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Additional Information

**Repression of ergosterol biosynthesis is essential for stress resistance and is mediated  
by the Hog1 MAP kinase and the Mot3 and Rox1 transcription factors**

Running title: Stress-mediated repression of ergosterol biosynthesis

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## **Abstract**

Hyperosmotic stress triggers a complex adaptive response which is dominantly regulated by the Hog1 MAP kinase in yeast. Here we characterize a novel physiological determinant of osmostress tolerance which involves the Hog1 dependent transcriptional down regulation of ergosterol biosynthesis genes (*ERG*). Yeast cells considerably lower their sterol content in response to high osmolarity. The transcriptional repressors Mot3 and Rox1 are essential for this response. Both factors together with Hog1 are required to rapidly and transiently shut down transcription of *ERG2* and *ERG11* upon osmoshock. Mot3 abundance and its binding to the *ERG2* promoter is stimulated by osmostress in a Hog1 dependent manner. As an additional layer of control, the expression of the main transcriptional activator of *ERG* gene expression, Ecm22, is negatively regulated by Hog1 and Mot3/Rox1 upon salt shock. Oxidative stress also triggers repression of *ERG2*, *11* transcription and a profound decrease in total sterol levels. However, this response was only partially dependent on Mot3/Rox1 and Hog1. Finally we show that the *upc2-1* mutation confers stress insensitive hyperaccumulation of ergosterol, overexpression of *ERG2*, *11* and severe sensitivity to salt and oxidative stress. Our results indicate that transcriptional control of ergosterol biosynthesis is an important physiological target of stress signaling.

## **Introduction**

Sterols are essential membrane compounds of eukaryotic cells. They are predominantly required for the function of the plasma membrane affecting its rigidity, fluidity and permeability. Sterol biosynthesis is a major metabolic commitment of the cell

and has been found to be tightly regulated at the expression level of the genes encoding the multiple biosynthetic steps (Espenshade & Hughes, 2007). Because of its medical interest, the regulation of cholesterol homeostasis has been heavily investigated in mammalian cells (Brown & Goldstein, 2009). Here, two transcriptional activators, the sterol response element binding proteins (SREBP), have been identified to up-regulate genes involved in cholesterol biosynthesis and -uptake in response to cholesterol deficiency (Goldstein *et al.*, 2006, Hua *et al.*, 1993, Wang *et al.*, 1994, Yokoyama *et al.*, 1993). The fungal equivalent of cholesterol is ergosterol. The ergosterol biosynthetic pathway shares many highly conserved steps with the cholesterol pathway, which demonstrates that budding yeast is an attractive model system to understand sterol homeostasis (Espenshade & Hughes, 2007, Henneberry & Sturley, 2005, Sturley, 2000). Additionally, the major antifungal therapies target ergosterol biosynthesis underlining the need to acquire a detailed knowledge about the mechanisms that control yeast ergosterol homeostasis (Carrillo-Munoz *et al.*, 2006).

The principle source of ergosterol in yeast is its internal biosynthesis as long as there is enough oxygen available, which serves as an essential cofactor of various enzymatic steps of the ergosterol pathway (Lorenz & Parks, 1991). Under strictly anaerobic conditions, ergosterol becomes essential for yeast cells and has to be taken up from the exterior in a process that is normally repressed in the presence of oxygen (Ishtar Snoek & Yde Steensma, 2007). In *Saccharomyces cerevisiae*, the transcriptional control of ergosterol biosynthesis genes (*ERG*) has been intensively studied by treatments that impair the efficient synthesis of ergosterol and thereby cause sterol depletion. This can be achieved by drug inhibition of particular biosynthetic enzymes or by lowering the concentration of oxygen. Two structurally related transcription factors, Ecm22 and Upc2, have been identified as the main activators of genes encoding enzymes for the late steps of ergosterol

biosynthesis such as *ERG2* and *ERG3* (Vik & Rine, 2001). Both factors bind to the so called Sterol Regulatory Element (SRE) which is present in many *ERG* gene promoters. Ecm22 is the main transcriptional activator of *ERG2* in normal growth conditions, whereas it is taken over by Upc2 upon sterol depletion (Davies *et al.*, 2005). Single point mutations have been reported for both transcription factors, namely *upc2-1* and *ecm22-1*, which increase their transcriptional activation capacity under normal conditions and, in the case of *upc2-1*, allow the uptake of external sterols even in the presence of oxygen (Crowley *et al.*, 1998, Davies *et al.*, 2005). Hap1 is an additional transcriptional repressor/activator which binds directly to *ERG* gene promoters conferring regulation dependent on the oxygen levels (Davies & Rine, 2006, Hickman & Winston, 2007). Finally, Mot3 has been identified as a direct transcriptional repressor of several *ERG* genes contributing to the control of the ergosterol pathway upon hypoxia (Hongay *et al.*, 2002) (Figure 9).

