The EMBO Journal Vol. 21 No. 15 pp. 4114–4124, 2002

Mot3 is a transcriptional repressor of ergosterol biosynthetic genes and is required for normal vacuolar function in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae MOT3* gene encodes a nuclear protein implicated in both repression and activation of transcription. However, a *mot3Δ* mutation causes only mild phenotypes under normal growth conditions. To learn more about Mot3 function, we have performed a synthetic lethal screen. This screen identified *PAN1*, a gene required for normal endocytosis, and *VPS41*, a gene required for vacuolar fusion and protein targeting, suggesting a role for Mot3 in the regulation of membrane-related genes. Transcriptional analyses show that Mot3 represses transcription of *ERG2*, *ERG6* and *ERG9*, genes required for ergosterol biosynthesis, during both aerobic and hypoxic growth. Chromatin immunoprecipitation experiments suggest that this repression is direct. Ergosterol has been shown to be required for endocytosis and homotypic vacuole fusion, providing a link between Mot3 and these processes. Consistent with these results, *mot3Δ* mutants have a number of related defects, including impaired homotypic vacuole fusion and increased sterol levels. Taken together, our data suggest that proper transcriptional regulation of ergosterol biosynthetic genes by Mot3 is important for normal vacuolar function and probably for the endocytic membrane transport system.

Keywords: ergosterol/Mot3/repression/transcription/vacuole

Introduction

MOT3 encodes a transcription factor that modulates the expression of a large number of genes in *Saccharomyces cerevisiae*. Mot3 is nuclear (Grishin *et al*., 1998), is rich in charged amino acids, has two Zn-fingers and binds DNA in a Zn-dependent manner (Madison *et al*., 1998). The Mot3 Zn-finger region is highly homologous to that of Msn2 and Msn4, two *S.cerevisiae* proteins involved in the stress-response pathway. *MOT3* was originally identified in a genetic screen for high-copy-number suppressors of the *spt3Δ mot1-24* double-mutant lethality (Madison *et al*., 1998) and in an unrelated screen for mutations that cause an altered response to α-factor (Grishin *et al*., 1998). More recently, Mot3 has been shown to repress the transcription of two genes during aerobic growth: *ANB1*, encoding a translation initiation factor; and *DAN1*, encoding a cell wall mannoprotein (Kastaniotis and Zitomer, 2000; Kastaniotis *et al*., 2000; Cohen *et al*., 2001). Mot3 has also been shown to activate transcription of *CWP2*, a gene encoding a cell wall protein (Abramova *et al*., 2001a). Thus, Mot3 can serve as both a repressor and an activator of transcription.

As described in this paper, Mot3 also plays a role key in the regulation of genes required for ergosterol biosynthesis. Ergosterol is the major sterol in *S.cerevisiae* membranes and serves a role similar to that of cholesterol in mammalian cells. Ergosterol is required for proper fluidity and function of cellular membranes (Sturley, 2000), and it plays a role in both endocytosis (Munn *et al*., 1999) and homotypic vacuole fusion (Kato and Wickner, 2001). Ergosterol biosynthesis is regulated by intracellular ergosterol and oxygen levels, and some regulation is known to occur at the transcriptional level for many *ERG* genes (Sturley, 2000). Both *cis*-acting promoter elements and *trans*-acting factors, including the heme activator proteins Hap1 and Hap2/3/4 as well as Rox1, have been identified that affect *ERG* gene transcription; these findings have suggested that transcription of *ERG* genes is sensitive to oxygen levels (Turi and Loper, 1992; Kennedy *et al*., 1999; Jensen-Pergakes *et al*., 2001; Kennedy and Bard, 2001; Leber *et al*., 2001; Vik and Rine, 2001). Most recently, Upc2 and Ecm22, two direct activators of *ERG2* transcription, have been identified and shown to bind to the Sterol Regulatory Element (SRE) in the *ERG2* promoter (Vik and Rine, 2001). However, the mechanisms that regulate transcription of *ERG* genes in response to changes in ergosterol and oxygen levels remain largely unknown.

The identification of Mot3 as a transcriptional regulator of ergosterol biosynthetic genes arose from efforts to understand the roles of Mot3. Mot3 is not essential for growth under several conditions tested, although *mot3Δ* strains exhibit many mild mutant phenotypes (Grishin *et al*., 1998; Madison *et al*., 1998). To learn more about Mot3, we conducted a screen for mutations that cause inviability in combination with a *mot3Δ* mutation. Mutations were identified in two genes: *PAN1*, involved in endocytosis; and *VPS41*, involved in vacuolar protein sorting and fusion (Radisky *et al*., 1997; Tang *et al*., 2000). Interestingly, we have found that *mot3Δ* mutants have several defects in vacuolar function as well. Although endocytosis and vacuolar protein-sorting and fusion are separate functions, they are all part of the general process known as the endocytic membrane transport system and, as such, they require many functions of the cell membranes (Munn, 2000). Therefore, we hypothesized a connection between Mot3 and genes required for membrane function. Transcription and chromatin immunoprecipitation experiments show that Mot3 is a repressor of at

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least three of the genes required for ergosterol biosynthesis, *ERG2*, *ERG6* and *ERG9*. These results provide strong evidence that Mot3 plays an important role in proper function of the endocytic membrane transport system.

Results

Mutations in PAN1 and VPS41 cause synthetic lethality with a mot3Δ mutation

To identify genes that are functionally related to *MOT3*, we performed a synthetic lethal screen for mutations that cause inviability in a *mot3Δ* genetic background. We screened ~87 000 colonies and identified two candidates. These mutants display additional mutant phenotypes even in a *MOT3*+ background, facilitating their genetic analysis: one of the synthetic lethal mutants is temperature sensitive for growth at 37°C and both mutants are sensitive to cadmium. Genetic analyses demonstrated that the synthetic lethality with *mot3Δ* and the additional phenotypes co-segregated 2:2 in each case (Materials and methods), demonstrating that all of the phenotypes are caused by a single mutation in each of the candidates. Additional genetic tests showed the two new mutations to be recessive and to complement each other, suggesting that they identify two separate genes. We confirmed that these mutations are synthetically lethal with *mot3Δ* by two tests. First, we performed plasmid-shuffle experiments to demonstrate that the viability of the double mutants depended upon a plasmid containing *MOT3*+. Secondly, we separated each mutation from *mot3Δ* by crosses to a *MOT3*+ strain and reconstructed the synthetic lethality by crossing each single mutant to a *mot3Δ* strain.

To clone the genes corresponding to the *mot3Δ* synthetic lethal mutations, plasmid library clones were isolated by complementation of the synthetic lethality or the temperature- and cadmium-sensitive phenotypes. The specific genes were identified by plasmid complementation with single genes and by linkage analysis. These experiments showed that the synthetic lethal mutation that causes temperature sensitivity is in *PAN1* (designated *pan1-151*), and the other synthetic lethal mutation is in *VPS41* (designated *vps41-298*) (Figure 1). *PAN1* is an essential gene required for endocytosis (Tang and Cai, 1996; Tang *et al*., 1997, 2000; Wendland and Emr, 1998). *VPS41* is a non-essential gene required for vacuolar protein targeting and homotypic vacuole fusion (Nakamura *et al*., 1997; Radisky *et al*., 1997; Darsow *et al*., 2001).

