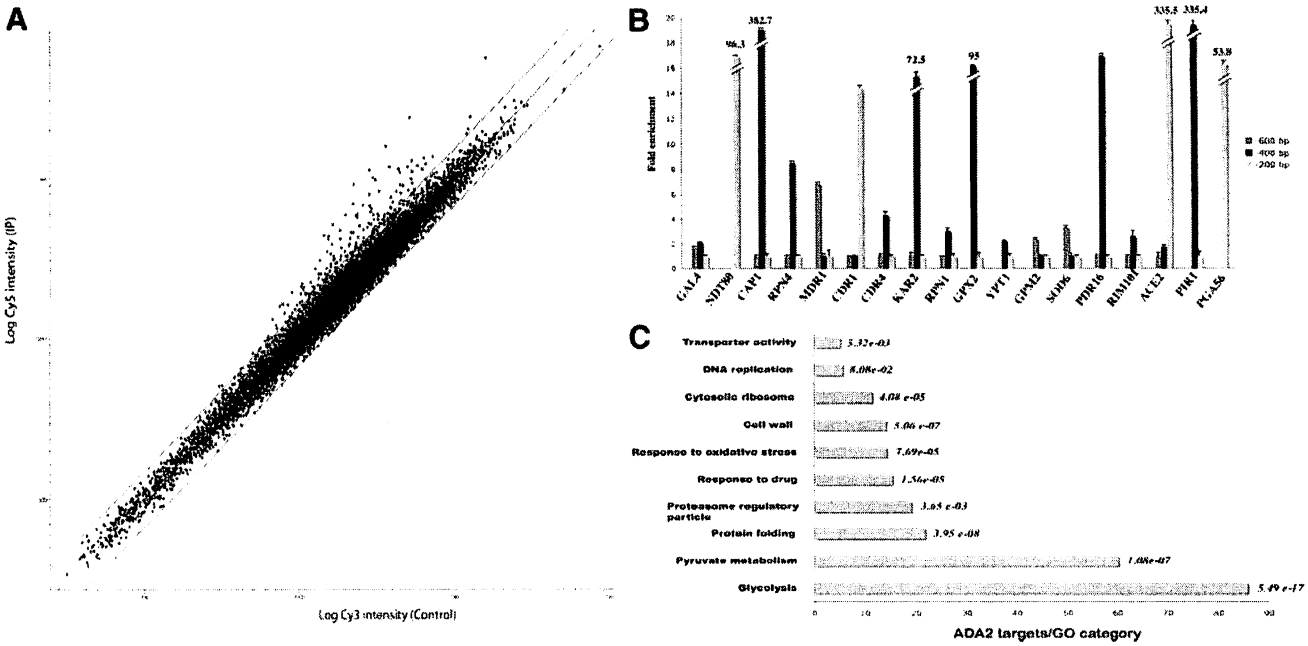


Figure 1. Genome-wide location of Ada2p. (A) Location analysis scatter plot. DNA fragments derived from ChIP were labeled with Cy5, compared with genomic DNA fragments from an untagged strain, and labeled with Cy3. Both samples were hybridized to a single array. Representative scatter plot for Ada2p includes lines representing the 2-SD cutoff (1.44-fold). (B) In vivo occupancy of Ada2p at various intergenic regions. TAP ChIP DNA was subjected to qPCR to validate ADA2 binding to 0 to −20-bp, −200- to −400-bp, and −400 to −600-bp promoter regions. SDs were based on data from two independent experiments. (C) GO biological process annotation of Ada2p bound promoters. The p value was calculated using hypergeometric distribution as described in the GO Term Finder Tool Web site (www.candidagenome.org/cgi-bin/GO/goTermFinder).

Ada2p Regulates the Expression of Drug, Oxidative Stress and Unfolded Protein–Responsive Genes

To assess whether hypersensitivity to oxidative stress, as well as Ada2p occupancy correlates with gene expression, RNA levels of oxidative stress–responsive genes was determined. We analyzed the expression level of the transcription factor *CAP1* as well as *CTA8* (homolog of *S. cerevisiae HSF1*), *SOD6*, *TRX1*, and the glutathione peroxidase *GPX2* in wt and the *ada2* mutant under normal growth conditions and after treatment with menadione. The data revealed that, for the wt, the transcript levels of the selected genes were noticeably increased when cells were challenged with menadi-