Despite the characterization of the above mentioned transcription factors which seem to adjust the expression of the *ERG* genes to sterol depletion, we lack knowledge as to the nature of signal transduction pathways that would target ergosterol homeostasis and to environmental conditions other than sterol depletion that cause differential expression of the *ERG* pathway. In the present paper we show that adaptation to hyperosmotic and oxidative stress involves the repression of *ERG* genes and the decrease in cellular ergosterol levels in a process which is controlled by the Mot3 and Rox1 transcription factors and the Hog1 MAP kinase. Hog1 is the terminal signaling kinase of the High Osmolarity Glycerol (HOG) MAP kinase pathway, which allows yeast cells to efficiently adapt to increasing osmolarity in their environment (de Nadal *et al.*, 2002, Hohmann, 2002, Hohmann *et al.*, 2007). Once activated by stress, Hog1 orchestrates a complex array of different physiological responses, which altogether guarantee survival and growth under

hyperosmolarity. Upon osmoshock, Hog1 directly targets plasma membrane Na<sup>+</sup> and K<sup>+</sup> transporters to lower the intracellular ion concentrations (Proft & Struhl, 2004), regulates cell cycle progression by the phosphorylation of Sic1 and Hsl1 (Clotet *et al.*, 2006, Escote *et al.*, 2004), modulates translation efficiency through the protein kinase Rck2 (Bilsland-Marchesan *et al.*, 2000, Teige *et al.*, 2001), and is responsible for the vast majority of transcriptional regulation in the nucleus (Posas *et al.*, 2000, Rep *et al.*, 2000). Osmotic stress causes the massive transcriptional activation of genes involved in the stress defense, like the production of the osmolyte glycerol (Albertyn *et al.*, 1994), ion homeostasis (Marquez & Serrano, 1996), redox metabolism (Schuller *et al.*, 1994) and mitochondrial function (Pastor *et al.*, 2009). Genomic profiling experiments also identify a subset of yeast genes which is repressed in response to osmotic shock and which are involved in translation, ribosome biogenesis and amino acid biosynthesis (Gasch *et al.*, 2000). Hog1 activates transcription by targeting different specific transcription factors including Sko1 (Proft *et al.*, 2001), Hot1 (Rep *et al.*, 1999) and Smp1 (de Nadal *et al.*, 2003), by indirect or direct recruitment of chromatin modifying and remodeling complexes (De Nadal *et al.*, 2004, Mas *et al.*, 2009, Proft & Struhl, 2002, Zapater *et al.*, 2007), and by association with the RNA polymerase II complex during elongation (Proft *et al.*, 2006). However, how Hog1 represses transcription in response to stress, is very poorly understood. Here, we demonstrate that the *ERG* pathway is targeted by HOG through the Mot3 and Rox1 transcription factors to decrease the cellular ergosterol content and that this negative regulation is physiologically important for stress resistance.

## Results

### *Downregulation of ergosterol levels is physiologically relevant for osmostress adaptation in yeast*

The sterol content is critical for the fluidity and permeability of biological membranes. We asked whether adaptation of yeast cells to hyperosmotic and salt stress involved the modulation of the ergosterol content. Therefore we measured the total ergosterol concentration of a wild type strain before and after adaptation to increasing concentrations of NaCl or sorbitol. As shown in figure 1A, yeast cells reduce significantly their total ergosterol content when faced with  $>0.7\text{M}$  NaCl or  $>1\text{M}$  sorbitol. We next employed specific inhibitors of the ergosterol biosynthesis pathway like fluconazole or ketoconazole to artificially lower the sterol content of the cells. We found that brief treatments (2 to 4 hours) with moderate drug concentrations decreased the total sterol content to about the half of the initial untreated levels (Figure 1B, data shown for fluconazole). Importantly, we found that this treatment did not affect the growth rate of the yeast cells. We then tested, whether artificially lowering the sterol levels by drug pretreatment of the cells affected the growth in high salt media. As depicted in figure 1C, fluconazole treated wild type cells recovered earlier from a NaCl shock indicating that inhibition of ergosterol synthesis conferred a growth advantage under high salinity. The beneficial effect of fluconazole treatment for NaCl tolerance was also detected in the salt sensitive *hog1* deletion strain lacking the activity of the Hog1 MAP kinase, which is responsible for the adaptation to hyperosmotic stress (Figure 1C). Taken together, we conclude that modulation of the sterol content is an important determinant for salt stress adaptation in yeast cells.

