ELM1 is required for multidrug resistance in *Saccharomyces cerevisiae*

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ABSTRACT

In *Saccharomyces cerevisiae*, transcription of several drug transporter genes including the major transporter gene *PDR5* has been shown to peak during mitosis. The significance of this observation, however, remains unclear. *PDR1* encodes the primary transcription activator of multiple drug transporter genes in *S. cerevisiae*, including *PDR5*. Here, we show that in synchronized *PDR1* and *pdr1-3* (multidrug resistant) strains, cellular efflux of a known substrate of ATP-binding-cassette transporters, doxorubicin (a fluorescent anti-cancer drug), is highest during mitosis when *PDR5* transcription peaks. A genetic screen performed to identify regulators of multidrug resistance revealed that a truncation mutation in *ELM1* (*elm1-300*) suppressed the multidrug resistance of *pdr1-3*. *ELM1* encodes a serine/threonine protein kinase required for proper regulation of multiple cellular kinases, including those involved in mitosis, cytokinesis, and cellular morphogenesis. *elm1-300* as well as *elm1Δ* mutations in a *pdr1-3* strain also caused elongated bud morphology (indicating a G2/M delay), and reduction of *PDR5* transcription under induced and non-induced conditions. Interestingly, mutations in several genes functionally related to *ELM1*, including *cla4Δ, gin4Δ* and *cdc28-C127Y*, also caused drastic reductions in drug resistance and *PDR5* transcription. Collectively, these data show that *ELM1*, and genes encoding related protein serine/threonine kinases, are required for regulation of multidrug resistance involving, at least in part, control of *PDR5* transcription.
INTRODUCTION

In *S. cerevisiae*, transcriptional up-regulation of transporters that belong to the ATP-binding-cassette (ABC) superfamily results in multiple or pleiotropic drug resistance (MDR/PDR). Transcriptional activation of many of these transporters is known to occur with drug exposure (e.g., cycloheximide) or in the presence of gain-of-function mutations in the transcriptional activators themselves (Balzi and Goffeau 1995; Moye-Rowley 2003; Wolberger et al. 2001). Transcription of most genes is known to be significantly reduced during mitosis, and this mitotic repression has been, at least in part, attributed to inactivation of the transcriptional machinery (Gottesfeld and Forbes 1997; Long et al. 1998). Surprisingly, microarray analysis indicates that transcription of several drug transporter genes, including *PDR5*, peaks during mitosis ((Spellman et al. 1998), reviewed in (Bahler 2005; Wittenberg and Reed 2005)). However, this finding has not been thoroughly investigated, and the impact of *PDR5* transcriptional up-regulation during mitosis on multidrug resistance remains unknown.

Two major transcriptional activators, Pdr1 and Pdr3, control the level of many drug transporters in *S. cerevisiae* ((Gao et al. 2004; Milgrom et al. 2005) and references therein). These homologous proteins belong to the Gal4 superfamily with Cys$_6$-Zn(II) DNA binding domains (Bauer et al. 1999; Kolaczkowski et al. 1998; Poch 1997). The DNA binding domain of Pdr1 targets over a dozen transport gene promoters (most notably *PDR5*) with the pleiotropic drug resistance element (PDRE) 5’-TCCGCGGA-3’ (Balzi and Goffeau 1995; Kolaczkowska et al. 2002). The functions of Pdr1 and Pdr3 overlap; however, Pdr3 but not Pdr1 is subject to auto-regulation (Delahodde et al. 1995). Several substitution mutations within Pdr1 result in a hyperactive activator (e.g., F815S in the Pdr1-3 hyper-activator protein encoded by the *pdr1-3* allele (Carvajal et al. 1997; Meyers et al. 1992)) that increases the
transcription of many genes encoding ABC transporters (including \textit{PDR5}), as well as permeases and enzymes involved in lipid and cell wall synthesis (DeRisi et al. 2000). Similar to Pdr1, several hyperactive Pdr3 activators, including that encoded by the \textit{pdr3-2} allele, were identified (Nourani et al. 1997).

The Pdr5 transporter is a major plasma membrane-associated ATPase regulated by Pdr1/Pdr3, and it is responsible for cellular detoxification of many agents including the anticancer drug doxorubicin (Rogers et al. 2001). Yeast Pdr5 exhibits functional homology to the mammalian P-glycoproteins (Pgp), and overexpression of Pdr5 confers multidrug resistance. Extensive efforts have been made to identify small molecules that could reverse the drug resistance phenotype, primarily by inhibiting transporter activities (Lewis 2001). In this regard, the function of Pdr5, as well as similar drug transporters of the pathogenic yeast \textit{Candida albicans}, are inhibited by the immunosuppressant FK506 (tacrolium, Prograf®) (Egner et al. 1998; Schueter-Muehlbauer et al. 2003).

We previously characterized the transcriptional regulation of \textit{PDR5} by comparing differences in the recruitment of activators and coactivators, and the nucleosome structure between isogenic \textit{PDR1} and \textit{pdr1-3} strains. We demonstrated that Pdr1 is constitutively bound to the \textit{PDR5} promoter. Cycloheximide induction in the wild-type \textit{PDR1} strain alters the nucleosome structure at the \textit{PDR5} upstream activating sequence (UAS) region harboring PDRE. These alternations reflect changes in the interactions between Pdr1 and PDRE, and are associated with \textit{PDR5} transcriptional activation (Gao et al. 2004). Moreover, we showed that proper interactions between histones and \textit{PDR5} coding sequences specifically require the transcription factor SAGA (Spt-Ada-Gcn5-Acetyltransferase) (Milgrom et al. 2005). However, factors other than Pdr1 and SAGA that are required for proper transcriptional regulation of \textit{PDR5} remain to be
In *S. cerevisiae*, mitotic entrance is coupled to morphogenesis (SAKCHAISRI et al. 2004). Entry into mitosis is initiated by activation of Clb2-Cdc28/Cdk1 cyclin-dependent kinase (CDK), which involves degradation of its inhibitor Swe1 kinase (MCMILLAN et al. 2002). Mechanistically, Swe1 phosphorylation by Cdk1 activates Swe1, and this phosphorylation is required for the formation of a stable Swe1-Cdk1 complex that maintains Cdk1 in an inhibited state (ASANO et al. 2005; HARVEY et al. 2005). Additional kinases are involved in the regulation of transition from G2 to mitosis. *ELM1* (elongated morphology 1) encodes a serine/threonine protein kinase, and cells harboring *elm1Δ* exhibit elongated filamentous growth, an indication of G2/M delay (KOELER and MYERS 1997). The function of Elm1 kinase in mitotic signaling (SREENIVASAN and KELLOGG 1999) has been linked, in part, to regulators of septin organization, a key set of protein serine/threonine kinases encoded by *GIN4* and *CLA4*, as well as an interactor of mitotic cyclin Clb2 encoded by *NAP1* (ALTMAN and KELLOGG 1997; BENTON et al. 1997; CARROLL et al. 1998; EDGINGTON et al. 1999; GLADFELTER et al. 2004; LONGTINE et al. 2000). Support of Elm1 involvement in the G2/M transition includes the fact that Elm1 is required for hyperphosphorylation of Swe1 during mitosis (SREENIVASAN and KELLOGG 1999). Elm1 is also required for the regulation of bud emergence at the G1 phase (SREENIVASAN et al. 2003). Independent of its roles in the cell cycle, Elm1 appears to function upstream of Snf1, a key AMP-dependent kinase pathway that regulates carbon metabolism in yeast (HONG et al. 2003; SUTHERLAND et al. 2003) and mammalian cells (WOODS et al. 2003).

In the present study, we show that *PDR5* transcription during cell cycle progression is inversely correlated with cellular accumulation of doxorubicin. A truncation and null mutation in *ELM1* were identified as suppressors of pdr1-3. Yeast strains harboring mutations in genes
encoding Elm1-related kinases (e.g., gin4Δ, cla4Δ and cdc28-C127Y) similarly reversed the multidrug resistance of pdr1-3, exhibited elongated bud morphology, impaired PDR5 transcription (without affecting Pdr1-independent transcription), and abolished cellular doxorubicin elimination. Epistasis analysis suggested that ELM1 functions upstream of Pdr1-mediated PDR5 transcription. This ELM1-PDR5 genetic connection is independent of the SNF1 pathway. We also show that elm1Δ alters nucleosome structure upstream of the established Pdr1 binding sites in the PDR5 promoter (Katzmann et al. 1996). However, Myc-tagged Elm1 is not detectable on the PDR5 promoter. In summary, our studies indicate a novel link between regulation of multidrug resistance and cell cycle progression in S. cerevisiae, involving genes encoding key serine/threonine kinases acting during mitosis.

MATERIALS AND METHODS

Chemicals and solutions: Doxorubicin HCl (MW 579.99) solution (3.45 mM) was obtained from GensiaSicor Pharmaceuticals (Irvine, CA). FK506 (tacrolimus, Prograf®, MW 822) solution (5 mg/ml = 6.08 mM) was obtained from Fujisawa (Deerfield, IL). The remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO). Sodium methanesulfonate solution used for HPLC was prepared from 15.4 M methane sulfonic acid by addition of one equivalent of sodium hydroxide and dilution to 4.0 M.

Yeast strains, genetic manipulations, agar plate drug resistance assays, and measurements of cellular respiration: Yeast cells were grown in rich (YPD) or synthetic media according to standard procedures (Sherman 1991). The genotypes of yeast strains used in this study are listed in Table 1 (Gao et al. 2004; Wolfger et al. 1997) and described previously for deletion
strains derived from BY4741 (Milgrom et al. 2005). The null alleles introduced into pdr1-3 were carried out by PCR-mediated allele transfer from deletion alleles of non-essential genes available from the collection of synthetic genetic arrays (Tong et al. 2001), or by PCR-based gene deletion using modification cassettes as previously described (Longtine et al. 1998). The strain with the upstream PDR5 promoter region replaced (WCS651, Table 1) was generated by using the following primers to amplify a TRP1 fragment from pRS404 vector: PDR5-F1, 5' CTTTTGTACGATTTTTAAACAGTAAAATCGATGCATATTAAGGGAGGCCCCGGATCCC CGGGTTAATTAA-3' (with underlined sequences being PDR5 specific), and PDR5-R1, 5' GGTAATTTGATGTTCTTTTTTTCTTTGATTTGAACTTTTGTTCTCTCTCTCTCTCTGGATTGACA GCTCGTTTAAAC-3'. The WCS651 strain generated bears a deletion from -726 to -1123 (relative to transcription start site) and replaced with TRP1 (1049 bps). Myc or GFP tags were introduced at the 3’ end of the PDR1 and PDR5 coding sequences by PCR-mediated modification (Longtine et al. 1998). PDR5 mRNA was induced by treatment of cells for 45 min in YPD medium containing 0.2 µg/ml (0.71 µM) cycloheximide (CYH) as described (Gao et al. 2004; Milgrom et al. 2005).