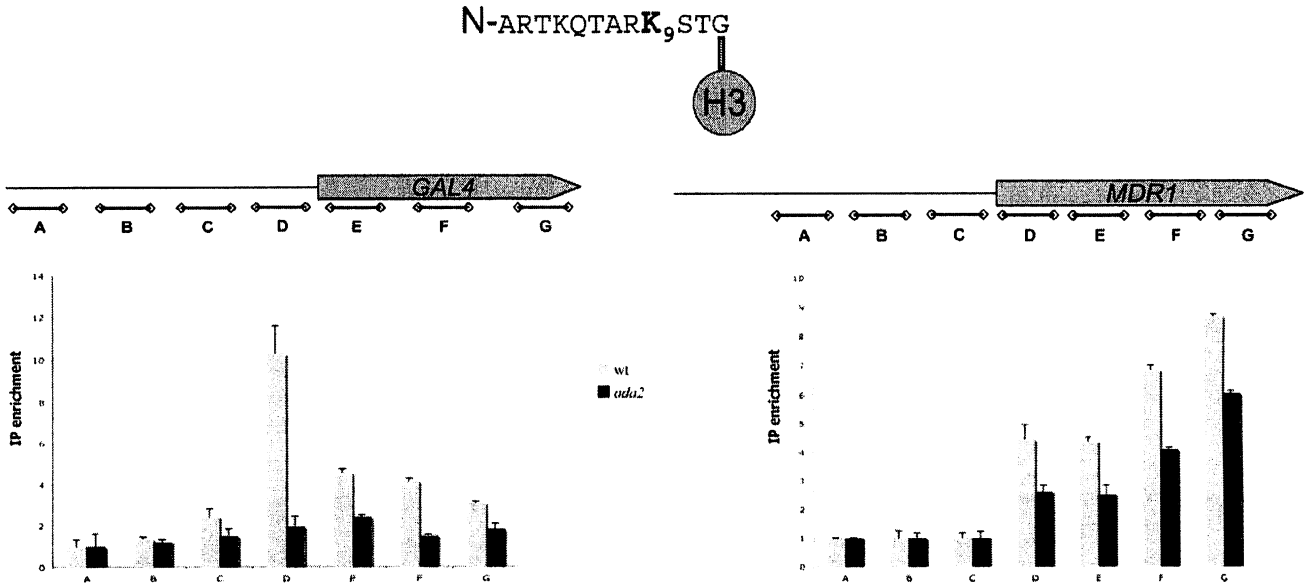


Figure 2. Ada2 deletion affects acetylation of H3K9 in vivo. Fine mapping of *GAL4* and *MDR1* H3K9 acetylation in the wt and *ada2* mutant using ChIP-qPCR. The IP enrichments represent the binding ratios that were all normalized to the *ACT1* ORF, which was set to 1.