*Mot3 and Rox1 are important for growth and modulation of ergosterol levels upon salt stress*

The observed changes in the sterol levels upon stress suggested that signaling pathways exist in yeast to down regulate ergosterol biosynthesis in response to salt stress. In our previous work we identified the *MOT3* and *ROX1* gene promoters as direct targets for the transcription factor Sko1, one of the key transcription factors in the response to osmostress in yeast (Proft *et al.*, 2005). Moreover, Mot3 is a direct repressor of some *ERG* genes encoding ergosterol biosynthesis enzymes (Davies & Rine, 2006, Hongay *et al.*, 2002) and Rox1 has been described as a synergistic repressor with Mot3 (Sertil *et al.*, 2003). We therefore investigated a possible function of Mot3 and Rox1 in the adaptation to hyperosmotic stress and tested whether both factors were necessary for efficient growth on high salt medium. We compared the growth kinetics of wild type, the *mot3* and *rox1* single mutants, and the *mot3rox1* double mutant in the presence of 1M NaCl. Of the single mutants *rox1* or *mot3*, only the loss of Mot3 caused a mild growth delay on high salt (data not shown). However, the double deletion *mot3rox1* caused a significant reduction in the growth yield under salt stress (Figure 2A). We next expanded the phenotypic analysis of the *mot3rox1* mutant to other toxic cations like tetramethylammonium (TMA), norspermidine and hygromycin b. We found a hypersensitivity of *mot3rox1* mutant cells to TMA, norspermidine and hygromycin b as compared to wild type cells (figure 2A and data not shown). We conclude that Mot3 and Rox1 contribute to the resistance to a variety of toxic cations.

We then addressed the question whether Mot3 and Rox1 were necessary for the observed changes in ergosterol levels upon salt stress. Therefore we compared the ergosterol content of wild type and the respective *mot3* and *rox1* mutant strains before and



after the adaptation to high salt. As depicted in figure 2B, all mutants affecting Mot3 and/or Rox1 function failed to efficiently repress ergosterol levels in response to salt stress. We next tested whether the increased salt sensitivity of *mot3* and *rox1* mutants was directly attributable to a major accumulation of Na<sup>+</sup> ions upon exposure to NaCl stress. Therefore we quantified the steady state intracellular Na<sup>+</sup> concentration of wild type and *mot3* and *rox1* deletion strains in the presence or absence of 1M NaCl. As shown in figure 2C, yeast wild type cells accumulate approximately 50mM Na<sup>+</sup> under these salt stress conditions, whereas *mot3*, *rox1* or the *mot3rox1* mutant strains showed a more than 2 fold increased intracellular Na<sup>+</sup> concentration. These results suggested that loss of repression of the ergosterol biosynthetic pathway upon stress in the *mot3* or *rox1* mutants leads to a hyperaccumulation of toxic Na<sup>+</sup> ions upon salt stress and subsequent stress sensitivity. Finally we confirmed that fluconazole treatment alleviated the stress sensitivity of *mot3rox1* mutants (shown for growth with hygromycin b in Figure 2D).

*Osmostress dependent repression of ERG gene expression depends on the Hog1 MAP kinase and the Mot3, Rox1 transcription factors*

Having found that both transcription factors Mot3 and Rox1 were necessary to lower ergosterol levels in response to salt challenge, we measured the expression levels of individual *ERG* genes along the osmotic shock by Northern analysis. In order to get a comprehensive view of the *ERG* gene expression under such stress conditions, we individually quantified the transcript levels of seven different ergosterol biosynthesis enzymes involved in the last, ergosterol specific, conversions from lanosterol to ergosterol. We found that the expression of the *ERG* genes tested did not uniformly respond to osmotic stress. As shown in figure 3A, *ERG11*, *ERG2* and *ERG3* were strongly downregulated

during the first minutes of NaCl stress, while *ERG6* and *ERG9* lacked such regulation. Specifically, *ERG2* and *ERG11* expression was repressed 3 to 6 fold in wild type cells within the first 20 min of exposure to mild salt stress. We next tested whether Mot3 and Rox1 were implicated in the fast repression of both *ERG* genes. Deletion of Mot3 caused a substantial loss of *ERG2* and *ERG11* repression upon osmostress. The lack of Rox1 function led to an increase of *ERG2* and *ERG11* basal expression (approximately 1.5 fold), however, it only affected the regulated repression in the case of *ERG11* (Figure 3B). A double mutant *mot3rox1* showed a loss of *ERG2* and *ERG11* down regulation which was similar to the *mot3* mutant. As a control we measured the expression of the salt inducible *GRE2* locus in the respective mutant strains. As expected, we found that *GRE2* was normally up regulated in the *mot3* and *rox1* mutants. Since the Hog1 MAP kinase is the master transcriptional regulator upon hyperosmotic stress, we next tested the function of the kinase in the observed *ERG* gene repression. As shown in Figure 3B, a *hog1* mutant strain completely failed to repress *ERG2* and *ERG11* transcription upon osmoshock. We next addressed the question of whether the downregulation of *ERG* gene expression was a transient adaptation to acute osmostress or a long term adaptation. Therefore we repeated the Northern analysis comparing unstressed with salt stress adapted cells. As shown in Figure 3C, the massive reduction of *ERG2* and *ERG11* mRNAs observed within the first minutes of the osmotic insult was no longer detected in cells that had adapted to the hyperosmotic environment. In the case of *ERG11* we detected a slight increase in expression levels in salt adapted wild type cells. Taken together, our results indicate that hyperosmotic stress triggers the rapid and transient transcriptional repression of some *ERG* genes through the Mot3 and Rox1 transcription factors and that this regulation is entirely dependent on Hog1.