The results from this synthetic lethal screen show that *mot3Δ* is synthetically lethal with mutations in genes required for both endocytosis and vacuolar function. Previous studies have shown that mutants defective for endocytosis are often synthetically lethal with mutations that affect vacuolar function (Munn and Riezman, 1994; Riezman *et al*., 1996; Wesp *et al*., 1997; Munn, 2000, 2001). Thus, these data suggest that Mot3 could be important for both normal endocytosis and vacuolar functions, or generally required for proper function of the endocytic membrane transport system.

mot3Δ mutants have defective vacuoles

To test the hypothesis that Mot3 is required for proper function of the vacuole, we examined vacuolar morphology and function, comparing wild-type and *mot3Δ* strains. First, to test whether *mot3Δ* mutants have a morphologically abnormal vacuole, we stained a *MOT3*+ strain and two different *mot3Δ* mutant strains with FM4-64, a vacuole-specific vital dye (Vida and Emr, 1995). Mutations that impair homotypic vacuole fusion result in an increase in the number of vacuolar structures per cell. The criteria used for classifying vacuole morphology phenotypes have been described previously (Banta *et al*., 1988). Briefly, cells with a class A or wild-type vacuolar morphology have one to three vacuoles per cell; cells with a class B morphology have more than three vacuoles per cell; and cells with a class C morphology lack any visible vacuole structure. From this analysis, the *MOT3*+ strain exhibited a wild-type (class A) vacuolar morphology (Figure 2), with a single vacuole visible in each cell. In contrast, the *mot3Δ* mutants displayed a mutant class B vacuolar morphology phenotype, with greater than three vacuoles per cell. We also stained the *vps41-298* mutant, which had no detectable vacuolar structures, suggesting a class C phenotype (Figure 2). We conclude from these experiments that *mot3Δ* mutants have abnormal vacuoles.

To test whether the aberrant vacuolar morphology exhibited by *mot3Δ* mutants correlated with a defect in vacuolar function, we assayed the ability of these mutants to grow in alkaline media. One important function of the yeast vacuole is to maintain a neutral intracellular pH (Klionsky *et al*., 1990). Therefore, mutants with a defective vacuole are unable to grow well in media buffered at either extreme acidic or alkaline pH (Klionsky *et al*., 1990). Indeed, the *mot3Δ* mutants grew poorly on solid alkaline buffered medium, similar to the defect we observed for the *vps41-298* mutant (Figure 3) and *vps41Δ* (data not shown). Therefore, *mot3Δ* mutants are defective

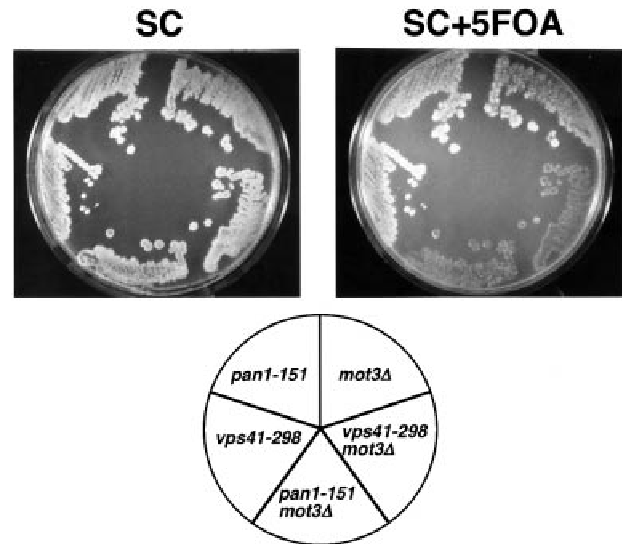

Fig. 1. A *mot3Δ* mutation is synthetically lethal with *pan1-151* and *vps41-298*. *mot3Δ* (FY2070), *mot3Δ vps41-298* (FY2073), *mot3Δ pan1-151* (FY2072), *vps41-298* (FY2075) and *pan1-151* (FY2074), each carrying a *URA3*+*MOT3*+ plasmid, were grown on a YPD plate for 2 days at 30°C. The YPD plate was then replica plated onto an SC and an SC + 5-FOA plate and photographs were taken after incubation for 1 day at 30°C.

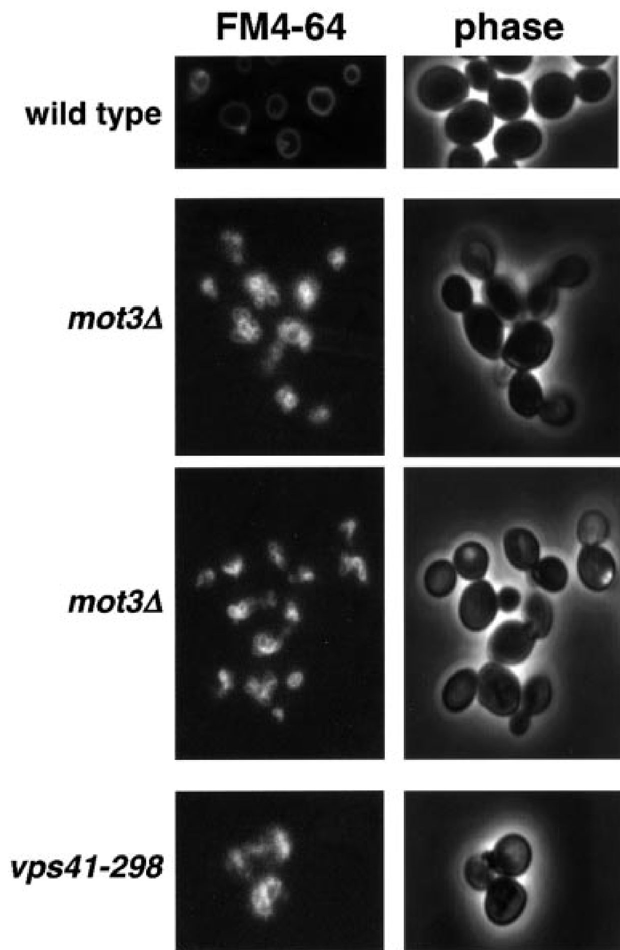

Fig. 2. A *mot3Δ* mutant has a defect in homotypic vacuole fusion. *Saccharomyces cerevisiae* strains were grown to 1–2 × 10^7 cells/ml in liquid YPD, stained with FM4-64 and visualized as described in Materials and methods. At least 100 cells were examined for each strain and the percentage that had the phenotype shown is as follows: wild type (FY2066), 95% class A; *mot3Δ* (FY2069), 78% class B; *mot3Δ* (FY2071), 82% class B; *vps41-298* (FY2075), 100% class C.