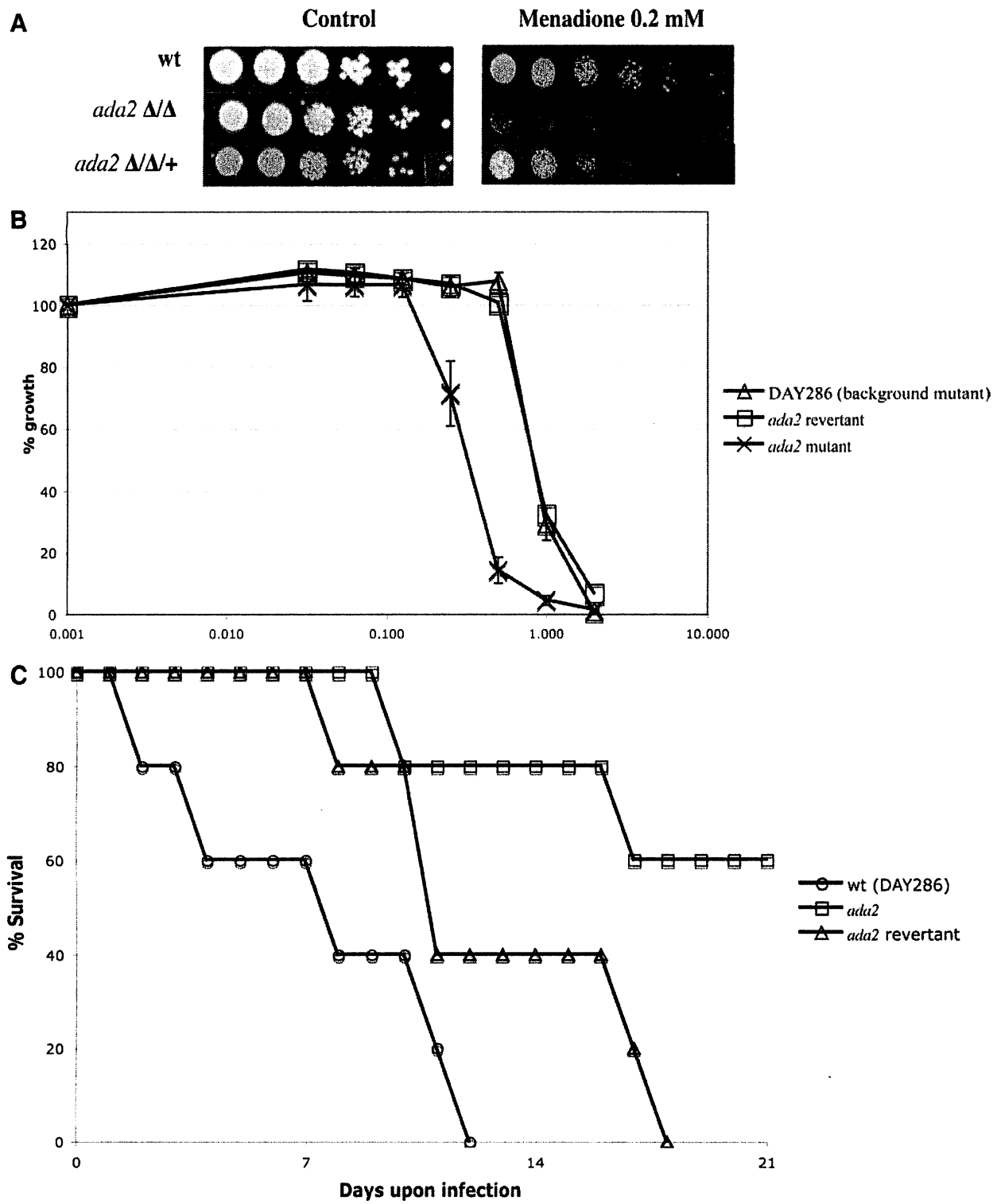


Figure 3. Phenotypic analysis of *ada2* mutant. (A) Absence of Ada2p causes hypersensitivity to menadione. Fivefold serial dilutions of wt, *ada2* mutant, and revertant strains were grown on YPD supplemented with menadione at 0.2 mM and grown at 30°C for 48 h. (B) Fluconazole sensitivity of *ada2* revealed using microtiter plate liquid assay. The data are presented as the percent relative growth of the cells in fluconazole-containing medium compared with the growth of the same strain in fluconazole-free medium. The values represent the means ± SDs of three independent experiments performed in duplicate. (C) Survival of mice infected with *C. albicans ada2* mutant (□), *ada2* revertant (△), and wt parental (○) strains. Mice were inoculated by tail vein injection, and survival was measured over a 21-d period. Average expression of oxidative stress–responsive genes is shown in wt and *ada2* cells 30 min after 0.2 mM menadione treatment. The reported values are the means ± SD of three independent experiments.

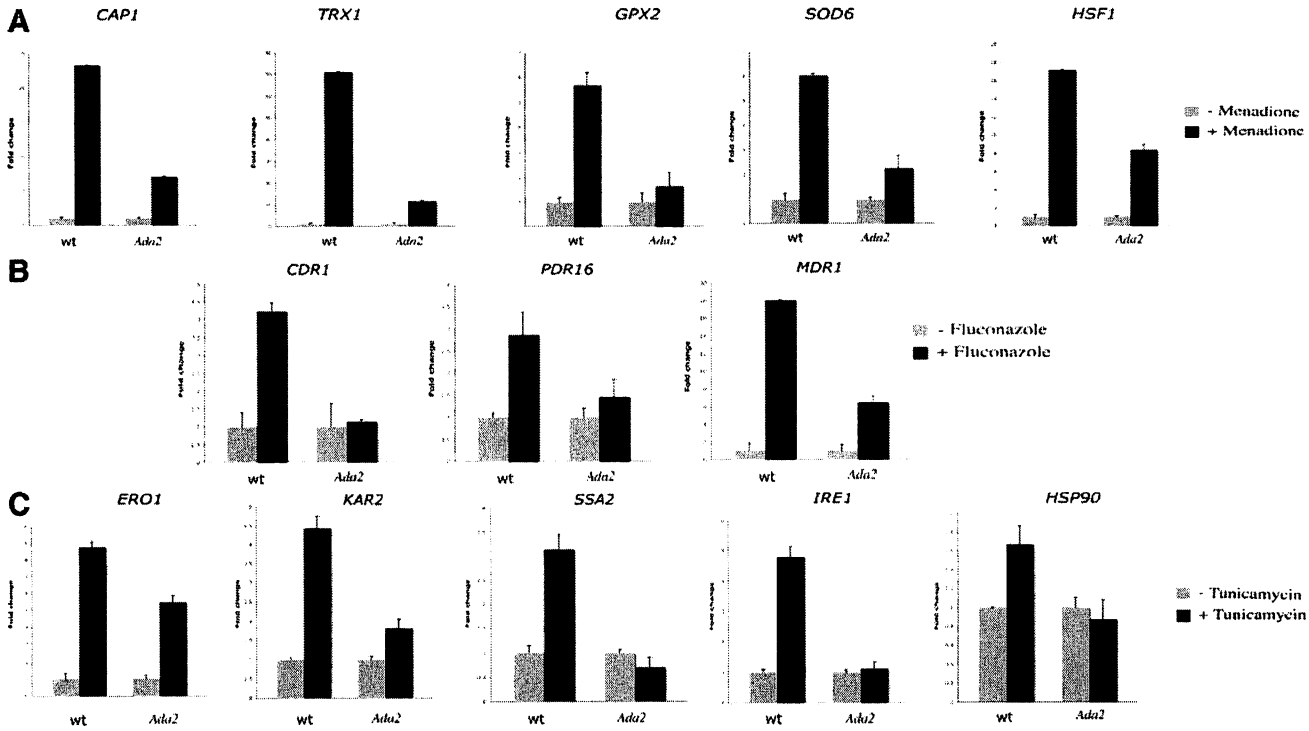


Figure 4. Ada2p regulates the expression of drug-, oxidative stress–, and UPR-responsive genes. Average expression of oxidative stress– (A), drug- (B), and UPR- responsive (C) genes is shown in wt and *ada2*. The reported values are the means ± SD of three independent experiments.

one (Figure 4A). This activation was significantly reduced but not fully abolished in the absence of Ada2p for all tested genes.