We next wanted to quantify on a global scale the impact of the loss of Mot3 and Rox1 function on the expression of *ERG* genes. Therefore we determined the gene expression profiles of wild type and *mot3rox1* mutants upon osmotic stress by microarray hybridization. For 7 out of the 14 *ERG* genes encoding the ergosterol specific biosynthetic enzymes we detected a modest (1.2 to 1.6 fold) but highly significant ( $p=0.002$ ) overexpression in the *mot3rox1* mutant (Table 2A). Among those genes we identified *ERG11*, 2 and 25, which are repressed upon NaCl shock (Figure 3A, B).

*Hog1 controls RNA PolIII recruitment both positively and negatively in response to osmostress.*

The Hog1 MAPK is responsible for the vast majority of transcriptional activation upon osmoshock. Here, we have described a negative regulation of gene expression which depends on stress and Hog1. Therefore we asked whether Hog1 could both stimulate and repress transcription depending on the specific stress-responsive promoter by controlling the density of RNA polymerase II (PolII). We measured PolII recruitment by chromatin immunoprecipitation (ChIP) in vivo using an HA-tagged Rpb3 subunit at two osmostress inducible loci, *GRE2* and *STL1*, and the osmostress repressed *ERG2* and *ERG11* genes. As expected, we found that PolII density was highly induced within the first minutes of osmoshock dependent on the function of Hog1 (Figure 4A). In contrast, PolII was rapidly lost from the *ERG2* and *ERG11* core promoters upon stress and this was dependent on the Hog1 MAPK. Since it has been found that Hog1 physically associates with the promoter regions of stress-activated genes, we asked whether the kinase would also interact with the repressed *ERG* promoters. Therefore we performed ChIP of HA-tagged Hog1 comparing the inducible loci *CTT1* and *GRE2* with the repressed loci *ERG2* and *ERG11*. As shown in

figure 4B, we found that Hog1 recruitment was transiently stimulated by osmostress at *CTT1* and *GRE2*, but no Hog1 occupancy could be detected in the case of the *ERG* genes. We conclude that stress-activated Hog1 stimulates transcription at inducible genes where the kinase itself gets stably recruited, whereas at the *ERG* loci Hog1 represses transcription without this stable recruitment.

*Adaptation of ergosterol biosynthesis upon salt stress implies the transcriptional down regulation of ECM22*

The expression of ergosterol biosynthesis genes is activated by the transcription factors Ecm22 and Upc2, with Ecm22 being the main transcriptional activator of *ERG* genes under favorable growth conditions. Since we found that adaptation to osmostress involves the transcriptional repression of *ERG2* and *ERG11*, we addressed the question whether Ecm22 was a target of osmostress signaling. We therefore quantified the expression of *ECM22* by Northern analysis along a brief osmoshock. As depicted in figure 5, *ECM22* transcription was rapidly repressed in wild type cells upon osmoshock. The observed repression was not significantly altered in either single mutant *mot3* or *rox1*. However, downregulation of *ECM22* upon stress was absent in either the double mutant *mot3rox1* or the *hog1* mutant (Figure 5). We conclude that expression of *ECM22* is shut down in response to salt stress via the Hog1 kinase and the Mot3 and Rox1 transcription factors. These results were also confirmed by our transcriptomic analysis showing that *ECM22* is more than 2 fold overexpressed in *mot3rox1* upon osmostress (Table 2B). Additionally, the array experiment identifies the second transcriptional activator of the *ERG* genes, *UPC2*, as overexpressed in the *mot3rox1* mutant.

### *Stress-regulated occupancy of the Mot3 repressor at ERG2*

We next addressed the question whether the stress-regulated repression of *ERG* genes was the effect of direct binding of Mot3. We therefore used ChIP to determine the occupancy of Mot3 at the stress responsive *ERG2* promoter and the constitutive *ERG6* promoter in response to osmotic shock. As shown in figure 6A, we found that Mot3 binding was enhanced by salt shock at *ERG2*, but not at the *ERG6* promoter. In the absence of Hog1, the stress activated binding of Mot3 to *ERG2* is significantly diminished indicating that the MAP kinase down regulates *ERG2* expression by stimulating the binding of Mot3 to its promoter. We additionally quantified the Mot3 and Rox1 protein levels in wild type and *hog1* mutant cells during adaptation to salt stress. We found that Mot3 protein abundance transiently increased during the first 40 min of NaCl stress (Figure 6B). This increase was absent in a *hog1* mutant. Unexpectedly, Rox1 levels decreased under the same conditions in a similar way in wild type and *hog1* mutants. These data suggest that the repression of *ERG* genes upon osmotic stress involves the Hog1 dependent up-regulation of Mot3 protein abundance and stimulated promoter binding of Mot3.