Agar plate drug resistance assays were carried out as follows. Strains were spotted (at sequential 10-fold dilutions) on plates containing either YPD or complete synthetic medium with indicated amino acids omitted. Images were taken after incubation at 30°C for three days. Plates containing 0.2 µg/ml CYH (Fig. 4E), 1.0 µg/ml CYH (Fig. 1A, 4A, 4C, 6B) or 15 µg/ml fluconazole (FLU, Fig. 1A) were used in the agar plate drug resistance assay.

Cellular respiration was measured at 25°C in sealed vials containing 10⁷ cells as described (Souid et al. 2003). The cells were suspended in 1.0 ml medium, containing 6.0 mM Na₂HPO₄, 10 mM glucose, 2.0 µM Pd phosphor [Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-
tetrabenzoporphyrin] and 1.0% (w/v) fat-free bovine serum albumin. The rate of cellular respiration was determined as the negative slope of the curve of \([O_2]\) versus time (in \(\mu\text{M} \text{O}_2 \text{min}^{-1}\) per \(10^7\) cells). These measurements reflected mainly mitochondrial respiration because addition of 1.0 mM NaCN completely inhibited oxygen consumption.

**Ethylmethane sulfonate (EMS) mutagenesis and identification of \(elm1-300\) mutation:** The EMS mutagenesis protocol was performed as described (Lawrence 1991; Milgrom et al. 2005), using a concentration of EMS (15 \(\mu\text{l}/\text{ml}\) of cell culture, final 15 mM) that gave 50% cell survival. Log phase \(pdr1-3\) cells (~5 x \(10^7\) cells per ml of WCS261, Table 1) were treated with EMS, and 8 x \(10^4\) cells were screened for mutants that failed to grow on 1.0 \(\mu\text{g}/\text{ml}\) CYH by replica plating. Seven mutants showed a clear loss of CYH resistance. These were mated with the opposite mating type of the \(pdr1-3\) strain (WCS347, Table 1). We noted that \(pdr1-3\) is dominant over \(PDR1\) in term of drug resistance (Wolfger et al. 1997). Tetrad analysis of the resulting diploid progeny indicated that two of the seven mutants (WCS343 and WCS345) harbored a single recessive mutation responsible for the loss of CYH resistance. Strain WCS345 was transformed with a YEpl3-based wide type genomic DNA library to clone the corresponding wild-type gene of the suppressor mutation. The resulting transformants were selected for restoration of CYH resistance. To exclude the possibility that recovery of CYH resistance in WCS345 was due to overexpression of genomic DNA inserted in the YEpl3 vector, we sub-cloned the insert into a low copy number pRS415 plasmid (Sikorski and Hieter 1989) and repeated the test for restoration of drug resistance in WCS345. Analysis of various sub-clones of genomic inserts confirmed \(ELM1\) as the wild-type gene corresponding to the single recessive allele introduced by EMS in the WCS345 strain. The \(elm1\) allele responsible for loss
of CYH resistance was cloned by PCR from genomic DNA of WCS345 strain and sequenced.

**Mapping PDR5 chromatin structure by micrococcal nuclease, Northern and Western blots:**

The detailed mapping of the PDR5 promoter in pdr1-3 and pdr1-3 elm1Δ strains (Fig. 8A) was essentially as described (GAO *et al.* 2004). The following modifications were made for mapping data presented in Fig. 8B. First, *Cla*I instead of *Hind*III digestion was used for mapping nucleosome structures upstream known PDREs. Second, 32P-labeled probes for Southern blot analysis were generated from genomic PCR product (273 bp) of primers 5'-CGATGCATATTAAGGGAGGCC-3' and 5'-CGCTTCCTTTGTATGATATC-3'. Preparation of total RNA and Northern blot analysis were performed as described (GAO *et al.* 2004; SHEN and GREEN 1997). Probes for PDR5, SWI5, PDR12 and ADH1 transcripts were obtained by PCR amplification from genomic DNA. Preparation of whole cell extract by glass bead disruption was performed as described (WALKER *et al.* 1997) and protein samples were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting (HARLOW and LANE 1988). The mouse monoclonal antibodies against c-Myc (9E10 clone), GFP antibody, Glucose-6-phosphate dehydrogenase (G6PD) antibody, and antibody α-tubulin (12G10 clone), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Roche Diagnostics Corporation, Sigma, and the Developmental Studies Hybridoma Bank at University of Iowa respectively. Quantification of relative mRNA and protein levels was performed using a phosphoimager (Molecular Dynamics, model 425).

**Cell synchronization:** Cell synchronization with nocodazole or α-factor was performed as described (AMON 2002). Yeast strains bearing the pdr1-3 allele or a 3’ Myc-tagged pdr1-3 allele
were grown in YPD at 30°C to $A_{600}$ of ~ 0.3. Samples were removed for the asynchronous controls. For metaphase arrest, nocodazole (20 µg/ml) was added to the cultures. When > 90% of the cells showed the characteristic arrest (equal size of budded mother-daughter cells), cells were collected by centrifugation, washed twice with two-volumes of YPD, and the pellets resuspended in equal volume of YPD. Samples were taken as time zero for RNA preparation, or incubated with 50 µM doxorubicin for 15 min for measurement of cellular doxorubicin. Microscopic examination of cells harvested throughout the experiments confirmed that cells sampled at time zero were arrested at mitosis and revealed approximation of various stages during a cell cycle. The synchronized cultures continued to grow at 30°C, and samples were taken at 15-min intervals for preparations of RNA, and measurement of intracellular doxorubicin after incubation with 50 µM doxorubicin for 15 min. There was no apparent cell cycle progression during the 15 min incubation with doxorubicin, consistent with the anti-proliferative effect of the drug in mammalian cells (HARISI et al. 2005) and yeast (our observation).

Growth of cultures for G1 arrest by α-factor was essentially the same as metaphase arrest by nocodazole. The concentration of α-factor used for synchronization was 3 µg/ml, and the characteristic schmoo phenotype indicating G1 arrest was confirmed microscopically.

**Chromatin immuno-precipitation (ChIP):** The condition for Formaldehyde-based *in vivo* cross-linking and ChIP was performed as described (GAO et al. 2004; MILGROM et al. 2005). The immunoprecipitations were performed using anti-Myc antibody. The following sets of primers were used for the PCR analysis: *PDR5* (upstream activating sequences, UAS), 5’-TCGTGATCAGATTCAGCACC-3’ and 5’-GGAGAGGCCTTGTTTGTATTGC-3’; *PDR5* (middle of the coding sequences, CDS), 5’-GAAAGCTCTGAAGAGGAATCC-3’ and 5’-
CCCTTTCCGGCCAAACAATCCA-3’; PDR1 (promoter), 5’-CACATTTTCTCGACGGTTC-3’ and 5’- GTAACGGGAAAACACAGAG-3’.

**Cellular doxorubicin determination:** For cellular doxorubicin efflux, log-phase cells were incubated at 30°C in YPD plus 50 µM doxorubicin for 60 min. The cells were collected by centrifugation, re-suspended in drug-free YPD (pre-warmed to 30°C) and incubated at 30°C for various periods of time. Aliquots of the cell suspension were then spun at specific time points. The pellets were suspended in 250 µl of 10% perchloric acid/2.0 M Na methanesulfonate. Equal volumes of glass beads were added, and the suspensions were vigorously vortexed for 3.0 min. The acid-soluble supernatants were separated on HPLC and analyzed as described below.

For cellular doxorubicin accumulation, log-phase cells were incubated in YPD media plus 50 µM doxorubicin at 30°C for various periods of time. The cells were collected by centrifugation and the pellets were immediately chilled on ice. The cold pellets were washed twice with ice-cold double distilled H₂O containing 20 µM FK506, a known inhibitor of the ABC transporters (EGNER *et al.* 1998). The washed pellets were then suspended in 250 µl of 10% perchloric acid/2.0 M sodium methanesulfonate. Equal volumes of glass beads were added, and the suspensions were vigorously vortexed as described above.

Doxorubicin peaks were detected by fluorescence (excitation, 480 nm and emission, 560 nm) as described (FOGLI *et al.* 1999). The analysis was performed on a Beckman HPLC system. The solvent used was 60% of 50 mM NaH₂PO₄ (pH ~3.5) and 40% of acetonitrile. The column (4.6 x 250 mm Beckman ultrasphere IP) was operated isocratically at 0.5 ml/min. Standards (10 µM doxorubicin in 10% perchloric acid/2.0 M sodium methanesulfonate) were included with each analytical run. Peaks were identified (doxorubicin retention time, ~13.8 min) and
quantitated using doxorubicin standards, with the minimal quantifiable level of doxorubicin (~10 pmol), giving a signal: noise ratio of 3:1. Cellular doxorubicin was expressed in pmol per 10⁷ cells based on microscopic enumeration. Standard deviations were derived from at least three sets of experiments. Treating of *PDR1* and *pdr1-3* cells with 50 μM doxorubicin for 15-120 min did not induce *PDR5* expression (our observation). Statistical significance between values in different strains was determined by paired Student’s *t*-test analyses. A value of *p* < 0.05 was considered significant.