Furthermore, we sought to determine whether the Ada2p-dependent transcriptional inducibility of drug-responsive genes contributes to azole tolerance. To this end, transcript levels of the two drug transporters Cdr1p and Mdr1p, as well as the phosphatidylinositol transfer protein Pdr16p, were evaluated in the wt and in the *ada2* mutant under normal condition and after challenge with fluconazole. In the wt background, the expression of *CDR1*, *PDR16*, and *MDR1* was significantly induced, whereas in the *ada2* mutant the inducibility of those genes was impaired (Figure 4B). Although Ada2p was also seen to interact with the promoter of the transcription factor Ndt80p, known to be required for drug tolerance by regulating *CDR1* expression (Wang *et al.*, 2006a), no differential expression of the *NDT80* gene was noticed in the wt or in the *ada2* mutant in response to fluconazole (data not shown).

In the budding yeast *S. cerevisiae*, SAGA/ADA was shown to play an important role in mediating the UPR (Welihinda *et al.*, 1997, 2000). In our study, we found that Ada2p binding was significantly enriched for gene promoters whose products are involved in protein folding (Figure 1A). Indeed, promoters for several chaperones such as Hsp90p, Hsp104p, Hsp60p, and Hsp31p as well as the endoplasmic reticulum (ER)-resident chaperone Kar2p and the ER-thioloxidase Ero1p were bound by Ada2p. To investigate whether Ada2p is required for the activation of the UPR in *C. albicans*, expression of the chaperones *HSP90*, *SSA2*, and *KAR2* as well as *ERO1* was assessed in the wt and the *ada2* mutant treated or not with the UPR-inducing agent tunicamycin. Additionally, the expression level of *IRE1*, widely known to mediate the UPR in eukaryotes by regulating Hac1p synthesis through *HAC1* mRNA splicing, was also monitored. Noticeably, Ada2p bound to the *IRE1* promoter with a moderate enrichment ratio of 1.31, which was just below the defined cutoff. In the wt background, the transcript level of all selected genes was induced in tunicamycin-treated cells. In the *ada2* mutant, although tunicamycin inducibility of *KAR2* and *ERO1* was partially impaired, *SSA2*, *IRE1*, and *HSP90* up-regulation was completely abolished (Figure 4C). The effect of the *ada2* deletion on the splicing of *HAC1* mRNA was also evaluated using qRT-PCR as outlined in *Materials and Methods*. The Hac1p splicing rate was assessed in the presence of tunicamycin, and our results revealed a reduction of 26% of *HAC1* splicing in the *ada2* mutant compared with the wt (data not shown). Despite the apparent role of Ada2p in regulating UPR gene expression and *HAC1* splicing, there was no significant difference in sensitivity to tunicamycin or DTT between the *ada2* mutant and wt cells (data not shown).

Specific Recruitments of Ada2p by Gal4p, Cap1p, and Mrr1p Transcription Factors

Recruitment by Gal4p to Glycolysis and Pyruvate Metabolism Gene Promoters. In the budding yeast *S. cerevisiae*, recruitment of the SAGA complex to regulate *GAL* genes through Gal4p has been broadly investigated. Indeed, many studies have shown that the transcriptional activation of *GAL* genes was completely dependent on SAGA (Sterner *et al.*, 1999; Bhaumik and Green, 2001; Larschan and Winston, 2001). Although the sequence and synteny of the Leloir pathway genes are highly conserved between *S. cerevisiae* and *C. albicans*, the regulatory circuit controlling this metabolic process is completely different (Martchenko *et al.*, 2007). *GAL4* is not required for control of

Table 3. Ada2p promoter occupancies of glycolysis and pyruvate metabolism genes in the wt and *gal4* mutant