### *Oxidative stress causes ERG gene repression largely independent on Mot3, Rox1 and Hog1*

We next investigated whether other stimuli than hyperosmotic stress would cause a transcriptional adaptation of ergosterol biosynthesis. We therefore investigated the impact of oxidative stress on the total ergosterol levels and on *ERG* gene expression. As shown in figure 7A, yeast wild type cells profoundly reduce their ergosterol content when they adapt to oxidative stress caused by menadione. Treatment with 75 $\mu$ M menadione caused an ergosterol reduction by a factor of 2.8. This decrease is comparable to the one observed by severe NaCl stress (Figure 1A). In the absence of both repressors Mot3 and Rox1, a

reduction in the ergosterol levels by a factor of 1.8 was still observed, however, the ergosterol content of menadione adapted *mot3rox1* mutant cells was significantly higher as compared to wild type. Mutation of the Hog1 MAPK caused an ergosterol content which was largely unresponsive to menadione (Figure 7A). We then quantified the effect of menadione treatment on the expression of the *ERG2* and *ERG11* genes. As illustrated in figure 7B, the expression of both *ERG* genes was rapidly repressed in the first 20 min of exposure to menadione. In the double mutant *mot3rox1*, as well as in the *hog1* mutant, *ERG2* and *ERG11* repression by oxidative stress was only weakly diminished. The expression of the menadione inducible *CCPI* gene was normally regulated in all mutant strains. We finally tested whether the observed alterations in ergosterol biosynthesis caused sensitivity to oxidative stress. As shown in figure 7C, the *mot3rox1* mutant strain showed a delayed growth in the presence of menadione as compared to wild type. This sensitivity was similar to the one measured for the *hog1* mutant strain (Figure 7C). Taken together, we show that menadione treatment causes a strong reduction in ergosterol levels and *ERG2, 11* gene expression, which is only partially dependent on Hog1 and the Mot3 and Rox1 transcription factors.

*The upc2-1 mutation causes unregulated ergosterol levels and ERG gene expression leading to severe osmotic and oxidative stress sensitivity*

We have seen that a regulated repression of ergosterol biosynthesis is required for a proper adaptation to salt and oxidative stress. As described above, downregulation of *ERG* gene expression can be interrupted by either eliminating the transcriptional repressors Mot3 and Rox1 or the Hog1 MAPK. We next wanted to investigate whether overactivation of ergosterol biosynthesis would interfere with the observed stress-regulated adaptation. We

therefore took advantage of a previously identified mutant allele in the transcriptional activator Upc2. This *upc2-1* point mutation renders the Upc2 transcription factor constitutively active and uncouples ergosterol uptake from its normal repression by oxygen. We first measured the ergosterol levels in the *upc2-1* mutant under normal and oxidative stress conditions and compared it to the wild type. As shown in figure 8A, we found that *upc2-1* cells accumulate much higher levels of ergosterol in normal conditions and, unlike the wild type, completely fail to lower their ergosterol content in response to menadione treatment. We then compared the sensitivities to menadione of *upc2-1* with wild type cells and found that the growth of *upc2-1* was strongly inhibited in the presence of menadione (figure 8B). A dramatic sensitivity was also detected in the case of salt stress, where moderate concentrations of Na<sup>+</sup> or K<sup>+</sup> proved to be much more toxic to *upc2-1* cells as compared to wild type (figure 8C). Accordingly, we also found severe sensitivities of the *upc2-1* mutant to other toxic cations like TMA, norspermine or hygromycin b (data not shown). Additionally, we quantified *ERG2* and *ERG11* expression in the *upc2-1* mutant and found that the mutant overexpressed both genes constitutively and independently on salt stress (figure 8D). Also, the hypersensitivity of *upc2-1* to salt stress was reverted by the treatment with ergosterol biosynthesis inhibitors, as shown in figure 8E for fluconazole and growth in high K<sup>+</sup>. Finally we took advantage of the ability of *upc2-1* cells to take up ergosterol aerobically and tested the effect of external sterol addition on stress tolerance. As shown in figure 8F, the salt (NaCl) and oxidative stress (menadione) sensitivity of *upc2-1* was aggravated in the presence of ergosterol. Taken together, we demonstrate that the *upc2-1* mutation confers constitutive and stress-independent overexpression of *ERG* genes, which results in hyperaccumulation of ergosterol and severe salt and oxidative stress

susceptibility, which is corrected by drug inhibition of ergosterol biosynthesis and increased by the addition of ergosterol.