RESULTS

Characterization of Pdr1-mediated multidrug resistance: a critical role for *PDR5* in cellular doxorubicin efflux: The transcription of several ABC transporter genes (including *PDR5*) is up-regulated in the *pdr1-3* strain (WCS261), which is resistant to CYH and the antifungal drug fluconazole (FLU). Introducing *pdr5Δ* into the *pdr1-3* strain gave loss of multidrug resistance, confirming *PDR5* as the major transporter target of Pdr1 (Fig. 1A). The impact of CYH treatment (0.2 μg/ml) in our study on the stability of *PDR5* mRNA was investigated in the *rpb1-1* strain, which carried a temperature-sensitive mutation in the largest subunit of RNA polymerase II. After thermal inactivation of the *rpb1-1* allele, *t₁/₂* for *PDR5* mRNA was about 20 min in either the presence or the absence of CYH condition (our observation), consistent with the estimation by microarray analysis (HOLSTEGE *et al.* 1998). Thus, our Northern blot analyses of *PDR5* reflect transcription levels rather than stability of mRNA.

Compared to wild-type *PDR1*, a ~10-fold increase in *PDR5* mRNA was found in the *pdr1-
3 strain (DE RISI et al. 2000; GAO et al. 2004), and further induction occurred with CYH treatment (GAO et al. 2004). To compare the level of Pdr5 protein in PDR1 versus pdr1-3, we introduced sequences encoding a 13xMyc-tag or GFP-tag at the 3’-end of the PDR5 coding sequence. The tagged version of PDR5 was functional, indicated by normal resistance to several drugs (not shown). An ~10-fold increase in Pdr5 protein was observed in pdr1-3 relative to PDR1 (Fig. 1B; note the 10-fold lower G6PD loading control in the pdr1-3 lane). Pdr5 protein was inducible by CYH in both pdr1-3 (Fig. 1C) and PDR1 strains (not shown). The comparable increases in both PDR5 mRNA and Pdr5 level in the pdr1-3 strain relative to the PDR1 strain confirmed transcriptional regulation as a crucial step for PDR5 mediated drug resistance in S. cerevisiae (MOYE-ROWLEY 2003).

Doxorubicin is a known substrate of many ABC transporters (EYTAN 2005) and deletion of PDR5 leads to hypersensitivity to doxorubicin and many other drugs (GOLIN et al. 2003; ROGERS et al. 2001). Transport activities for doxorubicin were determined by analytically detecting the fluorescence of the drug in acid soluble supernatants extracted from cells and separated by HPLC (Fig 2A). In the presence of FK506, a known inhibitor of ABC transporters including Pdr5 (EGNER et al. 1998), cells accumulated ~9-fold more doxorubicin than in its absence (compare doxorubicin peaks in Fig. 2A to 2B; please note the different cell number count in each condition). FK506 appears to prevent the interactions between Pdr5 and its substrates directly. Changes of a single residue of the Pdr5 transmembrane domain 10 (for example, S1360F, T1364F and T1364A) alter both substrate specificity and susceptibility to FK506 (EGNER et al. 1998; EGNER et al. 2000).

A time course study was used to compare the relative accumulation of doxorubicin by the PDR1 and pdr1-3 strains. During the first 15 min of incubation with the drug, doxorubicin
content was similar in the *PDR1* and *pdr1-3* cells (Fig. 2C). Thereafter, the difference between the two strains increased, with accumulation being higher in *PDR1*. Cellular doxorubicin reached a plateau after ~50 min, about twice higher in *PDR1* than *pdr1-3* (Fig. 2C). Based on this study, we pre-incubated the cells with 50 µM doxorubicin for 60 min to visualize the rates of drug elimination (Fig. 2D), while drug accumulation was measured by incubating cells with 50 µM doxorubicin for 15 min (Fig. 3B). The rate of drug efflux was determined by measuring the fraction of the drug remaining in the cells after incubation in drug free medium. The resulting best fit lines (Fig. 2D) gave $r^2$ values for *pdr1-3* of 0.975 and for *pdr1-3 pdr5Δ* 0.990, which is consistent with a kinetic study of P-glycoprotein (AMBUDKAR *et al.* 1997) and a process ensuring complete elimination of the drug. The zero-order rate constant ($k$), calculated as the negative slope of the best fit straight line was $\sim 48 \times 10^{-4}$ s$^{-1}$ for the *pdr1-3* and $< 0.1 \times 10^{-4}$ s$^{-1}$ in the presence of 20 µM FK506. Cellular doxorubicin elimination was therefore negligible in the presence of the inhibitor FK506. The $k$ values for *pdr1-3 pdr5Δ* and *PDR1* were comparable, $\sim1.4 \times 10^{-4}$ s$^{-1}$ and $\sim1.5 \times 10^{-4}$ s$^{-1}$, respectively, which were at least 30-fold lower than that for *pdr1-3* strain (Table 2). Significantly, the *pdr1-3 pdr5Δ* strain exhibited a drug efflux rate similar to that in *PDR1* (Fig. 2D), underscoring the pivotal role of Pdr5 in Pdr1-regulated cellular detoxification. The non-linear relationship between the increased doxorubicin efflux ($\sim$30-fold relative to *PDR1*, Table 2) and the up-regulation of *PDR5* expression ($\sim$10-fold relative to *PDR1*, Fig. 1) may reflect the collective activity of several transporters known to be overexpressed in the *pdr1-3* strain (DERISI *et al.* 2000). Other factors might include altered patterns of post-translational modifications on Pdr5, such as ubiquitination (EGNER and KUCHLER 1996), phosphorylation (CONSEIL *et al.* 2001), and glycosylation (JAKOB *et al.* 2001) in the *pdr1-3* strain, which in turn contributed to the effectiveness of the drug transporter functions.
Cyclic levels of *PDR5* mRNA and cellular doxorubicin during cell cycle progression: We next analyzed *PDR5* mRNA fluctuation during the cell cycle. We synchronized *pdr1-3* cells with nocodazole and monitored *PDR5* transcript levels and cellular doxorubicin concentration during cell cycle progression (Fig. 3). *PDR5* transcripts varied considerably during the cell cycle and peaked concurrently with *SWI5* mRNA accumulation, a known marker of mitosis (Fig. 3A). *PDR5* mRNA was ~4-fold higher at the M phase than the G1 phase (Fig. 3B, diamonds and solid line). Cellular doxorubicin, on the other hand, was about 4-fold lower at the M phase than at G1 phase (Fig. 3B, circles and dotted line). Similar phase related behavior was seen with *PDR5* mRNA and cellular doxorubicin during cell cycle progression in synchronized *PDR1* cells (not shown). These results indicated that *PDR5* mRNA accumulation was cell cycle controlled. Moreover, the inverse relationship between *PDR5* mRNA and cellular doxorubicin suggested that *PDR5* expression might alter cellular susceptibility to drugs during the cell cycle, with the greatest susceptibility during G1 phase and the least susceptibility during M phase. The possibility that regulatory mechanisms might modulate *PDR5* expression during cell cycle progression prompted us to search for genes affecting this aspect of *PDR5*-mediated drug resistance.

Mutation of *ELM1* suppresses *PDR5* transcription and drug resistance: We then performed a genetic screen for extragenic suppressors of CYH resistance in the *pdr1-3* haploid strain (WCS 261). We searched for factors that regulate drug resistance mediated by *PDR5*. Screening of ~80,000 EMS-treated colonies gave seven candidates showing loss of resistance. Two of the seven candidate haploid *pdr1-3* strains harbored additional single recessive mutations as indicated by CYH resistance of the homozygous diploid *pdr1-3* strains resulting from mating
candidate suppressor strains to an isogenic pdr1-3 strain of opposite mating type (WCS347), and by segregation of 2:2 resistant and sensitive spores derived from each tetrad. In one of the two mutants, WCS345, the corresponding wild-type gene of the extragenic suppressor allele was cloned by complementation from a high copy genomic library. Sequencing the clones identified a 6-kb fragment of chromosome XI, carrying the two complete genes ELM1 and CSE4. Microscopic examination of the WCS345 strain demonstrated elongated bud morphology, indicating a G2 delay (Fig. 4A, left panel, pdr1-3 elm1-300), a phenotype reminiscent of elm1 mutations (KOEHLER and MYERS 1997). A low-copy plasmid pRS415 (SIKORSKI and HIETER 1989) harboring ELM1 (pELM1) restored CYH resistance (Fig. 4A, lower left panel) and rescued the elongated bud morphology of pdr1-3 elm-300 (not shown). The mutant elm1 allele from the WCS345 strain was cloned and sequenced. The allele harbored a C to T mutation in the coding region, converting glutamine 301 (CAG) to a stop codon (TAG), and was therefore termed elm1-300 (Fig. 4A). This truncation deleted part of the kinase domain of Elm1, which span amino acid residues 88 to 406 ((KOEHLER and MYERS 1997); Fig. 4A). The Elm1 kinase domain contains two stretches of amino acid residues that match the consensus sequence of Ser/Thr kinases (BLACKETER et al. 1993; KOEHLER and MYERS 1997). The elm1-300 mutation eliminated completely the second consensus sequence, GTPAFIAPE (amino acid residues 309-317; Fig. 4A).

The level of PDR5 mRNA in pdr1-3 elm-300 was significantly lower than in pdr1-3, and CYH failed to fully induce PDR5 transcription (Fig. 4B), indicating that both non-induced (constitutive) and drug-induced transcriptions of PDR5 were reduced in pdr1-3 elm-300. These transcriptional defects were rescued by introducing wild-type ELM1 on a low copy plasmid, pELM1, into the pdr1-3 elm-300 strain (Fig. 4B). Therefore, ELM1 was required for proper
constitutive and drug-induced \( PDR5 \) transcription.