Orf19	Gene name	Description	Gal4p binding motif	Position	Binding ratio *wt*	Binding ratio *gal4*
Glycolysis						
orf19.4941	*TYE7*	bHLH transcription factor	GCC $_{(N11)}$ GGC	−1214	2.11	0.91
orf19.3575	*CDC19*	Putative pyruvate kinase	CGG $_{(N11)}$ TCG	−263	2.14	0.91
orf19.3967	*PFK1*	Alpha subunit of phosphofructokinase	TCC $_{(N11)}$ GGG	−753	2.21	0.95
orf19.6540	*PFK2*	Beta subunit of phosphofructokinase	CCC $_{(N11)}$ GGC	−586	2.01	1.00
orf19.3997	*ADH1*	Alcohol dehydrogenase	CGG $_{(N11)}$ CCG	−613	2.04	1,03
orf19.3888	*PGI1*	Glucose-6-phosphate isomerase	TCC $_{(N11)}$ GGC	−508	1.84	0.96
orf19.6745	*TPI1*	Triose-phosphate isomerase	CGT $_{(N11)}$ CCG	-291	1.79	0.84
orf19.4617	*FBA1*	FBA1 Putative fructose-bisphosphate aldolase	GCC $_{(N11)}$ GGT	−397	1.78	1.1
orf19.5338	*GAL4*	Transcription factor with zinc cluster DNA-binding motif	CGA $_{(N11)}$ CCG	−710	1.5	1.04
orf19.1067	*GPM2*	Phosphoglycerate mutase	CGA $_{(N11)}$CCG	−268	1.55	1.15
orf19.6814	*TDH3*	Glyceraldehyde-3-phosphate dehydrogenase	ACC $_{(N11)}$ GGC	−303	1.65	1.08
orf19.395	*ENO1*	ENO1 Enolase (2-phospho-D-glycerate-hydrolyase)	GCC $_{(N11)}$ GGT	−308	2.6	1.03
orf19.903	*GPM1*	Phosphoglycerate mutase	AGG $_{(N11)}$ CCG	−268	2.55	1.13
orf19.3651	*PGK1*	Phosphoglycerate kinase	ACC $_{(N11)}$ GGC	−456	2.5	0.98
Pyruvate metabolism						
orf19.2877	*PDC11*	Pyruvate decarboxylase	GCC $_{(N11)}$ GGG	−324	1.95	0.89
orf19.5021	*PDX1*	Dihydrolipoamide dehydrogenase (E3)-binding protein of the mitochondrial pyruvate dehydrogenase (PDH) complex	GCC $_{(N11)}$ GGT	−164	2.02	1.15
orf19.3097	*PDA1*	E1 alpha subunit of the pyruvate dehydrogenase (PDH) complex	GCC $_{(N11)}$ GGT	−141	1.86	0.87
orf19.5294	*PDB1*	E1 beta subunit of the pyruvate dehydrogenase (PDH) complex	GCC $_{(N11)}$ GGT	−196	1.87	0.92
orf19.6561	*LAT1*	Dihydrolipoamide acetyltransferase component (E2) of pyruvate dehydrogenase complex	GCC $_{(N11)}$ GGT	−242	1.8	0.93
Translation and ribosome assembly						
orf19.2935	*RPL10*	Cytoplasmic ribosomal subunits	—	—	1.72	1.71
orf19.6265	*RPS22A*	Cytoplasmic ribosomal suunits	—	—	1.56	2.15

Occupancy of *RPL10* and *RPS22A* promoters is shown as a control. Detection of Gal4p motif was performed as described by Hogues *et al.* (2008).

the Leloir pathway genes but instead participates in the regulation of genes, representing 2% of *C. albicans* genome, which are involved in a diverse range of cellular process including glycolysis.

In our study glycolysis stood out as the most significantly enriched category for Ada2p binding. This prompted us to ask whether Ada2p is required for Gal4p to regulate glycolysis genes in *C. albicans*. To test this hypothesis, we performed genome-wide mapping of Ada2p in a *gal4* deletion mutant. Our result revealed clearly that the occupancy of all Ada2p-bound glycolysis promoters was dramatically reduced in the absence of *GAL4* (Table 3). Ada2p occupancy at promoters of genes encoding the pyruvate metabolism genes Pdc11p, Pda1p, Pdx1p, Lat1p, and Pdb1p was also lost in *gal4* background (Table 3). These data suggest that Ada2p is exclusively recruited by Gal4p to regulate genes from the glycolysis pathway and pyruvate metabolism.

Cap1p Recruitment to the Promoters of Oxidative Stress–responsive Genes. Because many promoters bound by Ada2p were found upstream of genes previously reported to be regulated by Cap1p, we compared the Ada2p occupancy data with the published transcription profiling undertaken on the *cap1* mutant treated with H_2O_2 (Wang *et al.*, 2006b). A significant overlap (p = 1.04e-13) was found between Cap1p-dependant genes and Ada2p bound promoters consisting of 15 common genes (Figure 5). When Ada2p binding cutoff was reduced to 1.2 the overlap was expanded to 27 genes (p = 1.8e-32). Based on these results, it is possible that Cap1p is recruited by Ada2p to its target promoter genes as part of the oxidative stress response. To test this hypothesis, we mapped, using ChIP, Ada2-TAP in vivo occupancy in a *cap1* mutant strain. The −1- and −200-bp promoter regions of the Cap1p-dependant genes *TTR1*, *GRP2*, *GPX2*, *YCF1*, *MDR1*, and *CIP1* were targeted for occupancy enrichment using qPCR. Our result demonstrates clearly that Ada2p promoter binding for all tested genes was decreased in the *cap1* mutant compared with the parental strain (Figure 5).