## **Discussion**

Here we report the identification of a physiologically important stress adaptation that involves the regulated repression of a metabolic pathway in yeast. The biosynthesis of ergosterol is down regulated upon stress by targeting the expression of individual *ERG* genes, which encode specific enzymes of the ergosterol synthesis pathway. In the case of hyperosmotic stress, the modulation of ergosterol homeostasis dominantly involves the signaling MAP kinase Hog1 and the two transcriptional repressors Mot3 and Rox1 (Figure 9).

The negative transcriptional control of the *ERG* pathway is important for the correct adaptation to salt stress. This is shown here by two genetic manipulations, which impair the correct repression of *ERG* genes either by elimination of the transcriptional repressors in the *mot3rox1* mutant or by constitutive stimulation of activation in the *upc2-1* mutant. Both mutants show stress unresponsive overexpression of the *ERG2* and *ERG11* genes (Figures 3 and 8), hyperaccumulation of cellular ergosterol upon stress (Figures 2 and 8) and hypersensitivity to salt stress (Figures 2 and 8). Furthermore, inhibition of the ergosterol biosynthesis pathway by azole drugs like fluconazole or ketoconazole is generally beneficial for the tolerance to salt stress (Figures 1, 2, 8). It is important to note that both drugs cause ergosterol depletion by the specific inhibition of the Erg11 enzyme, which is shown here to be targeted at its expression level upon salt and oxidative stress. On the other



hand, an excess of sterols, as simulated here by the addition of ergosterol to *upc2-1* mutant cells, is detrimental for salt and oxidative stress adaptation. These results clearly identify the ergosterol content as a critical physiological parameter for stress tolerance. We postulate that artificial sterol depletion in mutant strains which otherwise are not able to adjust their ergosterol content, is beneficial for their resistance to stress.

The supply of ergosterol under normal growth conditions is essential and yeast employs two related transcriptional activators, Ecm22 and Upc2, to continuously activate transcription of the *ERG* genes (Vik & Rine, 2001). The stress situations reported here require the downregulation of *ERG* genes and therefore have to somehow interfere with the activated transcription maintained mainly by Ecm22. We found that *ECM22* expression is repressed by Mot3/Rox1 and Hog1 rapidly after salt stress (Figure 6). Therefore one mechanism to down regulate *ERG* gene expression seems to include the transcriptional repression of its main activator Ecm22. Another layer of control is the stimulated binding of Mot3 to *ERG* gene promoters, here experimentally addressed for the *ERG2* locus. The Mot3 occupancy at *ERG2* is stimulated by salt stress dependent on the Hog1 kinase. Additionally, Mot3 protein levels increase transiently during salt stress dependent on the HOG pathway. These data are in agreement with previous reports showing that *MOT3* expression is induced upon osmostress through Hog1 and the Sko1 transcription factor (Proft *et al.*, 2005). Although increased expression of *MOT3* and enhanced association of the repressor with target promoters would explain how the HOG pathway triggers gene repression upon salt stress, we realize that binding of Mot3 to *ERG2* is even more rapid than the increase in Mot3 protein levels. Thus, association of Mot3 with target promoters might not simply be the result of higher protein concentrations and, additionally, Mot3 could bind to different target genes upon stress with different kinetics. Taken together, it

seems that lowering the *ERG* gene expression is achieved by both impairing the Ecm22 activator function at the transcriptional level and by stimulating the presence of the Mot3 repressor at *ERG* gene promoters. The Mot3 function is in line with the observation that it directly represses *ERG2* and *ERG6* upon hypoxia (Hongay *et al.*, 2002). We currently do not know whether Rox1 also represses *ERG* genes directly bound to their promoters, since we were not able to detect stress-stimulated binding of Rox1 to *ERG2* or *ERG11* by ChIP. We show that the role of Rox1 differs from Mot3 in the response to osmotic stress. Unlike Mot3, Rox1 protein levels decrease after salt shock and, with the exception of *ECM22* expression, loss of Rox1 has less impact on gene repression as compared to Mot3. Furthermore, currently unidentified repressors act on the *ERG* genes upon oxidative stress. This is suggested by the Mot3/Rox1 independent regulation of *ERG* gene expression upon oxidative stress.