To further test the effect of \( elm1 \), we introduced the \( elm1\Delta \) allele into \( pdr1-3 \) (Fig. 4C) and \( PDR1 \) (Fig. 4D) strains. The \( pdr1-3 \ elm1\Delta \) strain was more sensitive to CYH than \( pdr1-3 \ elm1-300 \) (Fig. 4C), suggesting a residual function of \( ELM1 \) in the \( elm1-300 \) allele. Loss of CYH resistance was also observed when the \( elm1\Delta \) allele was introduced to another drug resistant strain \( pdr3-2 \) that overexpressed \( PDR5 \) (DELAVEAU et al. 1994) (Fig. 4C). These data indicated that the observed \( elm1\Delta \) effects were not \( pdr1-3 \) allele specific. Compared with the \( PDR1 \) wild type strain, the presence of the \( elm1\Delta \) drastically reduced \( PDR5 \) mRNA under both non-induced and induced conditions (Fig. 4D). We therefore conclude that \( ELM1 \) is required for Pdr1/Pdr3-regulated CYH resistance, which is mediated, at least partially, by \( PDR5 \). Epistasis analysis revealed that both \( pdr1-3 \ pdr5\Delta \ elm1\Delta \) and \( pdr1-3 \ pdr5\Delta \) strains failed to grow in the presence of 0.2 \( \mu \)g/ml CYH or 10 \( \mu \)g/ml fluconazole whereas \( pdr-1-3 \ elm1\Delta \) could survive under the same conditions (Fig. 4E). These results provided genetic evidence that \( ELM1 \) functions upstream of \( PDR5 \) in regulation of CYH resistance.

Elm1 is an upstream activator of Snf1 kinase (HONG et al. 2003; SUTHERLAND et al. 2003). Elm1 also regulates mitotic entrance, septin formation, and cytokinesis (BOUQUIN et al. 2000; GARRETT 1997; KOEHLER and MYERS 1997). To test the possibility that Elm1 affects \( PDR5 \) transcription via the Snf1-mediated pathway, \( PDR5 \) transcription (Fig. 4F, left panel) and CYH resistance (Fig. 4F, right panel) were analyzed in pair-wise isogenic wild type and \( snf1\Delta \) strains. Deletion of \( SNF1 \) had no effect on either \( PDR5 \) transcription or CYH resistance. These data indicate that \( ELM1 \) regulates \( PDR5 \) transcription via a pathway(s) independent of \( SNF1 \).
Mutation of genes required for mitotic progression represses PDR5 transcription: In S. cerevisiae, entry into mitosis is mediated by activating Clb2-Cdc28/Cdk1 cyclin-dependent kinase (CDK). As described in Introduction, additional serine/threonine kinases (e.g., ELM1, GIN4 and CLA4) are also involved in regulating the transition from G2 to mitosis. We next investigated the effects on PDR5-mediated drug resistance by other genes that are functionally related to ELM1 and required for proper mitotic progression. In addition to elm1Δ, we introduced individual null alleles of nap1Δ, gin4Δ and cla4Δ into haploid pdr1-3 strain, and analyzed their effects on cell morphology, CYH resistance, PDR5 mRNA accumulation (Fig. 5) and cellular doxorubicin efflux (Table 2). The nap1Δ, gin4Δ, cla4Δ and elm1Δ alleles resulted in various degrees of elongated bud morphology (Fig. 5A), decreased PDR5 transcription (Fig. 5B, left panel), loss of CYH resistance (Fig. 5B right panel), and reduced doxorubicin efflux rates (Table 2), with the general order of effect on drug resistance being: elm1 > cla4 > gin4 > nap1 in both pdr1-3 strain (Fig. 5B) and PDR1 strain (Fig. 5C). Consistent patterns of reduced growth at lower drug concentrations were also observed; the minimal inhibitory concentrations for three drugs are listed in Table 3. The pdr1-3 elm1Δ strain exhibited the most pronounced effects on elongated morphology, PDR5 transcription, and CYH resistance. The pdr1-3 nap1Δ strain exhibited relatively minor morphological changes, moderately decreased PDR5 transcription, and slightly reduced CYH resistance. It is worth noting that there was no detectable doxorubicin efflux in the pdr1-3 elm1Δ and pdr1-3 cla4Δ strains (Table 2), virtually identical to that observed in the presence of the inhibitor FK506 (Fig. 2D). This suggests that other drug transporter genes in addition to PDR5 are negatively affected by elm1Δ and cla4Δ, consistent with previous reports (Spellman et al. 1998). Significantly, however, elm1Δ, cla4Δ, gin4Δ, and nap1Δ did not affect sorbic acid-induced PDR12 mRNA (Fig. 5D, left panel) or growth in the presence of
sorbate (Fig. 5D, right panel). *PDR12* encodes a weak acid anion transporter, whose transcription is independent of Pdr1 and Pdr3 (*KREN et al. 2003; PIPER et al. 1998*). These results indicate a gene specific transcriptional defect in these mutant strains.

Cdc28 is a master regulator of cell division in *S. cerevisiae* that controls mitotic entrance (*MENDENHALL and HODGE 1998*). We hypothesized that a *cdc28* mutation impairing mitotic progression would suppress the multidrug resistance of *pdr1-3*, in a comparable manner to *elm1Δ*. The *cdc28-C127Y* allele was previously shown to cause elongated bud morphology (*EDGINGTON et al. 1999*). In contrast, the *cdc28-Y19F* allele (*MC MILLAN et al. 1999*) is insensitive to Swe1 kinase-imposed inhibition of mitotic entrance, and exhibits normal morphogenesis. We introduced these two mutant alleles into the *pdr1-3* strain, and investigated their effects on *PDR5* mRNA levels and CYH resistance. As anticipated, *pdr1-3 cdc28-C127Y* exhibited reduced *PDR5* mRNA (Fig. 6C), loss of CYH resistance (Fig. 6B), and elongated bud morphology (Fig. 6A). Like the *pdr1-3 elm1Δ* strain, doxorubicin efflux in *pdr1-3 cdc28-C127Y* strain was negligible (Table 2). In contrast, the *pdr1-3 cdc28-Y19F* strain behaved in a manner comparable to the *pdr1-3* strain (Table 2, the difference in the efflux rate between the two strains was within the standard error). Reduced CYH resistance due to the presence of the *cdc28-C127Y*, but not *cdc28-Y19F*, allele was also observed in the *PDR1* strain (Fig. 6D), indicating the effect is not *pdr1-3* allele specific. We further analyzed the effects of Swe1 (Cdc28 kinase) and Mih1 (Cdc28 phosphatase) on *PDR5* transcription. The *swe1Δ* strain can enter mitosis like wild-type cells, whereas the *mihΔ* strain exhibits a mitotic delay (*SIA et al. 1996*). Relative to *pdr1-3* and *pdr1-3 swe1Δ*, the level of *PDR5* mRNA in *pdr1-3 mih1Δ* was significantly reduced (Fig. 6E). As both *mihΔ* and *cdc28-C127Y* mutants were defective in mitotic entrance and exhibited marked reduction of *PDR5* transcription, these data reinforce the significance of *PDR5*
transcription peaking during normal mitosis (Fig. 2). Therefore, mitotic progression is required for optimal $PDR5$ expression and development of drug resistance.

We then examined the possibility that other non-cell cycle related changes in these mutants (for instance, general sickness) might account for the total loss of doxorubicin efflux (Table 2). For example, it was reported that loss of signaling between nuclei and mitochondria reduces the level of $PDR5$ expression and drug resistance in $rho^o$ cells (HALLSTROM and MOYEROWLEY 2000). We therefore measured the rates of cellular mitochondrial oxygen consumption (cellular respiration) in these mutant strains (strains shown in Fig. 5 and Fig. 6). The respiration was virtually identical, $4.6 \pm 0.8 \mu M \text{O}_2 \text{min}^{-1} \text{per} \ 10^7 \text{cells}$, in all mutants studied. These results rule out that alteration of mitochondrial functions in these mutants contribute to the observed defects in $PDR5$ transcription and related loss of drug resistance. Moreover, $\text{snf1} \Delta$, which exhibits a growth defect on a non-fermentable (respiratory) carbon source, did not affect $PDR5$ transcription and CYH resistance (Fig. 4F). Together, these data support the possibility that Elm1 and related serine/threonine kinases affect $PDR5$ transcription and drug resistance by a cell-cycle-derived mechanism.

**Recruitment of Pdr1 to the $PDR5$ UAS is cell cycle-independent:** We next explored the molecular events required for $PDR5$ transcriptional up-regulation during mitosis. We investigated whether the level of the Pdr1-3 activator and its binding to the $PDR5$ promoter varied during the cell cycle. We monitored the level of Pdr1-3 during cell cycle progression by integrating a Myc-epitope tag at the 3’-end of the $pdr1$-3 open reading frame. The induction of $PDR5$ in this strain was identical to the non-tagged parental strain (GAO et al. 2004). Myc-tagged Pdr1-3 was then analyzed during the cell cycle (Fig. 7A). Western blot analysis of
nocodazole synchronized cells showed constant Pdr1-3 levels during cell cycle progression (Fig. 7A). Therefore, the fluctuating PDR5 mRNA levels in the cell cycle (Fig. 3) were not due to fluctuating Pdr1-3 levels.

We then investigated how much Pdr1-3 activator associates with the PDR5 promoter. A chromatin immunoprecipitation (ChIP) assay was used to analyze the recruitment of Myc-tagged Pdr1-3 to the PDR5 promoter. As PDR5 transcription is sensitive to cell cycle progression, it is possible that different synchronization protocols might interfere with activator recruitment (SHEDDEN and COOPER 2002). To test this possibility, we conducted parallel ChIP assays from cells synchronized by nocodazole or α-factor treatment then released. The two treatments gave the same result. Successful synchronization and release at various time points thereafter by either nocodazole (Fig. 7A) or α-factor (Fig. 7B) were validated by microscopic examination of distinct morphologies throughout the experiments (not shown). Pdr1-3 was recruited specifically to the promoter region of PDR5, and no detectable Pdr1-3 was recruited to the PDR5 coding sequences (CDS) (Fig. 7B, synchronized by α-factor). Pdr1-3 recruitment to the PDR5 promoter was constitutive and independent of the cell cycle. In fact, there was no difference in the amount of Pdr1-3 activator bound to the PDR5 promoter as the cell cycle progresses (Fig. 7B). We also performed ChIP analysis on the recruitment of wild-type Myc-tagged Pdr1 activator to the PDR5 promoter and the results were virtually identical to the Pdr1-3 recruitment data presented in Fig. 7B (data not shown). Therefore, the recruitment of Pdr1 and Pdr1-3 activators to the PDR5 promoter was not responsible for the fluctuating levels of PDR5 mRNA in the cell cycle.