Mrr1p Recruitment to the Promoters of Genes Related to Fluconazole Clinical Resistance. Intriguingly, Ada2p was

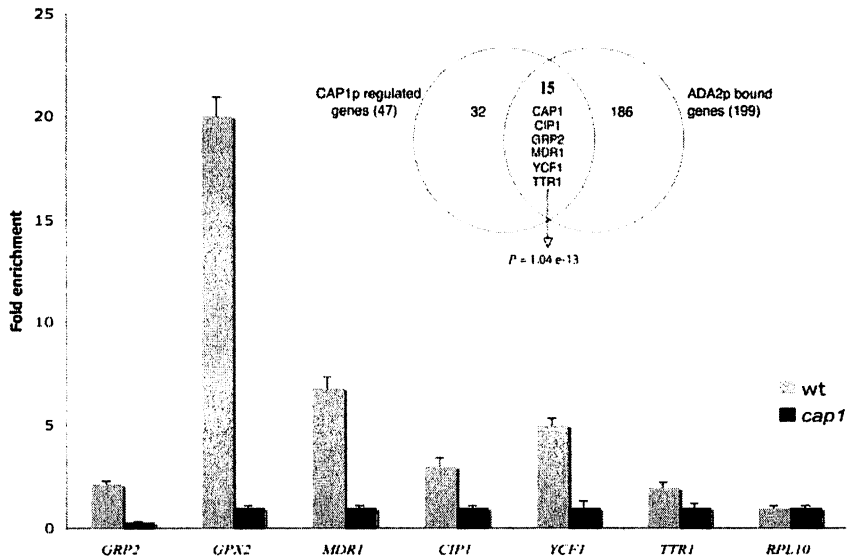


Figure 5. Cap1p recruits Ada2p to the promoter of oxidative stress–responsive genes. ChIP-qPCR occupancy analysis of Ada2p at the indicated promoter regions in the wt strain (▨) and *cap1* mutant (■). Error bars, SD of two biological replicates. Occupancy of *RPL10* promoters is shown as a control. Venn diagram depicting the overlap between Ada2p bound promoters and Cap1p-dependant genes is shown.

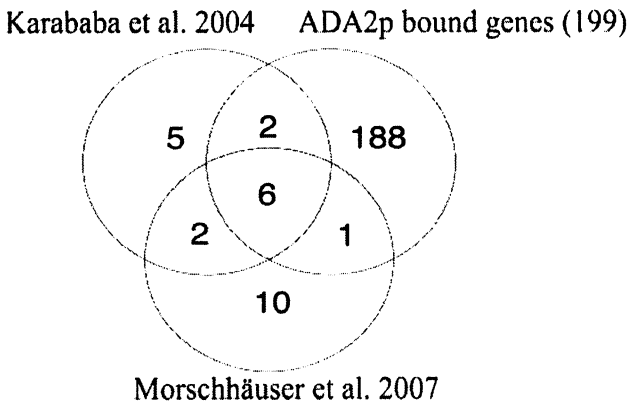
found to bind promoters of genes constitutively activated in azole-resistant clinical strains overexpressing Mdr1p (Karababa *et al.*, 2004). Recent work elucidated the mechanism controlling the overexpression of *MDR1* and multidrug resistance in those clinical isolates (Morschhauser *et al.*, 2007). Drug resistance was caused by a gain-of-function mutation in a zinc cluster transcription factor called Mrr1p. We identified a common core set of eight overexpressed genes, including *MDR1 (IFD5* and *IFD7* were not considered because they were removed from Assembly 21), which overlap with Ada2p-binding genes (Figure 6A). This finding suggests that Ada2p is recruited to the promoters of genes mediating azole resistance, most probably by its association with Mrr1p. To support this hypothesis we first used ChIP-qPCR to determine if Mrr1p binds to the *cis*-regulatory regions of these eight core genes mediating azole clinical resistance. As shown in Figure 6B, Mrr1p binding was significantly enriched for seven promoters among the eight resistance genes. The overlap with Ada2p-binding consisted of six resistance genes.

Taking into consideration the key role of Mrr1p as a master regulator of drug resistance, along with Ada2p cooccupancy of core resistance genes, Ada2p might function as a coactivator of Mrr1p. This was demonstrated by assessing Ada2-TAP in vivo occupancy in a *mrr1* mutant strain using ChIP-qPCR targeting the −1- and −200-bp promoter regions of the eight core resistance genes. Our result demonstrates clearly that Ada2p promoter binding for the six resistance genes cooccupied by both Ada2p and Mrr1p decreased in the *mrr1* mutant compared with the parental strain (Figure 6B).