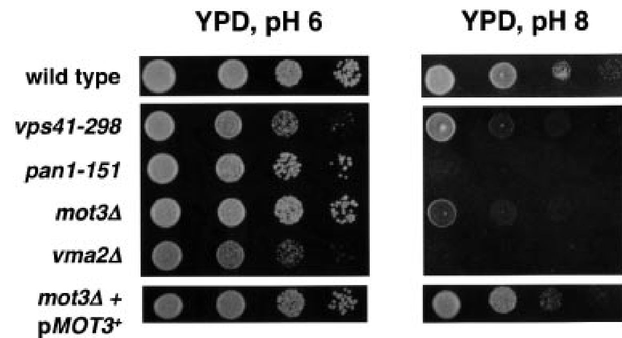

Fig. 3. A *mot3Δ* mutant is defective for growth at pH 8. Wild-type (FY2066), *vps41-298* (FY2075), *pan1-151* (FY2074), *mot3Δ* (FY2070), *vma2Δ* (FY2076) and *mot3Δ* (FY2070) with a *URA3*$^+$ *MOT3*$^+$ plasmid (p*MOT3*$^+$) were tested for growth on YPD and YPD pH 8 plates by spot tests (see Materials and methods). To prevent growth of sensitive strains due to media acidification by growth from neighboring resistant strains, wild-type and *mot3Δ*$^+$p*MOT3*$^+$ were grown on a separate YPD pH 8 plate. YPD plates were photographed after 2 days of incubation at 30°C. YPD pH 8 plates were photographed after 6 days of incubation at 30°C.

for vacuolar function. This phenotype of *mot3Δ* cells is fully complemented by providing *MOT3*$^+$ on a plasmid. In addition, the *pan1-151* mutant is unable to grow on alkaline buffered medium and its defect is as severe as that seen for a previously characterized vacuolar mutant, *vma2Δ* (Anraku *et al*., 1992). *VMA2* encodes the regulatory subunit of the vacuolar ATPase (Anraku *et al*., 1992). These results, taken together with the aberrant vacuolar morphology of *mot3Δ* mutants, strongly suggest that Mot3 is important for homotypic vacuole fusion and proper vacuolar functions.

Mot3 represses expression of ERG2, ERG6 and ERG9

Mot3 is nuclear (Grishin *et al*., 1998) and has been shown to act as a repressor and an activator of transcription (Grishin *et al*., 1998; Madison *et al*., 1998; Kastaniotis *et al*., 2000; Abramova *et al*., 2001a; Cohen *et al*., 2001). Therefore, the *mot3Δ* synthetic lethal phenotypes and defects in vacuolar function are probably due to improper transcription of genes, such as those encoding proteins required for vacuolar function or vacuolar membrane fusion. To assess this possibility, we performed microarray analyses comparing mRNA from *mot3Δ* and wild-type strains. The results from these experiments showed minor (<1.5-fold) effects on the mRNA levels of many genes involved with vacuolar function (*VPS*, *VAM* and *VMA* genes; data not shown) and cell wall biosynthesis (*PAU*, *DAN* and *TIR* genes; data not shown). However, a more significant effect was observed on the mRNA levels of *ERG2*, *ERG6* and *ERG9*, genes encoding ergosterol biosynthetic enzymes. The mRNA levels of these genes were increased in the *mot3Δ* mutant (data not shown). To confirm the microarray results, northern analyses were performed. These results show that *ERG2*, *ERG6* and *ERG9* mRNA levels were elevated ~2.5-fold in *mot3Δ* mutants compared with wild-type strains (Figure 4), suggesting that Mot3 is a repressor of these *ERG* genes.

Previous studies suggest that *ERG* genes are tightly regulated at the transcriptional level, particularly in the later steps of the ergosterol biosynthetic pathway, through an uncharacterized feedback loop that ensures proper ergosterol levels (Sturley, 2000). Consistent with this model, it has been shown that mutations in some *ERG* genes affect the transcription of other *ERG* genes (Lees *et al*., 1995; Arthington-Skaggs *et al*., 1996; Smith *et al*., 1996; Kennedy *et al*., 1999). Therefore, we also tested for double-mutant phenotypes when *mot3Δ* was combined with *erg2Δ* and *erg6Δ* mutations (*ERG9* is essential for aerobic growth, preventing similar tests with an *erg9Δ* mutation). As shown in Figure 4, *ERG9* mRNA levels were increased in *erg2Δ* and *erg6Δ* mutants compared with wild-type levels. In the *erg2Δ mot3Δ* and *erg6Δ mot3Δ* double mutants, there is an additive effect as *ERG9* mRNA levels are higher than in the single mutants. In addition, since *ERG2* and *ERG6* have been shown to be required for endocytosis (Munn *et al*., 1999; Munn, 2001), vacuolar fusion (Kato and Wickner, 2001) and growth in alkaline buffered media (Figure 5A), we tested for other phenotypes in the double mutants. Our results show that *erg2Δ mot3Δ* and *erg6Δ mot3Δ* double

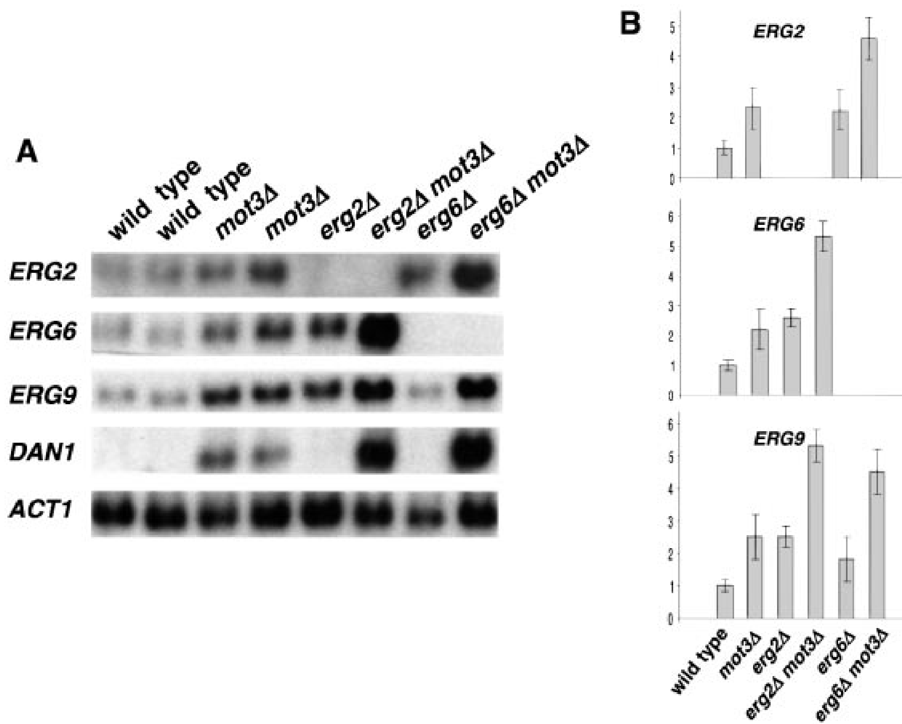

Fig. 4. *ERG2*, *ERG6* and *ERG9* mRNA levels are increased in *mot3Δ* mutants. (**A**) Representative northern analyses are shown of RNA prepared from wild-type (FY2066 and FY2068), *mot3Δ* (FY2069 and FY2071), *erg2Δ* (FY2077), *erg2Δ mot3Δ* (FY2078), *erg6Δ* (FY2079) and *erg6Δ mot3Δ* (FY2080) strains, probed for *ERG2*, *ERG6*, *ERG9*, *DAN1* and *ACT1* mRNAs. (**B**) Histograms showing the relative average mRNA levels for the genes indicated, normalized to the level of *ACT1* mRNA. The values and standard errors are from seven independent experiments.