We then analyzed Pdr1 and Pdr1-3 recruitment to the PDR5 promoter in the presence of an elm1Δ allele, in both PDR1 and pdr1-3 strains (Fig. 7C). In the elm1Δ strains, which the recruitment of Pdr1-3 onto the PDR5 promoter was slightly higher than that of Pdr1 (Fig. 7C),
the results are virtually indistinguishable from our previous observation of Pdr1-3/Pdr1 recruitment in the presence of wild type ELM1 (Gao et al. 2004). We conclude, therefore, that the recruitment of Pdr1 is not a rate-limiting step accounting for decreased PDR5 transcription in the elm1Δ strains. Collectively, the data presented in Fig. 7 indicate that cell cycle dependent PDR5 transcription is regulated at steps that are independent of Pdr1 recruitment.

**Altered nucleosome structure at the PDR5 promoter region in elm1Δ strains:** Our previous micrococcal nuclease (MNase) mapping of PDR5 nucleosome structure in PDR1 and pdr1-3 strains demonstrated that changes in PDR5 transcription levels were associated with alterations in PDR5 nucleosome structure even though Pdr1 was constitutively bound (Gao et al. 2004; Milgrom et al. 2005). This is consistent with the notion that regulation of chromatin structure plays a major role in gene activation (reviewed by (Bernstein and Allis 2005; Boeger et al. 2005)). As the elm1Δ allele drastically reduced PDR5 transcription (Fig. 4D and 5B), we next examined the nucleosome structure at the PDR5 promoter region and compared pdr1-3 and pdr1-3 elm1Δ strains under either non-induced or CYH-induced conditions (Fig. 8A). In both pdr1-3 and pdr1-3 elm1Δ strains, CYH induction did not significantly change the pattern or intensities of bands corresponding to MNase hypersensitive sites throughout the PDR5 promoter region (Fig. 8A, compare - and + CYH). It is worth noting that, unlike PDR5 coding sequences, the absence of typical well-positioned nucleosomes in the PDR5 UAS region may reflect the GC-rich nature of PDR5 PDREs sequences (Gao et al. 2004). In contrast, the pdr1-3 elm1Δ strain showed significant reductions in the intensity of several bands (marked with asterisks) clustered in the region from -700 to -900, extending to approximately -1100 (Fig. 8A, labels on the right) relative to transcription start site (+1). The loss of other bands marked from
-900 to -1,100 in the pdr1-3 elm1Δ strain may reflect restoration of positioned nucleosome structure in this region due to reduced PDR5 transcription. These nucleosomal alterations were unexpected because this region is located well upstream of the known Pdr1 binding sites (PDREs) and TATA box, and there were no significant differences within the PDREs or TATA region for the pdr1-3 and pdr1-3 elm1Δ strains (Fig. 8A). To test the generality of these observations, we performed a set of MNase mapping experiments comparing PDR1 with PDR1 elm1Δ strains and found consistent results (data not shown).

We then investigated whether PDR1 and/or PDR3 are required for regulation of nucleosome structure upstream of the PDREs. We examined the latter by using a set of isogenic strains: WT, pdr1Δ, pdr3Δ, pdr1Δ pdr3Δ (WCS 265-WCS268, Table 1). As expected, a strong band located close to the TATA box was observed in wild type strain (Fig. 8B, (Gao et al. 2004)). Neither pdr1Δ nor pdr3Δ alone significantly changed the PDR5 promoter region (including TATA box and PDREs), consistent with overlapping roles of Pdr1 and Pdr3 as transcriptional activators (Wolfger et al. 1997). Interestingly, a pdr1Δ pdr3Δ double deletion resulted in changes not only in the PDR5 promoter (marked with # signs) and coding sequences (marked with bracket signs) as expected, but also in the region upstream of the PDREs, including sequences from -700 to -900 (Fig. 8B, marked with asterisks at lower part of the figure). The increased site-specific MNase digestion presumably reflects less dynamic nucleosome structure in the absence of PDR1 and PDR3 (Fig. 8B). These data indicate that Pdr1 or Pdr3 is required for regulation of PDR5 nucleosome structure upstream of the PDREs, corresponding to the region where elm1Δ strain exhibited altered nucleosome structure. However, Myc-tagged Pdr1 was not recruited to the -700 to -900 region of PDR5 promoter (data not shown). This raises the possibility that PDREs occupied by Pdr1 and Pdr3 propagate altered nucleosome structure from
their binding sites to sequences further upstream. Such a long distance effect on nucleosomal structure resulting from changes in the interactions of transcription factors and DNA has been reported previously (FLEMING and PENNINGS 2001).

We then analyzed whether sequences upstream of the PDREs affect PDR5 transcription. We engineered a wild-type strain with the sequences from ~ -700 to ~ -1100 replaced by TRP1 (diagramed in Fig. 8C, the resulting strain named pdr5 promoter Δ400 strain). PDR5 transcription in the pdr5 promoter Δ400 strain was significantly reduced compared to its parental strain PDR1 under both non-induced and induced conditions. As expected, the pdr5 promoter Δ400 strain is hypersensitive to CYH (Fig. 8C). Not surprisingly, a reduced mRNA level of adjacent open reading frame YOR152C was detected in the pdr5 promoter Δ400 strain; however, deletion of YOR152C in PDR1 or pdr1-3 strains did not change their resistance to drugs (data not shown). Taken together, the data presented in Fig. 8 identify sequences important for PDR5 transcription located considerably upstream of the PDREs. Significantly, the nucleosome structure in this region requires ELM1 as well as PDR1/PDR3.

We then tested whether Elm1 directly regulates PDR5 transcription. We analyzed the recruitment of Myc-tagged Elm1 onto the PDR5 promoter and upstream region (up to and including the promoter of adjacent ORF YPR152C) in the PDR1 and pdr1-3 strains. In Fig. 8C, the promoter region of ORF YPR152C is depicted as iYOR152-0 (HARBISON et al. 2004; HORAK et al. 2002). No Elm1-Myc recruitment was detected on the promoter of either PDR5 or YOR152C (data not shown). This result is not surprising since Elm1 has been shown to be located primarily, if not exclusively, at the bud neck between mother and daughter cells (HUH et al. 2003; THOMAS et al. 2003). It is worth noting that the Myc-tagged Elm1 is functional in these strains based on their normal cell morphology and growth. Therefore, the results suggest
an indirect mechanism for Elm1 affecting \( PDR5 \) transcription.

**DISCUSSION**

In this study, we investigated the regulation of multidrug resistance involving the transcription of \( PDR5 \). We show that in a \( pdr1-3 \) strain, increased \( PDR5 \) transcription (GAO *et al.* 2004) correlates with increased levels of Pdr5 protein (Fig. 1). These results are consistent with microarray analysis, revealing \( PDR5 \) as the major target in a \( pdr1-3 \) strain (DERISI *et al.* 2000). These results also underscore the notion that transcriptional up-regulation is the predominant mechanism for development of yeast drug resistance. This is distinct from the development of mammalian drug resistance, in which mechanisms unrelated to transcription, such as gene amplification, play a crucial role in addition to transcriptional regulation (GOTTESMAN *et al.* 1995).

Employing the anticancer drug doxorubicin as a substrate, we demonstrated that cellular doxorubicin elimination follows zero-order kinetics (Fig. 2D and Table 2), indicative of catalysis, that is, its rate depends on the concentration of the catalyst (ABC transporters in the present case) and not the reactant (cellular doxorubicin concentration) (TINOCO *et al.* 1985). These findings are similar to the elimination of viblastine via P-glycoprotein, in which the results fit to Michaelis-Menten kinetics, \( V=V_{\text{max}} \frac{[S]}{K+[S]} \). When the concentration of substrates like vinblastine or doxorubicin (S) exceeds K and the rate (V) approaches Vmax, the efflux catalyzed by P-glycoprotein or Pdr5 will show zero-order kinetics and the number of transporters becomes limiting (AMBUDKAR *et al.* 1997). Such kinetics ensures efficient and almost complete cellular detoxification.

We demonstrated a striking inverse correlation between \( PDR5 \) mRNA levels and cellular
doxorubicin accumulation during cell cycle progression (Fig. 3). In these experiments, cellular doxorubicin at any given time point reflected the collective activities of many drug transporters. However, it became apparent that Pdr5 was the most important cell cycle regulated transporter, at least for doxorubicin and CYH. It is worth noting that, with equivalent treatments, doxorubicin content in the \textit{pdr1-3} cells during the G1 phase (2.0 pmol per $10^7$ cells, Fig. 3B) was higher than that in the unsynchronized \textit{PDR1} cells (~1.0 pmol per $10^7$ cells, Fig. 2C, 15 min). Therefore, G1-synchronized \textit{pdr1-3} cells exhibited even greater sensitivity than unsynchronized \textit{PDR1} cells with respect to detoxification of doxorubicin. The generality of this observation for different drugs and to what degree cell cycle-dependent drug sensitivity occurs in pathogenic yeast and cancer cells remain to be thoroughly investigated.

We searched for the most appropriate substrate to measure Pdr5 efflux in the various mutants. Ideally, an inert substrate is preferable. Fluconazole, an inhibitor of ergosterol biosynthesis (KONTOYIANNIS et al., 1999) was separated on HPLC and detected by absorbance. However, the detection sensitivity was too low (~5 nmoles). Doxorubicin, on the other hand, was detected by fluorescence; its lowest detection limit (with a signal-to-noise ratio greater than 3) was lower than 5 pmoles.