It is important to note that different *ERG* genes are not uniformly regulated upon salt stress (Figure 3). Genes such as *ERG2* or *ERG11* seem to be specifically targeted under those conditions. Transcription profiling experiments generally support our findings and identify several *ERG* genes including *ERG2*, *3*, *11* to be >2 fold downregulated upon NaCl stress (Krantz *et al.*, 2004, Ni *et al.*, 2009, Rep *et al.*, 2000). However, additional complexity is indicated by the fact that stress-adapted cells show different *ERG* gene expression patterns as compared to acute stress (Figure 3C) and it has been reported that osmotic stress, when applied under anaerobic conditions, can induce a subset of *ERG* genes (Krantz *et al.*, 2004). Taken together, the different extent of stress-regulated repression for different *ERG* genes might indicate that it is not just the shutdown of ergosterol biosynthesis, but the modulation of specific sterol species, which is important for stress adaptation. A detailed study of the sterol composition and its changes upon osmotic stress

might therefore reveal important insights into the sterol homeostasis and its stress protective effects.

It is worth noting that although Hog1 mediated transcriptional control of *ERG* genes accounts for most, if not all, of the downregulation of sterol levels upon osmotic stress, this is not the case for oxidative stress. *ERG2* and *ERG11* expression is still significantly regulated in *hog1* or *mot3rox1* mutants upon oxidative stress, which could be explained by the contribution to *ERG* gene repression by specific transcription factors others than Mot3 and Rox1 upon oxidative stress. Additionally, non-transcriptional mechanisms might operate under such conditions. Indeed, studies in fission yeast revealed that Erg9 is targeted for inhibition at the post-translational level by oxidative stress (Tafforeau *et al.*, 2006).

What is the physiological meaning of the regulation of ergosterol homeostasis in response to saline and oxidative stress? Here we show that lowering the ergosterol content is a physiologically relevant response to salt and oxidative stress. The *ERG* pathway and its importance for stress resistance has been traditionally investigated by the use of null mutants in genes of the last non-essential biosynthetic steps of the sterol. These mutants are viable because although they are unable to synthesize ergosterol, they accumulate different precursors of the *ERG* pathway. The majority of these mutants show pleiotropic sensitivities to different drugs, Na<sup>+</sup>, Li<sup>+</sup>, or hydrogen peroxide (Abe & Hiraki, 2009, Branco *et al.*, 2004, Emter *et al.*, 2002, Mukhopadhyay *et al.*, 2002, Welihinda *et al.*, 1994). However, the studies using *erg* mutants cannot reveal the mechanisms of ergosterol homeostasis upon naturally occurring stimuli. Here we show that regulatory mutants *mot3* and *rox1* with a defect in *ERG* gene repression hyperaccumulate Na<sup>+</sup> in the cell interior upon salt stress. This could be due to an increased uptake of Na<sup>+</sup> and/or a decreased Na<sup>+</sup> extrusion in a plasma membrane environment with elevated levels of ergosterol. In this

scenario, the most important transport systems are the Pma1 plasma membrane H<sup>+</sup>-ATPase and the Trk K<sup>+</sup> uptake system as the generator and consumer of the membrane potential, respectively, and the Ena1 Na<sup>+</sup>-ATPase as the main Na<sup>+</sup> extrusion system (Rodriguez-Navarro, 2000, Serrano & Rodriguez-Navarro, 2001). It will be very important in the future to define the impact of the ergosterol content on these ion transport activities. In this regard, the *mot3rox1* mutant should be an especially useful tool as it disconnects ergosterol biosynthesis from its natural stress regulation and does not interrupt the *ERG* system.

## **Experimental Procedures**

### *Yeast strains*

All strains used in this study are listed in table 1.

### *Total ergosterol quantification*

Ergosterol levels were determined photometrically at 281.5 nm using the method described in (Arthington-Skaggs *et al.*, 1999, Breivik & Owades, 1957). Cells were grown over night in the indicated growth medium to OD<sub>600</sub>=0.8. For each quantification three or more independent culture aliquots were processed, the ergosterol content measured in duplicate and normalized for the number of cells.

### *Chromatin Immunoprecipitation*

ChIP was performed as described previously (Kuras & Struhl, 1999). Quantitative PCR analyses at the indicated chromosomal loci were performed in real time using an Applied Biosystems 7500 sequence detector with the *POL1* (+1796/+1996) coding sequence as an internal control. Each immunoprecipitation was performed twice with different chromatin

samples. All occupancy data are presented as fold IP efficiency over the *POLI* control sequence. All primer sequences are available upon request.

#### *Continuous growth assays*

For sensitivity assays in continuous growth fresh overnight precultures of the indicated strains were diluted in triplicate in multiwell plates to the same initial OD. Growth was then constantly monitored in a Bioscreen C system (Thermo) for the indicated times. In the cases of fluconazole or ketoconazole treatment, the overnight cultures were diluted into fresh YPD medium alone or YPD supplemented with 2 $\mu$ M ketoconazole or 20 $\mu$ M fluconazole. The drug treatment was performed for 2 hours. The cultures were then diluted to the same starting OD in the indicated medium and growth was continuously monitored. For the external feeding experiments, yeast wild type and *upc2-1* mutants were diluted to the same OD in SD medium alone or SD medium which contained 50 $\mu$ M menadione or 0.8M NaCl. Ergosterol was supplemented to a final concentration of 10 $\mu$ g/ml from a stock solution in chloroform.