DISCUSSION

The absence of a complete sexual cycle in *C. albicans*, in addition to its diploid nature, limits the use of classical genetic approaches to dissect mechanisms controlling its virulence. In the model yeast *S. cerevisiae* the usefulness of ChIP-CHIP has been demonstrated in several studies that revealed unexpected regulatory functions or features

A



B

Gene name	Orf19 accession	Mrr1p binding[c]	Ada2p binding	ADA2 binding in *mrr1*[c]
IFD1	orf19.1048	9.3 ± 0.6	2.9	1.1 ± 0.1
IFD5	-- [(a)]	-	-	-
IFD7	orf19.629 [(b)]	-	-	-
CSH1	orf19.4477	6.3 ± 0.3	1.5	0.8 ± 0.2
HSP31	orf19.251	5.8 ± 0.4	3.3	0.9 ± 0.2
IPF5987	orf19.7306	8.5 ± 0.7	1.3	0.9 ± 0.1
GRP2	orf19.4309	5.2 ± 0.9	3	1.2 ± 0.1
MDR1	orf19.5604	4.3 ± 0.5	3.9	1.9 ± 0.4
OYE32	orf19.3131	1.4 ± 0.2	2.4	1 ± 0.2
GPX1	orf19.86	1.1 ± 0.4	1.1	0.9 ± 0.1
RPL10	orf19.2935	0.8 ± 0.1	1.7	2 ± 0.3

Figure 6. Mrr1p recruits Ada2p to the promoter of core genes related to fluconazole clinical resistance. (A) Relationship between Ada2p bound genes and the core genes associated with drug resistance of clinical strains overexpressing MDR1 as revealed by two independent profiling studies. (B) Ada2p and Mrr1p occupancy of the eight core resistance genes are listed in addition to *RPL10* used as a negative control. Primers were designed to amplify 200 base pairs upstream the ATG. [a] ORF not physically mapped; [b] ORF deleted from Assembly 20; [c] significantly enriched for seven promoters among the eight resistance genes.

(Harbison *et al.*, 2004; Pokholok *et al.*, 2006). Recently, ChIP-CHIP has been used in *C. albicans* to study TFs regulating drug resistance (Liu *et al.*, 2007; Znaidi *et al.*, 2008) and to describe the unexpected rewiring of transcriptional regulatory networks controlling the choice of mating type (Tsong *et al.*, 2006) or the expression of ribosomal protein genes (Hogues *et al.*, 2008). In our study, the use of this genomic tool allowed us to gain insights into the roles of Ada2p as a transcriptional regulator of many biological processes. Indeed, the functions of the promoter targets of Ada2p guided our experiments into its roles into the regulation of stress-dependent gene expression and azole sensitivity.

The finding that Ada2p occupies a broad range of *C. albicans* promoters suggests that this activator plays a global role in transcriptional regulation, as was already established for the SAGA/ADA coactivator complex in *S. cerevisiae*. This observation complements expression-profiling data that showed a wide variety of gene expression alterations in the *C. albicans ada2* mutant (Bruno *et al.*, 2006). These results, together with the fact that the deletion of *ADA2* leads to a significant decrease in H3K9 acetylation, imply that Ada2p is a general transcriptional regulator that operates through chromatin acetylation. This finding is consistent with the general model proposing that actively transcribed genes are correlated with increased histone acetylation mediated by enzymes with HAT activity (Roth *et al.*, 2001).

In eukaryotic cells, transcriptional coactivator complexes such as SAGA/ADA have been shown to facilitate the activity of sequence specific gene activators (TFs). This functional feature emphasizes that SAGA/ADA specificity is determined by the TF that recruits the coactivator complex to its target genes (Naar *et al.*, 2001). In the current study, the direct dependence of Ada2p recruitment on three TFs, Cap1p, Mrr1p, and Gal4p, was investigated. Ada2p occupancy of glycolysis as well as pyruvate metabolism gene promoters was almost completely lost in the *gal4* mutant (Table 3). This dependency was also demonstrated for Cap1p and Mrr1p directing Ada2p to the promoters of oxidative responsive and core resistance genes, respectively (Figures 5 and 6). This suggests the specific role of *C. albicans* Ada2p depending on the TFs Cap1p, Mrr1p and Gal4p and therefore uncovers the functional conservation of mechanism by which SAGA/ADA operates in this pathogen.

Identification of Ada2p target genes in *C. albicans* provided insights into different stress response categories controlled by this coactivator. In *S. cerevisiae*, SAGA was found to specifically effect the activation of genes that are commonly up-regulated in response to a variety of environmental stresses, such a heat, oxidation, acidity, DNA damage, starvation, and the presence of unfolded proteins (Huisinga and Pugh, 2004). These genes form what has been called the environmental stress response and are regulated by the TFs Msn2p and Msn4p (Gasch and Werner-Washburne, 2002). Even though the closest *C. albicans* homologues of *MSN2* and *MSN4* are not involved in modulating the response to global stress (Nicholls *et al.*, 2004), we nevertheless show that Ada2p binds the promoters of similar stress-response genes that are up-regulated after treatment with oxidative agents, heat, and inhibitors of protein folding (Figure 1C). Promoters of genes involved in vacuolar acidification (*RIM101*, *VMA7*, and *RBF1*), as well as DNA replication (*RNR1*, *RNR21*, *RNR22*, and *DLS1*), were also bound by Ada2p. Taking into consideration the high degree of functional similarity between SAGA/ADA in both *S. cerevisiae* and *C. albicans*, it seems likely that this complex is evolutionarily conserved and controls the general stress-response pathway in both species.