The doxorubicin-induced DNA damage results in cell cycle arrest at the G2/M phase (SIU \textit{et al.} 1999). This process is mediated by inhibiting dephosphorylation of the p$34^{\text{cdc2}}$ kinase (mammalian homolog of the \textit{S. cerevisiae} Cdc28) and by inducing cyclin B1 accumulation (LING \textit{et al.} 1996). Since doxorubicin effect of the cell cycle is expected to be similar in all strains studied, the loss of doxorubicin efflux in \textit{pdr1-3 cla4Δ}, \textit{pdr1-3 elm1Δ}, and \textit{pdr1-3 cdc29-C127Y} strains (Table 2) reflects primarily the effects of the genetic mutations on G2/M transition.
We provided several lines of evidence that establish a genetic connection between *PDR5* and *ELM1* or *ELM1*-related genes required for proper mitotic progression. First, cells harboring *elm1Δ* exhibit G2/M delay, reduction of *PDR5* transcription, and loss of CYH resistance (Fig. 4-5). Second, epistasis analysis indicates that although the *elm1Δ* mutation does not increase CYH susceptibility of the *pdr1-3 pdr5Δ* strain, the *pdr1-3 pdr5Δ elm1Δ* strain becomes more sensitive to CYH than the *pdr1-3 elm1Δ* strain (Fig. 4D). These data suggest that *ELM1* functions as an upstream regulator of the *PDR5*-mediated CYH resistance. Third, mutations of genes causing defective mitotic progression decrease *PDR5* transcription and CYH resistance (Fig. 5-6). Fourth, doxorubicin efflux in the *pdr1-3 elm1Δ*, *pdr1-3 cla4Δ* and *pdr1-3 cdc28-C127Y* strains is virtually undetectable (Table 2). The drug efflux rates in these strains are ~2 orders of magnitude lower than that in the *pdr1-3* strain (Table 2). These data provide a quantitative estimate of the functional consequences of down regulating drug transporter genes in these mutants. The expression of *PDR5* and perhaps other cell cycle regulated transporter genes is severely diminished by these mutations. This finding is consistent with the genome-wide analysis showing the expression of several ABC transporter genes (including *TPO1*, *TPO2*, *TPO3* and *TPO4*) peaks during the M phase, while the transcription of other well established transporter genes peak at other stages of the cell cycle (e.g., *SNQ2* peaks at G2 and *FLR1* at S phase) (SPELLMAN *et al.* 1998). Fifth, *elm1Δ* leads to detectable alteration in nucleosome structure upstream of known PDREs at the *PDR5* promoter region (Fig. 8). The alteration of *PDR5* nucleosome structure by *elm1Δ*, however, appears independent of the recruitment of Pdr1 to the PDREs (Fig. 7). Identification of a new region whose nucleosome structure undergoes conformational changes is consistent with the initial characterization of the *PDR5* promoter, which indicated that sequences of ~1.1 kb upstream of *PDR5* translation start site were required.
for maximal PDR5 expression (KATZMANN et al. 1996).

We note that the biochemical connection between ELM1 and PDR5 transcription is most likely via an indirect mechanism. This conclusion is based on the fact that Elm1 is a key cellular regulatory kinase and predominately located at the bud neck, and is not detectable on the PDR5 promoter by ChIP. The mechanism by which Elm1 regulates the nucleosome structure of the PDR5 promoter, therefore, remains to be determined. The fact that both Pdr1 and Pdr3 are phosphorylated (MAMNUN et al. 2002), raises the possibility that phosphorylation of Pdr1/Pdr3 by either Elm1 or its related kinases may affect the activity of Pdr1/Pdr3. In this regard, differential phosphorylation has been proposed to regulate the activity of Gal4 (MYLIN et al. 1990), the founding member of Cys6-Zn(II) DNA binding transcription factor family to which Pdr1 and Pdr3 belong (POCH 1997). It is interesting to note that the sequences SPVR (amino acids 942-945) and SPLK (amino acids 890-893) of Pdr1 and Pdr3, respectively, are located within the C-terminal transcription activation domains. These are consensus target sequences (S/T-P-X-K/R) for Cdc28 kinase. It is tempting to speculate that Cdc28 could participate in transcriptional regulation of PDR5 by modulating the activities of constitutively bound Pdr1/Pdr3 activators. Elm1, then, may be linked to Cdc28 activity via regulating Swe1 phosphorylation state during mitosis (SREENIVASAN and KELLOGG 1999). Consistent with this possibility, a pdr1-3 elm1Δ swe1Δ strain partially restores transcriptional defect of PDR5 observed in the pdr1-3 elm1Δ strain (Fig. 5B and data not shown). A potential requisite tie between Elm1 (or Cdc28), Pdr1/Pdr3 phosphorylation states, and PDR5 transcription level remains to be investigated.

The nucleosome structure affected by elm1Δ is located ~900 bp upstream of the PDR5 transcription start site (134 nucleotides upstream of translation start codon ATG). This region
encompasses the predicted promoter of the open reading frame YOR152C, which is oriented in the opposite direction of \textit{PDR5} (Fig. 8C) and encodes a putative membrane bound protein of unknown function (Terashima \textit{et al.} 2002). Interestingly, microarray studies indicated that YOR152C mRNA level was significantly up-regulated in the \textit{pdr1-3} strain (DeRisi \textit{et al.} 2000). However, deletion of YOR152C did not affect drug resistance of \textit{PDR1} or \textit{pdr1-3} strain (our unpublished data). Further characterization of YOR152C and the divergent promoter between the \textit{PDR5} and YOR152C open reading frames should elucidate molecular aspects of their transcriptional co-regulation.

The connection between cell cycle progression and drug transporter gene expression reported here in not unprecedented in other eukaryotes. For instance, in addition to regulation at DNA and mRNA levels, expression of P-glycoprotein is regulated at the protein level. The turnover of P-glycoprotein in multidrug resistant ovarian cells was shown to be cell-cycle-dependent (Zhang and Ling 2000). Furthermore, it was reported that colon cancer cells overexpressing P-glycoprotein showed a reduced sensitivity to doxorubicin during G2/M (Toffoli \textit{et al.} 1996). It is therefore suggestive that prior synchronization of drug-resistant cells to the cell cycle stage in which the expression of drug transporters is the lowest (such as G1 for \textit{PDR5}) could improve the efficacy of treatments of fungal infections and cancers.

The transcription factors that are required for cell-cycle-dependent \textit{PDR5} transcription remain to be explored. Factors known to be recruited to the \textit{PDR5} promoter (e.g., Pdr1, Pdr3, SAGA, Mediator and SWI/SNF complexes) are potential candidates (Ga\textit{o} \textit{et al.} 2004). Another possibility is the transcription factor Tos4, a known substrate of Cdc28 (Ubersax \textit{et al.} 2003) that regulates cell cycle progression (Iyer \textit{et al.} 2001) and binds to the intergenic region iYOR152C-0 (Horak \textit{et al.} 2002). Moreover, the transcription factor Sok2 also binds to
iYOR152C-0 (Harbison et al. 2004) and negatively regulates pseudohyphal differentiation/elongated morphology (Pan and Heitman 2000). Interestingly, the iYOR152C-0 region overlaps with the –700 to –1100 region that requires Elm1 and Pdr1/Pdr3 for its proper nucleosome structure (Fig. 8).

One intriguing question raised by our studies is why a drug transporter gene such as PDR5 is specifically expressed during mitosis. It is possible that cell-cycle-regulated drug transporter genes may reflect physiological functions of these transporters other than their roles as drug transporters (Jungwirth and Kuchler 2006; Schmitt and Tampe 2002). It has been shown that steroids, important components of the cell membrane, are physiological substrates of Pdr5 (Kolaczkowski et al. 1996). Moreover, transport of phosphatidylethanolamine is shown to be controlled by the transcription regulators PDR1 and PDR3 (Kean et al. 1997). As buds grow, biosynthesis and transportation of cell membrane components increase. Drug transporters thus may facilitate proper localization of steroids and other molecules in the newly formed daughter cell membranes. Consistent with this notion, the mammalian P-glycoprotein has been shown to transport, or "flip", short-chain lipids between the leaflets of the cell membrane (Romsicki and Sharom 2001). Interestingly, the connection between lipid metabolism, drug resistance, and cellular morphogenesis was also demonstrated by the functional analysis of a sphingolipid biosynthetic gene CaIPT1 of Candida albicans showing its involvement in both multidrug resistance and cellular morphogenesis (Prasad et al. 2005).

In conclusion, we provide genetic, kinetic and molecular evidence that ELM1 and functionally related kinase genes are required for multidrug resistance in S. cerevisiae. The mechanism for this regulation may include alteration of the nucleosome structure upstream of the PDR5 PDREs. The proposed mechanism is valid for both pdr1-3 and PDR1.

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Acknowledgments

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# TABLE 1. List of *Saccharomyces cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<td>WCS261 (YALF-A1): MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3</td>
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<td>Wolfger et al., 1997</td>
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<td>Wolfger et al., 1997</td>
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<td>Wolfger et al., 1997</td>
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<td>WCS267 (FY1679-28C/pdr1::TRP1 pdr3::HIS3): MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63 pdr1::TRP1 pdr3::HIS3</td>
<td>Wolfger et al., 1997</td>
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<td>WCS268 (FY1679-28C/pdr1::KanMx6): MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63 pdr1::Kan-Mx6</td>
<td>Wolfger et al., 1997</td>
<td></td>
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<td>WCS345: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 elm1-300</td>
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<td>WCS355: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 elm1-300 [pRS415-ELM1]</td>
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<td>WCS489: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 nap1::LEU2</td>
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<td>WCS491: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 cla4::KanMX</td>
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<td>WCS496: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 mih1::KanMX</td>
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<td>WCS497: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 swe1::TRP1</td>
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<td>WCS501: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 pdr5::KanMX</td>
<td>This study</td>
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<td>WCS502: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 pdr5::KanMX elm1::LEU2</td>
<td>This study</td>
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<tr>
<td>WCS503: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 cdc28::LEU2 [pRS316-CDC28]</td>
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<td>WCS504: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 cdc28::LEU2</td>
<td>This study</td>
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</table>
[pRS316-cdc28-Y19F]

WCS505: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 cdc28:LEU2 [pRS316-cdc28-C127Y]  This study

WCS506: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3-Myc-HIS3  Gao et al., 2004

WCS507: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 PDR1-Myc-HIS3  Gao et al., 2004

WCS530: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 PDR5-Myc  This study

WCS531: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 PDR1 PDR5-Myc  This study

WCS532: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 pdr1-3 PDR5-GFP  This study

WCS533: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 PDR1 PDR5-GFP  This study

WCS534: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 PDR1 cdc28:LEU2 [pRS316-CDC28]  This study