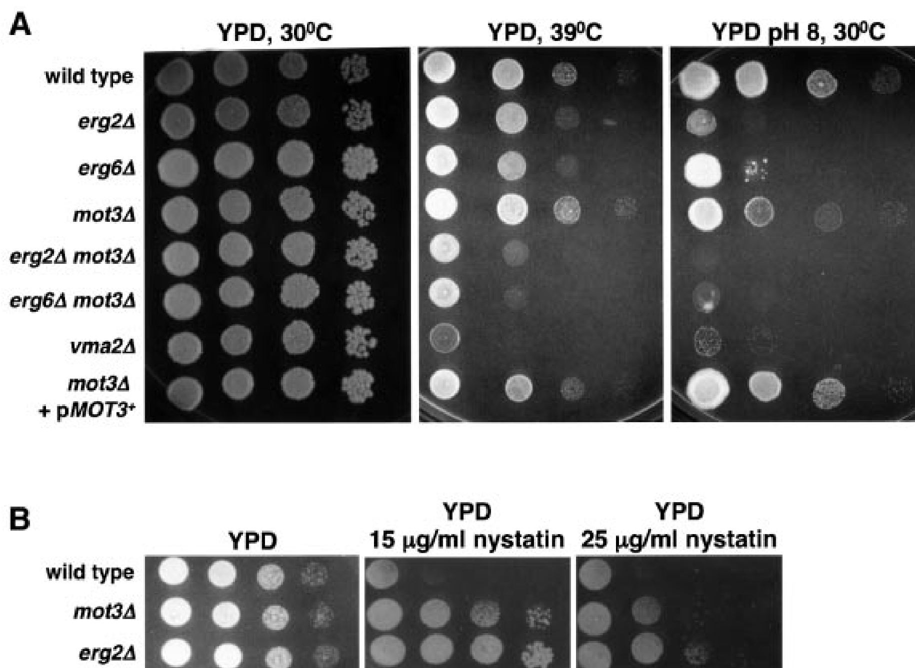

Fig. 5. Genetic interactions of *mot3Δ* with *erg2Δ* and *erg6Δ*. (**A**) Wild-type (FY2066), *erg2Δ* (FY2077), *erg6Δ* (FY2079), *mot3Δ* (FY2069), *erg2Δ mot3Δ* (FY2078), *erg6Δ mot3Δ* (FY2080), *vma2Δ* (FY2076) and *mot3Δ* (FY2069) carrying a *URA3*⁺ *MOT3*⁺ plasmid (p*MOT3*⁺) strains were tested for growth on YPD and YPD pH 8 by spot tests (see Materials and methods). YPD plates were photographed after 2 days of incubation. YPD pH 8 plates were photographed after 6 days incubation. (**B**) Nystatin resistance of *mot3Δ* mutants. Wild-type (FY2066), *mot3Δ* (FY2069) and *erg2Δ* (FY2077) strains were tested for growth on YPD and YPD-nystatin plates. The YPD plate was photographed after 1 day of incubation at 30°C and the YPD-nystatin plates were photographed after 2 days of incubation at 30°C.

mutants had a more severe growth defect than the single mutants on YPD at 39°C and on alkaline buffered YPD (Figure 5A). In conclusion, there is a strong genetic interaction of *MOT3* with *ERG2* and *ERG6*, supporting a role for Mot3 in the normal transcriptional regulation of these genes required for proper ergosterol homeostasis.

Ergosterol is not synthesized during anaerobic growth because oxygen is a cofactor for some of the ergosterol biosynthetic enzymes (Kennedy *et al.*, 1999; Sturley, 2000). There is also evidence that oxygen levels control transcription of some of the genes required for ergosterol biosynthesis (Thorsness *et al.*, 1989; Kennedy *et al.*, 1999; Sturley, 2000; Kennedy and Bard, 2001). Therefore, we tested whether *ERG2*, *ERG6* and *ERG9* mRNA levels are altered during hypoxic growth and whether Mot3 plays a role under this growth condition. Our results show that, indeed, there was a >10-fold repression of *ERG2* and *ERG6* mRNA levels under hypoxic conditions (Figure 6). *ERG9* mRNA levels were only decreased 2-fold during hypoxic growth (data not shown), in agreement with previous studies (M'Baya *et al.*, 1989; Kennedy *et al.*, 1999). In a *mot3Δ* background, the repression of *ERG2* and *ERG6* under hypoxic conditions was almost completely abolished (Figure 6). These results, then, show that Mot3 is required for the strong hypoxic repression of *ERG2* and *ERG6* transcription.

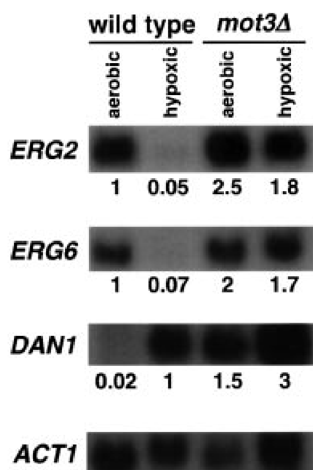

Fig. 6. Mot3 is required for hypoxic repression of *ERG2* and *ERG6*. A representative northern analysis is shown of RNA prepared from aerobically and hypoxically grown wild-type (FY2066) and *mot3Δ* (FY2071) cells, probed for *ERG2*, *ERG6*, *DAN1* and *ACT1* transcripts. The numbers indicate the average values from three independent experiments, normalized to either the wild-type strain grown aerobically (for *ERG2* and *ERG6*) or to the wild-type strain grown hypoxically (for *DAN1*). Standard deviations were <2%.

mot3Δ mutants have increased sterol levels

To test whether the increased levels of *ERG2*, *ERG6* and *ERG9* mRNAs in aerobically grown *mot3Δ* mutant cells affect sterol levels, we performed total sterol analyses on wild-type and *mot3Δ* cells. As shown in Table I, *mot3Δ* mutants have 21% more total sterols and 15% more ergosterol than wild-type cells. Despite this modest increase in total sterols and ergosterol, *mot3Δ* mutants are slightly resistant to nystatin (Figure 5B), a phenotype indicative of reduced ergosterol in plasma membrane (Lorenz and Parks, 1991). As nystatin primarily binds ergosterol, most *erg* mutants unable to synthesize ergosterol are resistant to nystatin (McLean-Bowen and Parks, 1982; Kovac *et al.*, 1987). Taken together, these results suggest that there is a positive correlation between increased mRNA levels of these *ERG* genes and an increase in total sterol production in *mot3Δ* mutants; however, *mot3Δ* mutants probably have a reduced level of ergosterol in their plasma membranes (see Discussion).

Overexpression of *UPC2* and *ECM22* causes a defect in vacuolar morphology

We observed a homotypic vacuole fusion defect in *mot3Δ* mutants, which have elevated *ERG2*, *ERG6* and *ERG9* mRNA levels compared with wild-type cells. To test whether the increased *ERG* gene transcription observed in *mot3Δ* mutants is responsible for the vacuolar defect, we increased *ERG* mRNA levels in a *MOT3*$^{+}$ background. To do this we took advantage of the recent finding that overexpression of *UPC2* and *ECM22* causes increased *ERG2* expression (Vik and Rine, 2001). Our results (Figure 7) show that a wild-type strain that contains *UPC2*, *ECM22*, or both genes on high-copy-number plasmids, had abnormal (class B) vacuolar morphology. In these experiments, *ERG2* and *ERG9* mRNA levels were increased ~2-fold, while *ERG6* mRNA levels were not significantly affected (data not shown). Thus, the increased expression of these *ERG* genes in *mot3Δ* mutants is likely to contribute to the *mot3Δ* vacuolar defect.