Because the genome-wide occupancy of Ada2p revealed association with specific functions in *C. albicans*, we evaluated the consequence of *ada2* deletion on different physiological responses. The sensitivity of *ada2* mutants was examined in different stress conditions such as heat (42 and 45°C), excess of unfolded protein (tunicamycin and DTT), pH (acid and alkaline), genotoxic agents (MMS and EMS), oxidative agents (H_2O_2 and menadione), and osmotic stress (NaCl). No obvious phenotypic aberrations were recorded except for the oxidative agents. The absence of apparent phenotypes could be explained by the presence of other redundant coactivator complexes that can be recruited as a substitute of SAGA/ADA. Indeed, this assumption is supported by the results of studies undertaken in *S. cerevisiae* that demonstrated overlapping functions between the TFIID and SAGA complexes (Lee *et al.*, 2000; Huisinga and Pugh, 2004). Thus, genes could depend equally on both complexes, and a promoter that is dominated by a particular complex could still use the other to a lesser degree. However it is important to note that Ada2p appears to be the major contributor to the response to oxidative stress in *C. albicans*. This implies that SAGA/ADA in *C. albicans* is the bona fide coactivator complex mediating oxidative tolerance. Consequently, our observation that *ada2* mutants are much less virulent in a systemic mouse infection model could be related to its high sensitivity to oxidative stress. This might result in reducing the protection of fungal cells against different ROS that are generated by phagocytes and dendritic cells (Urban *et al.*, 2006).

The mechanisms of antifungal resistance in *C. albicans* remain an area of intense scientific investigation (Cannon *et al.*, 2007). Interestingly, Ada2p binds the promoters of several drug transporters, namely *MDR1*, *CDR1*, *CDR4*, *QDR1*, *YCF1*, *FLU1*, *ORF19.4531*, and *ORF19.301* as well as the phosphatidylinositol transfer gene *PDR16*. A moderate increase in sensitivity to fluconazole was observed in cells lacking *ADA2* in addition to impaired inducibility of *MDR1*, *CDR,1* and *PDR16* after the addition of fluconazole. Thus, Ada2p plays a role in *C. albicans* azole tolerance, most probably by its recruitment as a coactivator by TFs that control the expression of at least *MDR1*, *CDR1*, and *PDR16*. Constitutive activation of the transcriptional regulators of efflux pumps such as Tac1p, Upc2p, or Mrr1p have been associated with clinical azole resistance in *C. albicans* (Coste *et al.*, 2007; Morschhauser *et al.*, 2007; Znaidi *et al.*, 2008), and those TFs could use Ada2p as a coactivator to activate their targets. The moderate phenotype of *ada2* mutants observed in the presence of fluconazole might be the consequence of the use of alternative transcriptional coactivators. Meanwhile, Ada2p was found to occupy promoters of core genes mediating azole resistance of clinical isolates overexpressing *MDR1*. Recently, the mechanism of this type of resistance has been shown to include a gain of function mutation in the TF Mrr1p, thus resulting in the overexpression of its target genes. In our work, we have shown that Mrr1p and Ada2p cooccupied six gene promoters among the eight core resistance genes, including *MDR1*. Ada2p occupancy of these genes was completely dependent on Mrr1p, demonstrating that Ada2p functions as a coactivator for Mrr1p. To investigate the role of Ada2p in mediating drug resistance in *C. albicans*, *ADA2* was deleted from a resistant clinical strain overexpressing *MDR1* (data not shown). No obvious alteration of fluconazole sensitivity was noticed. This can be explained again by the presence of redundant coactivator complexes that can be recruited by Mrr1p as a substitute for SAGA/ADA. However, we have also considered in the current study that the impaired inducibility of *CDR1* and

PDR16 in an *ada2* mutant could also be a cause of the reduced sensitivity toward fluconazole. The latter two resistance genes were shown to be under the control of Tac1p or Upc2p rather than Mrr1p (Liu *et al.*, 2007; Znaidi *et al.*, 2008). It is possible that these resistance regulators might share the recruitment of the coactivator Ada2p to their target genes.

Recent evidence describing the transcriptional rewiring of the Leloir pathway genes in *C. albicans* suggests that the *GAL* genes responsible for the breakdown of galactose are not under the control of the closest Gal4p homolog in this organism (Martchenko *et al.*, 2007). This TF was rather shown to be required for transcriptional activation of genes involved in glucose metabolism. Global location mapping of Gal4p binding demonstrates a significant enrichment at gene promoters involved in glycolysis as well as pyruvate metabolism (C. Askew and M. Whiteway, unpublished data). In the present study, we have demonstrated that Ada2p is recruited by Gal4p to regulate genes involved in glycolysis and pyruvate metabolism. Taking into consideration the complete dependency of transcriptional activation of *GAL* genes on SAGA/ADA in *S. cerevisiae*, our finding highlights the fact that even though *C. albicans* Gal4p has distinct functions, and even a different transcriptional activation domain, its intimate interaction with SAGA/ADA has been conserved in both organisms. This evidence further emphasizes the functional conservation of the SAGA/ADA coactivator complex in which Ada2p is a crucial constituent. Thus, it seems that transcriptional rewiring is not restricted to TF but also to their coactivators.

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