WCS535: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 PDR1 cdc28:LEU2 [pRS316-cdc28-Y19F]  This study

WCS536: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 PDR1 cdc28:LEU2 [pRS316-cdc28-C127Y]  This study

WCS651: MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 PDR1 pdr5 promter Δ400  This study
### TABLE 2

**Doxorubicin efflux rates in yeast mutant strains**

<table>
<thead>
<tr>
<th></th>
<th>Efflux rate</th>
<th>Relative efflux rate</th>
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<tr>
<td><em>PDR1</em></td>
<td>$1.5 \times 10^{-4}$ s$^{-1}$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>pdr1</em>-3</td>
<td>$48 \times 10^{-4}$ s$^{-1}$</td>
<td>32</td>
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<tr>
<td><em>pdr1</em>-3 <em>pdr5Δ</em></td>
<td>$1.4 \times 10^{-4}$ s$^{-1}$</td>
<td>0.93</td>
</tr>
<tr>
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<td></td>
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<tr>
<td><em>pdr1</em>-3 <em>nap1Δ</em></td>
<td>$1.2 \times 10^{-4}$ s$^{-1}$</td>
<td>0.8</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>pdr1</em>-3 <em>gin4Δ</em></td>
<td>~ $0.4 \times 10^{-4}$ s$^{-1}$</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>pdr1</em>-3 <em>cla4Δ</em></td>
<td>&lt; $0.2 \times 10^{-4}$ s$^{-1}$</td>
<td>&lt; 0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>pdr1</em>-3 <em>elm1Δ</em></td>
<td>&lt; $0.2 \times 10^{-4}$ s$^{-1}$</td>
<td>&lt; 0.13</td>
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<tr>
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</tr>
<tr>
<td><em>pdr1</em>-3 <em>cdc28-C127Y</em></td>
<td>&lt; $0.2 \times 10^{-4}$ s$^{-1}$</td>
<td>&lt; 0.13</td>
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<tr>
<td><em>pdr1</em>-3 <em>cdc28-Y19F</em></td>
<td>$46 \times 10^{-4}$ s$^{-1}$</td>
<td>31</td>
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</table>

The efflux rates of indicated strains were the negative slopes derived from time course experiments as described in Fig. 2D in the absence of FK506. Relative efflux rates were normalized to the *PDR1* strain.
TABLE 3

Minimal inhibitory concentrations of drugs in the yeast mutant strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>CYH (µg/ml)</th>
<th>CHL (mg/ml)</th>
<th>RHO (µg/ml)</th>
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<tbody>
<tr>
<td>PDR1</td>
<td>&lt; 0.2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>pdr1-3 pdr5Δ</td>
<td>&lt; 0.2</td>
<td>3</td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td>pdr1-3</td>
<td>&gt; 1.0</td>
<td>7.5</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>pdr1-3 elm1Δ</td>
<td>&lt; 0.2</td>
<td>&lt; 2</td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td>pdr1-3 cla4Δ</td>
<td>0.2</td>
<td>2</td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td>pdr1-3 gin4Δ</td>
<td>0.4</td>
<td>5</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>pdr1-3 nap1Δ</td>
<td>0.8</td>
<td>5</td>
<td>&gt; 20</td>
</tr>
</tbody>
</table>

Agar plate drug resistance assays were performed with various concentrations of cycloheximide (CYH, 0.2, 0.4, 0.6, 0.8 and 1.0 µg/ml), chloramphenicol (CHL, 1, 2, 3, 5, 6 and 7.5 mg/ml) and rhodamine (RHO, 2.5, 5.0, 7.5, 8, 10, 15 and 20 µg/ml). The PDR5 gene has been shown to be involved in the regulation of resistance to these three drugs (ROGERS et al. 2001). Each experiment was repeated at least twice.
FIGURE LEGENDS

Fig. 1. Role of PDR5 in multidrug resistance of pdr1-3 strain. (A) Agar plate drug resistance assay showing the drug susceptibility of pdr1-3, PDR1 and pdr1-3 pdr5Δ. Cells (10^7 cells and sequential 10-fold dilutions of each strain) were spotted on YPD plates with and without CYH (1.0 µg/ml) or fluconazole (FLU, 15 µg/ml). Images were taken after 30°C incubation for three days. (B) Western blot analysis of Pdr5 (Myc-tagged) in the PDR1 (40 µg of the whole cell lysate) and pdr1-3 (4 µg of the whole cell lysate) strains. Glucose-6-phosphate dehydrogenase (G6PD) served as a loading control. (C) Western blot analysis of Pdr5 (GFP-tagged) in pdr1-3 strain in the presence and absence of CYH (0.2 µg/ml for 45 min). Forty µg of the whole cell lysate were loaded in each lane.

Fig. 2. Kinetics of doxorubicin accumulation in PDR1 and pdr1-3 strains. Representative HPLC chromatograms for the pdr1-3 strain treated at 30°C with 50 µM doxorubicin alone (A) or 50 µM doxorubicin plus 20 µM FK506 (B). Doxorubicin peaks had a retention time of ~13.8 min. The volume (60 µl) injected into HPLC for doxorubicin alone corresponded to ~8.9 x 10^7 cells and for doxorubicin plus FK506 to ~2.0 x 10^7 cells. The first peak corresponded to the solvents. (C) Doxorubicin accumulation as a function of time in PDR1 and pdr1-3 strains. Cellular doxorubicin content was expressed as pmol per 10^7 cells. The cells were incubated with 50 µM doxorubicin at 30°C for the indicated time. (D) Doxorubicin efflux rates in the pdr1-3 (+/- FK506), PDR1 (– FK506), and pdr1-3 pdr5Δ (– FK506) strains. The cells were incubated with 50 µM doxorubicin at 30°C for 60 min. The cells were then washed and incubated in drug-free media for the indicated time periods. The fraction of doxorubicin retained by the cells was
plotted against incubation time in drug free medium. The efflux rate constants were determined as the negative slopes of the best-fit lines and summarized in Table 2.

**Fig. 3. PDR5 mRNA and doxorubicin efflux both peak at mitosis.** (A) Northern blot analysis of *PDR5* showing mRNA levels during the cell cycle. The blot was also probed for *SWI5* and *ADH1* mRNA. The pdr1-3 strain was synchronized in media containing 20 μg/ml nocodazole and then released. Total RNA was harvested at the indicated time post release. Morphologically, the period between 45 min and 135 min after nocodazole release corresponded to a complete cell cycle. AS, asynchronous. (B) Levels of doxorubicin during the cell cycle. In a parallel experiment to (A), cells were harvested at the indicated time and immediately placed in medium containing 50 μM doxorubicin for 15 min. Dotted lines indicate cellular doxorubicin; solid lines indicate relative *PDR5* mRNA. Cellular doxorubicin content was expressed as pmol per 10^7 cells. The level of mRNA at 135 min is denoted as 1.0.

**Fig. 4. Mutations in ELM1 suppressed CYH resistance mediated by the pdr1 and pdr3 mutations in a SNF1 independent manner.** (A) Top left panel, elongated morphology (an indication of G2 delay) of pdr1-3 elm1-300 strain, the white bars correspond to 5 μm; Lower left panel, loss of CYH resistance in pdr1-3 elm1-300. Pdr1-3 elm1-300 was transformed with a single copy pRS415 plasmid derivative containing wild-type *ELM1* [pELM1], which complemented the *elm1-300* mutation. The pRS415 was a centromere-based plasmid marked with *LEU2*. Images were taken after growth on complete synthetic medium minus leucine with and without 1.0 μg/ml CYH at 30°C for three days. A diagram depicts the kinase domain of Elm1 and truncation of Elm1-300. (B) Northern blot analysis of *PDR5* mRNA in pdr1-3, pdr1-3
elm1-300 and pdr1-3 elm1-300 [pELM1] strains in the presence and absence of CYH (0.2 µg/ml for 45 min). ADH1 served as a loading control. The strains were grown in complete synthetic medium minus leucine for plasmid retention. (C) Agar plate drug resistance assays of pdr1-3 and pdr3-2 strains harboring either elm1-300 or elm1Δ mutation on YPD with or without 1.0 µg/ml CYH. Cells harboring plasmid were grown in synthetic selective medium before spotting. Images were taken after 30°C incubation for three days. (D) Northern blot analysis of PDR5 mRNA in PDR1 and PDR1 elm1Δ strains in the presence and absence of CYH (0.2 µg/ml for 45 min). (E) Agar plate drug resistance assays of pdr1-3, pdr1-3 pdr5Δ, pdr1-3 elm1Δ, and pdr1-3 pdr5Δ elm1Δ strains on YPD with or without 0.2 µg/ml CYH, 10 µg/ml fluconazole (performed as described in C). (F) Left panel, Northern blot analysis of PDR5 mRNA in PDR1 and PDR1 snf1Δ strains in the absence and presence of CYH induction; Right panel, agar plate drug resistance compared CYH resistance of PDR1, PDR1 pdr5Δ, and PDR1 snf1Δ strains on YPD medium with and without 0.2 µg/ml CYH; the three strains are BY4741 background.

Fig. 5. Mutations causing a defective mitotic progression gave elongated bud morphology, loss of drug resistance, and reduced PDR5 transcription. (A) Elongated bud morphology of the strains bearing individual null mutations of NAP1, GIN4, CLA4 and ELM1. The white bars correspond to 5 µm. (B) Left panel, Northern blots of PDR5 mRNA in pdr1-3 and the strains bearing the indicated null mutations. ADH1 served as a loading control. PDR5 transcription was induced by 0.2 µg/ml CYH for 45 min; Right panel, agar plate drug resistance assays (performed as described in Fig. 4C) show loss of CYH resistance as a result of the indicated null mutations. (C). Agar plate drug resistance assays (performed as described in Fig. 4C) show loss of CYH and RHO (rhodamine) resistance as a result of the indicated null mutations in a PDR1 strain. (D)
Left panel, Northern blots of PDR12 mRNA in PDR1, pdr1-3 and the strains derived from pdr1-3 bearing the indicated null mutations. PDR12 transcription was induced by 1.0 mM sorbic acid for 45 min; Right panel, agar plate weak acid resistance assays of indicated strains grown in the absence or presence of 9 mM sorbate.