Mot3 binds to the promoter regions of *ERG2* and *ERG6* in vivo

To test whether Mot3 acts directly in repression, we performed chromatin immunoprecipitation experiments. The strongest cases of Mot3 repression that we have observed are of *ERG2* and *ERG6* during hypoxic growth, and we found a cluster of putative Mot3 consensus binding sites in the regulatory regions of these genes (Figure 8A). Therefore, we examined Mot3 binding to the *ERG2* and *ERG6* regulatory regions in cells grown in hypoxic conditions. In these experiments, we used a version of Mot3 fused to the myc-epitope tag. Our results (Figure 8B

Table I. Sterol levels in *mot3Δ* mutants

Relevant genotype	Total sterol	% increase	Total ergosterol	% increase
MOT3^{+}	6.75 ± 0.26	–	4.27 ± 0.17	–
mot3Δ	8.16 ± 0.23	21	4.92 ± 0.24	15

The total sterol and ergosterol levels are reported as µg sterol/g yeast dry weight (see Materials and methods) of wild-type (FY2066) and *mot3Δ* (FY2069) cells. Values represent the average and standard deviations from three independent experiments.

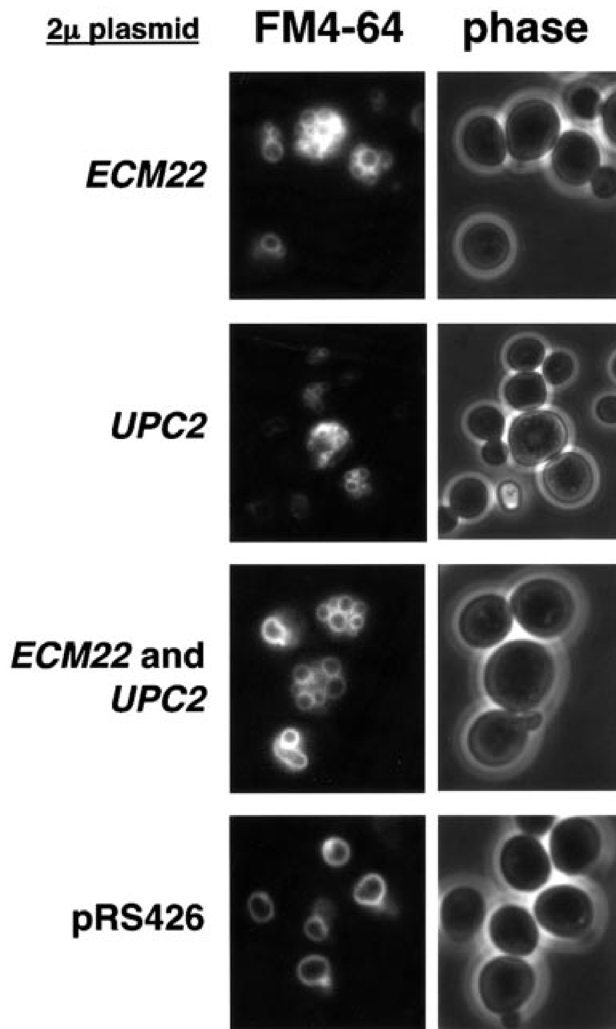

Fig. 7. Overexpression of *UPC2*, *ECM22*, or both, causes a vacuolar defect. High-copy-number plasmids carrying either *UPC2*, *ECM22* or no insert were used to transform the *MOT3*$^+$ strain FY2066 to either Ura$^+$, Leu$^+$ or Ura$^+$ Leu$^+$, respectively. Transformants were selectively grown and then stained with FM4-64 (see Materials and methods). The percentage of plasmid-containing cells that had the vacuolar phenotype shown above is as follows: *ECM22*, 71% class B; *UPC2*, 70% class B; *EMC22* and *UPC2*, 75% class B; the plasmid control, 78% class A.

and C) demonstrate specific chromatin immunoprecipitation of the *ERG2* and *ERG6* promoter regions. These chromatin immunoprecipitation results, combined with the DNA binding properties of Mot3, the presence of Mot3 binding sites, and derepressed *ERG2* and *ERG6* transcription in *mot3Δ* mutants, strongly suggest that Mot3 directly represses *ERG2* and *ERG6* transcription.

Discussion

Our studies have demonstrated two new and related findings about the transcription factor Mot3: (i) Mot3 is required for normal vacuolar morphology and function; and (ii) Mot3 is a transcriptional repressor of the *ERG2*, *ERG6* and *ERG9* genes. These conclusions are based on several results. First, a genetic screen discovered synthetic lethality between *mot3Δ* and mutations in *PAN1* and in *VPS41*, genes required for endocytosis (Tang and Cai, 1996; Wendland *et al*., 1996; Tang *et al*., 1997, 2000; Wendland and Emr, 1998) and for vacuolar protein targeting and fusion (Nakamura *et al*., 1997; Radisky *et al*., 1997), respectively. Previous studies have shown that mutations that affect vacuolar functions are frequently lethal in combination with mutations that impair endocytosis (Munn and Riezman, 1994; Munn, 2000, 2001). Secondly, *mot3Δ* mutants have defects in vacuolar function and morphology. Thirdly, *mot3Δ* mutants have increased levels of the *ERG2*, *ERG6* and *ERG9* mRNAs under both aerobic and hypoxic conditions. Fourthly, the repression of *ERG2* and *ERG6* by Mot3 appears to be direct, as chromatin immunoprecipitation experiments have shown Mot3 to be physically present at their promoters. Finally, *mot3Δ* mutants have increased sterol levels, consistent with the conclusion that Mot3 represses the transcription of these *ERG* genes. Taken together, these results suggest that Mot3 plays a critical role in a transcriptional regulatory system that modulates ergosterol levels to ensure a normal endocytic membrane transport system.

Although Mot3 has previously been implicated in the regulation of several different classes of genes (Grishin *et al*., 1998; Kastaniotis *et al*., 2000; Abramova *et al*., 2001a,b; Cohen *et al*., 2001), our results suggest that it is the defective repression of *ERG* genes that causes the vacuolar defects observed in *mot3Δ* mutants. This conclusion is based on our finding that when *ERG* genes are overexpressed in a *MOT3*$^+$ genetic background, we observed vacuolar defects similar to those observed in *mot3Δ* mutants. Although we cannot rule out that the defect in homotypic vacuolar fusion caused by *mot3Δ* or by overexpression of *UPC2* and *ECM22* is due to other pleiotropic effects, we do observe similar effects on *ERG2* and *ERG9* mRNA levels in the two different situations. Recent studies have shown that ergosterol is required for homotypic vacuolar fusion (Kato and Wickner, 2001). Our work provides the first evidence that transcriptional regulation of genes required for ergosterol biosynthesis plays a significant role in vacuolar functions. The fact that ergosterol is also required for normal endocytosis (Munn *et al*., 1999) suggests that Mot3 is also required for this aspect of the endocytic membrane transport system.

Increased ERG gene expression in mot3Δ mutants and vacuolar defects

Our results have demonstrated that in *mot3Δ* mutants there is an increased level of *ERG* gene mRNAs and an increased level of sterols. However, *mot3Δ* mutants have a vacuolar defect similar to those observed in mutants defective in ergosterol synthesis (Kato and Wickner, 2001), an apparently opposite situation. The mild nystatin resistance of *mot3Δ* mutants suggests that the increased expression of *ERG* genes in *mot3Δ* mutants may actually result in a reduced level of ergosterol in the plasma membrane. There are several possibilities for how increased *ERG* gene expression might reduce the level of functional ergosterol and impair vacuolar morphology and function. For example, the increased level of ergosterol might accumulate in an aberrant form, thereby reducing the level of ergosterol available for membranes. This

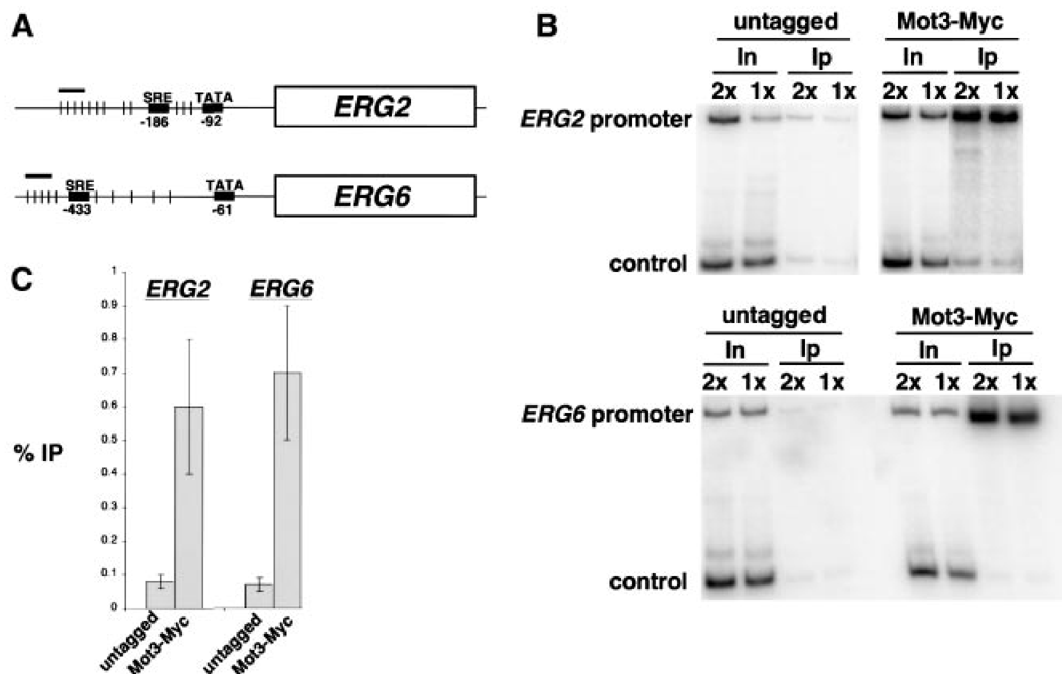

Fig. 8. Mot3 binds to the promoter region of *ERG2* and *ERG6 in vivo*. (**A**) Diagram of the *ERG2* and *ERG6* promoter regions. The short-thin vertical lines represent the positions of the Mot3 consensus binding site sequences [(C/T/A)AGG(T/A/C/G)(T/A)] and the short thick horizontal bar represents the position of the PCR product assayed in the chromatin immunoprecipitation experiments. (**B**) Chromatin immunoprecipitation was performed with extracts prepared from hypoxically grown Mot3-Myc18 (FY2081) and untagged (FY1339) cells. The abbreviations used indicate the input DNA (In) and immunoprecipitated DNA (Ip) used for PCR using primers that amplify the regions defined in (A). For input DNA, 2× and 1× indicate 1/500 and 1/1000 dilutions of the chromatin solution, respectively. For immunoprecipitated DNA, 2× and 1× indicate 1/10 and 1/20 dilutions of the immunoprecipitated chromatin solution, respectively. The non-specific control used in the reactions is described in Materials and methods. (**C**) A histogram showing the average values and their standard errors (% IP), calculated as previously described (Larschan and Winston, 2001), from three independent experiments.

possibility is supported by the observation that *mot3Δ* mutants have an elevated level of lipid particles (M.Valachovic and M.Bard, unpublished results). Previous studies have shown that a common mechanism for disposing of excess intracellular sterol is sterol esterification and deposition into such lipid particles (Soustre *et al*., 1998; Zweytick *et al*., 2000). Therefore, the increased level of sterols in *mot3Δ* mutants might activate this mechanism of disposal, resulting in abnormal sterol trafficking, which has been correlated with an abnormal sterol composition of vacuole and plasma membranes (Tinkelenberg *et al*., 2000). Alternatively, increased *ERG* gene expression might lead to an accumulation of ergosterol precursors, resulting in an aberrant incorporation of sterols other than ergosterol into the membrane.

The role of Mot3 during hypoxic and aerobic growth

Our results have shown that Mot3 represses *ERG2*, *ERG6* and *ERG9* during both hypoxic and aerobic growth. The strongest effect that we observed on repression by Mot3 occurs at *ERG2* and *ERG6* during hypoxic growth. Previous studies showed that, under hypoxic growth conditions, *S.cerevisiae* is unable to synthesize ergosterol due to the unavailability of oxygen to function as a cofactor for ergosterol biosynthetic enzymes (Kwast *et al*., 1998). Our results now show that hypoxic control of ergosterol biosynthesis also occurs at the transcriptional level by Mot3 repression of *ERG* gene transcription. Results from two genome-wide expression studies that compared mRNA levels during aerobic and anaerobic growth differ from our results and from each other with respect to expression of *ERG* genes during anaerobic growth. One study did not detect a difference in mRNA levels for *ERG2*, *ERG6* and *ERG9* (ter Linde *et al*., 1999), and the other found a modest increase in *ERG6* and *ERG9* mRNA levels and no change for *ERG2* (Kwast *et al*., 2002). Of note, the latter study also shows increased *CWP1* and *CWP2* mRNA levels during anaerobic growth, a condition in which these genes have been shown to be repressed (Abramova *et al*., 2001a). The reasons for the differences between our results and the microarray studies are not known, but could be due to different genetic backgrounds, growth conditions or the experimental techniques used. Regardless of the reason, our results have demonstrated that Mot3 is required for the hypoxic repression of *ERG2* and *ERG6* transcription.

During aerobic growth, Mot3 is also required for transcriptional repression of *ERG2*, *ERG6* and *ERG9*, although the degree of repression is significantly less than during hypoxic growth. However, the modest repression of *ERG* genes by Mot3 during aerobic growth is clearly important, because under aerobic conditions *mot3Δ* mutations cause synthetic lethality with *pan1* and *vps41* mutations, defects in vacuolar morphology and function, and increased levels of total sterols and ergosterol. Furthermore, during aerobic growth, *mot3Δ erg2Δ* and

mot3Δ erg6Δ double mutants have several synthetic phenotypes, suggesting that they impair a common pathway. Therefore, during aerobic growth, Mot3 may ensure that *ERG* gene transcription is modulated appropriately to achieve normal ergosterol levels.

Although there is previous evidence for regulation of ergosterol biosynthesis at the transcriptional level (Dimster-Denk *et al*., 1999), only a small number of regulatory factors have been identified. Some studies linking oxygen sensing and *ERG* gene transcription have identified Rox1 as a repressor and Hap1/2/3/4 as activators of *ERG11* and *HMG1* (Kwast *et al*., 1998). Recently, Vik and Rine (2001) identified two activators of *ERG2*, Upc2 and Emc22, which promote *ERG2* transcription under conditions of sterol starvation. A recent study has identified Sut1 as a putative transcription factor involved in the regulation of sterol uptake during aerobiosis, suggesting a link between sterol uptake and ergosterol biosynthesis (Ness *et al*., 2001). Our studies have added to our understanding of *ERG* gene regulation by demonstrating that Mot3 is an important repressor of *ERG2*, *ERG6* and *ERG9* gene transcription. The role of Mot3 with respect to these other positive and negative regulators of *ERG* gene transcription has not yet been addressed.