**Fig. 6. Cdc28-C127Y mutation gave elongated bud morphology, loss of CYH resistance, and reduced PDR5 transcription.** (A) Elongated morphology was exhibited by the pdr1-3 cdc28-C127Y strain but not in pdr1-3 and pdr1-3 cdc28-Y19F strains. The white bars correspond to 5 µm. (B) Agar plate drug resistance (performed as described in Fig. 4C, except that complete synthetic media minus uracil plates were used for plasmid retention) showed loss of CYH resistance in pdr1-3 cdc28-C127Y. (C) Northern blots of PDR5 mRNA in the three strains showed down regulation of PDR5 transcription in pdr1-3 cdc28-C127Y, but not in pdr1-3 cdc28-Y19F. PDR5 transcription was induced by 0.2 µg/ml CYH for 45 min. (D) Agar plate drug resistance (performed as described in Fig. 4C, except that complete synthetic media minus uracil plates were used for plasmid retention) showed loss of CYH resistance in PDR1 cdc28-C127Y. (E) Down-regulation of PDR5 transcription occurred in pdr1-3 mih1Δ strain but not in pdr1-3 swe1Δ strain. The CYH treatments were at 0.2 µg/ml for 45 min.

**Fig. 7. Recruitment of Pdr1 activator to the PDR5 promoter was independent of cell cycle progression and ELM1.** (A) Western blot of Myc-tagged Pdr1-3 activator showed constant levels during the cell cycle. The synchronization and release procedures were done as described in Fig. 3A. The same blot was also probed with antibodies against α-tubulin as a control. (B) Chromatin immuno-precipitation (ChIP) using antibodies against Myc-tagged Pdr1-3, showed
constitutive recruitment of the Myc-tagged Pdr1-3 activator to the upstream activating sequences (UAS) region of the \textit{PDR5} promoter. No recruitment to the coding sequences (CDS) of \textit{PDR5} was observed. The tagged strain was synchronized in YPD medium containing 3 µg/ml α-factor and then released at the indicated time points. The amount of whole cell lysate used in the Input PCR was 1/200 of that used for ChIP. (C) Myc-tagged Pdr1 and Pdr1-3 activators were constitutively recruited to the \textit{PDR5} upstream activating sequences (UAS) in the strains bearing \textit{elm1Δ} allele. Top panel: recruitment of C-terminally Myc-tagged activators Pdr1 and Pdr1-3 to the UAS of the \textit{PDR5} promoter was analyzed by ChIP. PCR was used to amplify \textit{PDR5} promoter DNA recovered in the anti-Myc antibody immunoprecipitation (IP) products. The recruitment signals of Pdr1 or Pdr1-3 to \textit{PDR12} promoter served as negative controls. Bottom panel: The recruitment signals of Pdr1 or Pdr1-3 to \textit{PDR5} UAS were presented as relative IP/Input ratio by a histogram. The histogram does not take into account the input PCR used 1/200th the amount of the whole cell lysate than that used for ChIP. Error bars are standard deviations between three independent experiments.

\textbf{Fig. 8. Location of altered chromatin structure of the \textit{PDR5} promoter in the \textit{elm1Δ} and \textit{pdr1Δpdr3Δ} strains.} (A) Analysis of micrococcal nuclease (MNase) susceptibility of the \textit{PDR5} promoter region in \textit{pdr1-3} versus \textit{pdr1-3 elm1Δ} strains, with and without CYH induction. Distinctive differences between \textit{pdr1-3} and \textit{pdr1-3 elm1Δ} strains in the hypersensitive sites located at \text~700 to \text~1100 bp upstream of transcription start site are marked with asterisks or brackets. The \textit{PDR5} transcription start site is marked as +1. The increasing concentrations of MNase marked by triangles represent 25, and 50 and 100 units per ml of the enzyme in MNase digestion. ND stands for naked DNA. MNase (10 units) was used to digest naked DNA. Solid
lined ovals depict positioned nucleosome structures, whereas dotted lined ovals depict more dynamic and less well-positioned nucleosome structures. Adjacent ORF YOR152C (768 bps) oriented in the opposite direction of PDR5 was indicated, in a different scale. (B) Analysis of micrococcal nuclease (MNase) susceptibility of the upstream PDR5 promoter region in the wild type (WT), pdr1Δ, pdr3Δ, and pdr1Δ pdr3Δ strains without CYH induction. Differences in the intensity of bands corresponding to MNase hypersensitive sites located at the established PDR5 promoter and coding sequences are marked with # signs and bracket respectively; asterisks mark sites located further upstream. MNase was used at 25 and 50 units. Solid and dotted lined ovals were as defined in (A). In (B), PDR5 is mapped in the opposite direction of that in (A) for better resolution of the region encompassing ~ -700 to -900. More details are described in Materials and Methods and as described previously (GAO et al. 2004). (C) The diagram depicts the construction of the pdr5 promoter Δ400 strain, which harbors a TRP1 replacement of the sequences located between -726 to -1123 nucleotides relative to the PDR5 transcription start site. The arrows denote the directions of the transcription of the PDR5 (4533 bps), TRP1 (1049 bps), and open reading frame YOR152C (768 bps). The intergenic region iYOR152C containing the promoter of YOR152C was indicated. Top inset is a Northern blot analysis of PDR5 mRNA in wild type strain and the pdr5 promoter Δ400 strain, which indicated decreased PDR5 transcription in the pdr5 promoter Δ400 strain under both non-induced and CYH induced conditions. Bottom inset is the agar plate drug resistance assay for the wild type and pdr5 promoter Δ400 strains on YPD in the absence or presence of CYH.
Figure 1

A.  
YPD  CYH  FLU  
\begin{align*}
\text{pdr}1-3 \\
PDR1 \\
pdr1-3 \ pdr5\Delta
\end{align*}

B.  
PDR1  pdr1-3  

C.  
\begin{align*}
pdr1-3 \\
\text{CYH} \\
Pdr5 \\
G6PD
\end{align*}
Figure 2

A. 

B. 

C. 

D.
Figure 3

(A)

<table>
<thead>
<tr>
<th>AS</th>
<th>0</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>105</th>
<th>120</th>
<th>135</th>
<th>150</th>
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</thead>
</table>

min after release from nocodazole

PDR5

SWI5

ADH1

Morphology of majority of cells

(B)

![Graph showing cellular doxorubicin and relative PDR5 mRNA levels over time]

Cellular doxorubicin (pmole per 10^7 cells) vs. min after release from nocodazole
A.

$pdr1-3$  $pdr1-3\ nap1\Delta$  $pdr1-3\ gin4\Delta$  $pdr1-3\ cla4\Delta$  $pdr1-3\ elm1\Delta$

B.

YPD  + 1.0 $\mu$g/ml CYH

C.

YPD  + 0.2 $\mu$g/ml CYH  + 20 $\mu$g/ml RHO

D.

YPD  + sorbate
Figure 6

A.  
\[ \text{pdr1-3} \quad \text{pdr1-3 cdc28-C127Y} \quad \text{pdr1-3 cdc28-Y19F} \]

B.  
- CYH  + 1.0 µg/ml CYH

\[ \begin{array}{ccc}
\text{pdr1-3} & \text{pdr1-3 cdc28-C127Y} & \text{pdr1-3 cdc28-Y19F} \\
\text{CYH} & - & - \\
PDR5 & - & + \\
ADH1 & + & + 
\end{array} \]

D.  
- CYH  + 0.2 µg/ml CYH

\[ \begin{array}{ccc}
PDR1 & \text{pdr1-3 cdc28-C127Y} & \text{pdr1-3 swel} \Delta \\
PDR1 & \text{pdr1-3 swel} \Delta & \text{pdr1-3 mktl} \Delta \\
PDR1 cdc28-C127Y & PDR5 & - \\
PDR1 cdc28-C127Y & PDR5 & + \\
PDR1 cdc28-Y19F & ADH1 & + \\
PDR1 cdc28-Y19F & ADH1 & + 
\end{array} \]
Figure 7

A.

<table>
<thead>
<tr>
<th>0</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>105</th>
<th>120</th>
<th>135</th>
<th>150</th>
</tr>
</thead>
</table>

min after release from nocodazole

Pdr1-3-Myc

α-tubulin

B.

**PDR5 UAS**

| 0  | 20 | 40 | 60 | 80 | 100 | 120 | 140 | 160 | 180 | 200 | 220 |

min after release from α-factor

Input

ChIP

Pdr1-3-Myc recruitment

**PDR5 CDS**

Input

ChIP

Pdr1-3-Myc recruitment

C.

<table>
<thead>
<tr>
<th>IP</th>
<th>Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
</tr>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

| pdr1-3 | PDR1 | pdr1-3 | PDR1 |
| elm1Δ  | elm1Δ| elm1Δ  | elm1Δ|

CYH

Pdr1-Myc/Pdr1-3-Myc

PDR5 promoter

PDR12 promoter

Relative IP/Input ratio

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<th>Relative IP/Input ratio</th>
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<tr>
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Pdr1-Myc Pdr1-3-Myc

61
Figure 8

A.

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<td>+</td>
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MNase

CYH

YOR-152C

1 ~ 1100

~ 900

~ 700

PDRE 1

PDRE 2

PDRE 3

TATA

+1

PDR5
Figure 8

B.

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<th>pdr3Δ</th>
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</table>

(bp) MW

Morse

PDR5

+1
TATA
PDRE 3
PDRE 2
PDRE 1

~ - 700
~ - 900
Figure 8

C.

\[ \text{iYOR152C-0} \]

\[ -1123 - 726 \]

\[ PDR5 \]

\[ \text{PdREs} \]

\[ TRP1 \]

\[ \text{WT \ pdr5 promoter} \]

\[ \Delta 400 \]

\[ \text{-} \quad + \quad - \quad + \quad \text{CYH} \]

\[ \text{PDR5} \]

\[ \text{ADH1} \]

\[ \text{YPD} \quad + \text{CYH 0.1 \mu g/ml} \quad + \text{CYH 0.2 \mu g/ml} \]