Two important and related aspects of Mot3 repression of *ERG2*, *ERG6* and *ERG9* remain to be studied. First, the mechanism of repression by Mot3 at these genes is not understood. A previous study showed that, during aerobic growth, Mot3 may repress *ANB1* and other genes by recruiting the Ssn6–Tup1 repressor complex (Kastaniotis *et al*., 2000). There is as yet no evidence that repression of *ERG* genes is dependent on Ssn6–Tup1, histone deacetylases or nucleosome remodeling complexes. Secondly, the difference in the degree of Mot3 repression of *ERG* gene transcription in cells grown hypoxically or aerobically is not yet understood. Since there are two clusters of putative Mot3 binding sites, 5′ and 3′ of the SRE elements in *ERG2* and *ERG6*, it is possible that differential binding by Mot3 provides a means to control the level of repression in response to oxygen levels or sterol levels. Consistent with this idea, Mot3 binds to its four sites in the *DAN1* promoter with different affinities (Cohen *et al*., 2001). Our future work will be aimed at elucidating the mechanism by which Mot3 represses expression of *ERG* genes during both hypoxic and aerobic growth.

Materials and methods

Yeast strains, genetic methods and media

The *S.cerevisiae* strains used in this study are listed in Table II. Strains designated FY are isogenic to S288C and are *GAL2*$^+$ (Winston *et al*., 1995). Lower case letters indicate a recessive mutant allele and upper case indicates the wild-type allele. Strain construction and other genetic manipulations were carried out by standard methods (Guthrie and Fink, 1991). The *mot3Δ2::HIS3* null allele, and the *erg2Δ::kanMX* and *erg6Δ::kanMX* null alleles have been described previously (Madison *et al*., 1998; Winzeler *et al*., 1999). The *MOT3-Myc18::TRP1* allele was constructed as described previously (Knop *et al*., 1999). All yeast media, including YPD, synthetic complete (SC), omission media (SC–), sporulation media and media containing 5-FOA were prepared as described previously (Rose *et al*., 1990). For scoring vacuolar phenotypes, YPD was buffered at pH 8.0 by adding NaOH and a phosphate buffer consisting of 157 mM K_2HPO_4 and 4 mM KH_2PO_4 (final concentrations). For testing nystatin resistance, nystatin (Sigma) was dissolved in *N*,*N*-dimethylformamide and added to YPD to 15 or 25 µg/ml final concentration (YPD-nystatin). YPD-nystatin plates were kept at room temperature and used within 48 h after preparation. Cadmium sensitivity was tested on YPD supplemented with 100 µM cadmium. For growth under hypoxic conditions, in which *S.cerevisiae* requires ergosterol in the growth medium (Parks *et al*., 1985, 1995), liquid YPD medium was supplemented with a 1% final concentration of a stock solution containing 5 ml of Tween-80, 5 ml of ethanol and 20 mg of ergosterol (Sigma) (YPD-ergosterol), and cells were grown in 50 ml tightly capped polypropylene conical tubes, in a shaker at 30°C, to a density of 1–2 × 10^7 cells/ml. The strong induction of *DAN1* expression (Figure 6) verifies that these conditions are hypoxic. Yeast transformants were selected on the appropriate SC media. All strains used for serial dilution plating were grown in the appropriate liquid media to a starting density of 1–2 × 10^7 cells/ml. Each row of spots represents a series of 10-fold dilutions in sterile water of the respective starting culture.

Bacterial strains and plasmids

Plasmids were constructed, amplified and isolated from *Escherichia coli* MH1 or DH5α, according to standard procedures (Sambrook *et al*., 1989). Plasmids were recovered from yeast as described previously (Robzyk and Kassir, 1992). The pRS series of vectors has been described previously (Sikorski and Heiter, 1989). Plasmid pCH2 was constructed by ligating a 4.3 kb *Bam*HI–*Xho*I fragment containing *MOT3* from pJM142 (Madison *et al*., 1998) into the *Bam*HI–*Xho*I sites of pRS416. Plasmid

Table II. *Saccharomyces cerevisiae* strains

Strain	Genotype
FY84	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 lys2-128δ*
FY85	*MATα ura3-52 his3Δ200 leu2Δ1 lys2-128δ*
FY1339	*MAT***a** *ura3Δ0 his3Δ200 trp1Δ63*
FY2066	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ1*
FY2067	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 lys2-128δ*
FY2068	*MATα ura3-52 his3Δ200 leu2Δ1 lys2-128δ*
FY2069	*MATα ura3-52 his3Δ200 lys2-128δ trp1Δ1 mot3Δ2::HIS3*
FY2070	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 lys2-173R2 mot3Δ2::HIS3*
FY2071	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 mot3Δ2::HIS3*
FY2072	*MATα ura3-52 his2Δ200 lys-128δ trp1Δ1 mot3Δ2::HIS3 pan1-151 <pCH2>*
FY2073	*MAT***a** *ura3-52 his3Δ200 lys2-1732 leu2Δ1 mot3Δ2::HIS3 vps41-298 <pCH2>*
FY2074	*MATα ura3-52 his3Δ200 leu2Δ1 lys2-173R2 pan1-151*
FY2075	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 lys2-128δ vps41-298*
FY2076	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ1 vma2Δ::kanMX*
FY2077	*MATα ura3-52 his3Δ200 leu2Δ1 lys2-128δ erg2Δ::kanMX*
FY2078	*MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ erg2Δ::kanMX mot3Δ2::HIS3*
FY2079	*MATα ura3Δ0 his3Δ200 leu2Δ1 lys2-128δ erg6Δ::kanMX*
FY2080	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 lys2-128δ met25Δ0 erg6Δ::kanMX mot3Δ2::HIS3*
FY2081	*MAT***a** *ura3Δ0 his3Δ200 trp1Δ63 MOT3-Myc18::TRP1*

pJM188 is a *MOT3 LEU2 CEN* plasmid (Madison *et al*., 1998). Plasmid pRL81 is the original *PAN1* plasmid isolated from a yeast genomic *URA3 CEN* library (Rose *et al*., 1987). Plasmid pHL6 is the original *VPS41* plasmid isolated from a yeast *LEU2 CEN* library (Spencer *et al*., 1988). Plasmid pAS249 is a *PAN1 URA3 CEN* plasmid (Sachs and Deardorff, 1992) (kindly provided by A.Sachs). Plasmid JK0024 is a *VPS41 URA3* 2μ plasmid (Radisky *et al*., 1997) (generously provided by J.Kaplan). Plasmid pCH7 is a 3.5 kb *Eco*RI fragment from pRL81 containing *PAN1* subcloned into the *Eco*RI site of pRS416. Plasmid pCH9 is the 3.5 kb *Eco*RI fragment from pCH7 containing *PAN1* subcloned into the *Eco*RI site of pRS305. Plasmid pCH11 is a 5.4 kb *Bam*HI–*Not*I fragment from JK0024 containing *VPS41* subcloned into the *Bam*HI–*Not*I site of pRS315. Plasmid pCH12 is a 5.4 kb *Bam*HI–*Not*I fragment from JK0024 containing *VPS41* subcloned into the *Bam*HI–*Not*I site of pRS305. Plasmid pJR2330 is an *ECM22 LEU2* 2μ plasmid (Vik and Rine, 2001) and plasmid pJR 2341 is a *UPC2 URA3* 2μ plasmid (both plasmids generously provided by B.Davies and J.Rine). Restriction enzymes were purchased from Boehringer Mannheim Biochemicals and used as recommended by the manufacturer.

Isolation of mot3Δ synthetic lethal mutations

Plasmid pCH2 (*MOT3 CEN URA3*) was used to transform two *mot3Δ* strains, FY2069 and FY2070 (Table II). The resulting strains were mutagenized with ethyl methanesulfonate by standard methods (Ausubel *et al*., 1988). Mutagenized cells were plated on YPD and incubated at 30°C. Approximately 42 000 colonies from FY2069 and 45 000 colonies from FY2070 were screened for the inability to grow without pCH2 by replica plating to 5-FOA plates. Those colonies that did not grow on 5-FOA but grew on SC-Ura and SC were chosen for further study. To demonstrate that the synthetic lethality was dependent on *mot3Δ*, plasmid shuffle experiments were conducted (Guthrie and Fink, 1991) using pJM188 (*MOT3 LEU2 CEN*) or the vector alone. Two mutants, FY2073 (from FY2069) and FY2072 (from FY2070), were able to lose pCH2 upon transformation with pJM188, but not when transformed with vector alone. To demonstrate that a single gene was responsible for the synthetic lethality with *mot3Δ*, FY2073 and FY2072 were crossed to FY2069 and FY2070, respectively. 5-FOA-sensitive double mutants were crossed to FY84 or FY85 to isolate the new mutations in a *MOT3*$^+$ background. These single mutations were verified to cause synthetic lethality with *mot3Δ* by crossing each candidate to FY2069 or FY2070 carrying pCH2 and reconstructing the synthetic lethality by the inability to lose pCH2 on 5-FOA plates.

Cloning of synthetic lethal genes

To clone the synthetic lethal genes, a yeast genomic plasmid library in a *URA3*-marked vector (Rose *et al*., 1987) or a *LEU2*-marked vector (Spencer *et al*., 1988) was used to transform the mutant strains FY2074 and FY2073, respectively. Approximately 43 000 Ura$^+$ transformants were screened by replica-plating for those that grew at 37°C and on YPD cadmium media. One transformant complemented both phenotypes. Approximately 30 000 Leu$^+$ transformants were screened by replica-plating for those that grew on SC-Leu + 5-FOA. One transformant complemented the 5-FOA-sensitive phenotype. Library plasmids were isolated from these candidates and their insert DNAs were analyzed by sequence analysis of the ends of the inserts, followed by identification of the cloned sequence by a computer database search. Complementation of the mutation in FY2074 was achieved by transforming FY2074 with either pAS249 or pCH7, identifying *PAN1* as the gene corresponding to the mutation, subsequently renamed *pan1-151*. Complementation of the mutation in FY2073 was achieved by transforming FY2075 with JK0024 or pCH11, identifying *VPS41* as the gene corresponding to this mutation, subsequently renamed *vps41-298*. Linkage analysis using the integrating plasmids pCH9 and pCH12 showed that *PAN1* and *VPS41* were linked to the corresponding synthetic lethal mutations and were not unlinked suppressors.

RNA preparation and northern hybridization analyses

Total RNA was isolated by a hot-phenol method (Ausubel *et al*., 1988) and 20 μg of RNA were used in each sample. Northern transfer and hybridization were performed as described previously (Swanson *et al*., 1991). ^{32}P-labeled probes were generated either with a Boehringer Mannheim Biochemical nick-translation kit or by random hexamer labeling (Ausubel *et al*., 1988). Northern results were quantitated using PhosphorImager screen and ImageQuant software (Molecular Dynamics).

Fluorescence microscopy

Yeast cells were grown in YPD or SC, supplemented with the appropriate amino acids for plasmid selection, to 1–2 × 10^7 cells/ml. Cells were harvested, resuspended in YPD and stained with FM4-64 (Molecular Probes), as described previously (Vida and Emr, 1995). Cells were viewed on a Zeiss AxiosKop2 microscope equipped with epifluorescence using a rhodamine filter, and images were obtained with a digital camera and IPlab software. The staining with FM4-64 is performed in YPD medium. Therefore, for strains carrying plasmids, the percentage of plasmid retention during the staining procedure was calculated by plating dilutions on YPD and SC lacking the appropriate amino acids after completion of the staining protocol.

Quantitative sterol extractions and sterol analysis

To determine the percentage of total cellular mass represented by sterol, quantitative sterol analysis was performed as described previously (Molzahn and Woods, 1972). To calculate the percentage of sterols per milligram of cell mass, 20 ml of the original culture, grown in SC medium, were harvested by vacuum filtration onto a pre-weighed 0.45 μm Millipore filter. The filters were pre-dried in a 105°C oven overnight and subsequently placed in a desiccator for 4 h before weighing. After vacuum fitration of harvested cells the filters were again placed into a 105°C oven overnight, followed by desiccation at room temperature for 4 h. The weight of the cells was determined immediately after opening the desiccator. The amount of individual sterols was calculated based on the area under each peak of the chromatograph relative to a known amount of ergosterol loaded onto the gas chromatograph using the Hewlett-Packard sterol quantitation program. Each sample was injected twice and the value reported for each sterol quantity was the average of two injections. Sterols were analyzed by gas chromatography using a Hewlett-Packard HP5890 series II chromatograph equipped with the Hewlett-Packard CHEMSTATION software package. The capillary column (DB-1) was 15 m × 0.25 mm × 0.25 μm (film thickness) (J&W Scientific, Folsom, CA) and was programmed from 195 to 280°C (1 min at 195°C and then an increase at 20°C/min to 240°C, followed by an increase at 2°C/min until the final temperature of 280°C was reached). The linear velocity was 30 cm/s, nitrogen was the carrier gas and all injections were run in the splitless mode.

Chromatin immunoprecipitation

For chromatin isolation, cells were grown in 50 ml of YPD-ergosterol medium in 50 ml tightly capped polypropylene conical tubes in a shaker at 30°C to a density of 1–2 × 10^7 cells/ml. All chromatin immunoprecipitations were performed as described previously (Dudley *et al*., 1999). Chromatin was sonicated to an average of 400 bp with a size range of 200–900 bp. A two-step immunoprecipitation was performed as described previously (Harlow and Lane, 1999). The rabbit polyclonal A14 anti-Myc antibody was used (Santa Cruz). Quantitative radioactive PCR was used to determine the percentage of *ERG2* and *ERG6* promoter DNA that co-immunoprecipitated with Mot3-Myc18, as described previously (Larschan and Winston, 2001). The linearity of all PCR reactions was assayed as described previously (Larschan and Winston, 2001). The relative enrichment for the specific *ERG2* and *ERG6* PCR products is reported after normalization to a control PCR product amplified within the same reaction. The control PCR product is a 150 bp region of chromosome V that is outside of any open reading frames (Komarnitsky *et al*., 2000). Quantitation was performed by PhosphorImager analysis (Molecular Dynamics). All calculations were carried out as described previously (Larschan and Winston, 2001). The primers used for the control PCR were described previously (Komarnitsky *et al*., 2000). All other primers used were created for this study and primer sequences are available upon request.

Acknowledgements

We thank Beverly Wendland, Dan Fraenkel, Brandon Davies and Jasper Rine for helpful discussions and advice. We also thank Martin Steffen, Aimée Dudley and George Church for help with microarray experiments. We are grateful to Beverly Wendland for providing FM4-64 and the staining protocol. We are also grateful to Alan Sachs, Jerry Kaplan, Brandon Davies and Jasper Rine for providing strains and plasmids. We thank Erica Larschan and Mary Bryk for critical reading of the manuscript. C.H. was supported by NIH Minority Predoctoral Fellowship 1F31GM19933-03. This work was supported by NIH grant GM45720 to F.W. and NIH grant GM62104 to M.B.

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Received May 9, 2002; revised and accepted June 14, 2002