#### *Northern blot*

Northern analysis was carried out as described previously (Proft et al., 2001). Approximately 30 micrograms of hot phenol-extracted total RNA was resolved on agarose-formaldehyde gels and blotted on nylon membranes. RNA was probed with gene specific <sup>32</sup>P-labeled DNA fragments. Signals were quantified using a Fujifilm BAS-1500 phosphoimager.

#### *Microarray analysis*

For the transcriptomic comparison of yeast wild type and *mot3rox1* mutants the cells were grown in YPD medium until mid-log phase and then subjected to a brief osmotic shock

(0.4M NaCl, 20 min). Total RNA was prepared from three independent culture aliquots for each strain using the RNeasy kit (Qiagen). Biotin labeling of the samples, hybridization to Affymetrix GeneChip® Yeast Genome 2.0 arrays and data analysis was performed at the VIB Microarray Facility (Leuven, Belgium; [www.microarrays.be](http://www.microarrays.be)).

#### *Intracellular ion measurements*

Extracts for the determination of intracellular Na<sup>+</sup> and K<sup>+</sup> concentrations were essentially prepared as described in (Mulet *et al.*, 1999). Briefly, five independent culture aliquots were washed in ice-cold washing solution (20mM MgCl<sub>2</sub> and isoosmotic sorbitol addition). The cells were finally resuspended in 0.5ml of 20mM MgCl<sub>2</sub>, the number of cells was determined and ions were extracted by heating the cells for 15min to 95°C. After centrifugation, aliquots of the supernatant were appropriately diluted and analyzed in an ICP optical emission spectrometer against Na<sup>+</sup> and K<sup>+</sup> standards.

#### *Immunodetection*

Mot3 and Rox1 protein levels were assayed by the use of strains expressing Tap-tagged versions of both proteins from their chromosomal loci. Protein extracts were prepared by boiling equal cell number aliquots in Laemmli SDS-PAGE loading buffer for 5 min. Proteins were transferred to PVDF membranes, stained with DB71 to visualize total protein and Tap-tagged proteins were detected using anti-PAP antibody (Sigma) and ECL-Plus (Amersham Biosciences).

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## Figure legends

Fig. 1. The modulation of ergosterol content and its effect on salt stress tolerance.

A. Yeast cells profoundly reduce their ergosterol content in response to salinity and hyperosmotic stress. Yeast wild type cells (BY4741) were grown to exponential growth phase ( $OD < 0.8$ ) in YPD containing or not the indicated concentrations of NaCl or Sorbitol. Total ergosterol levels were determined in duplicate from three independent cultures. The ergosterol concentration in normal growth medium was arbitrarily set to 1.

B. The effect of fluconazole treatment on the total ergosterol levels. Yeast wild type cells (BY4741) were either mock treated for the indicated times in YPD alone (control) or YPD + 20 $\mu$ M fluconazole. The cellular ergosterol content was determined as in A.

C. The effect of fluconazole treatment on salt stress adaptation. Yeast wild type (BY4741) or *hog1* mutants were either pretreated for 2h in YPD alone or YPD + 20 $\mu$ M fluconazole immediately before the growth assay. The growth curves are derived from the mean values of three independent cultures for each time point. The variance between triplicates is <10%.

Fig. 2. Mot3 and Rox1 are important for the modulation of ergosterol levels and growth upon stress.

A. The *mot3rox1* mutant is hypersensitive to diverse toxic cations. The growth of the wild type (BY4741) and the *mot3rox1* mutant was continuously monitored in YPD medium containing or not 1M NaCl, 0.7M TMA or 40 $\mu$ g/ml hygromycin b as indicated. The growth curves are derived from the mean values of three independent cultures for each time point. The variance between triplicates is <10%.

B. Mot3 and Rox1 are necessary for the down regulation of the ergosterol content upon salt stress. Yeast wild type cells (BY4741) and the indicated mutant strains were grown to exponential growth phase ( $OD < 0.8$ ) in YPD containing or not 1M NaCl. Total ergosterol levels were determined in duplicate from three independent cultures. The ergosterol concentration of the wild type in normal growth medium was arbitrarily set to 1.

C. *mot3* and *rox1* mutants hyperaccumulate  $Na^+$  upon salt stress. The indicated yeast wild type and mutant strains were continuously grown in YPD or YPD containing 1M NaCl. Intracellular  $Na^+$  concentrations were quantified as described in Materials and Methods for five independent cultures. The mean values are depicted with the standard deviation.

D. The growth of wild type and the *mot3rox1* mutant was monitored in YPD medium containing 40 $\mu$ g/ml hygromycin b. Cells were either pretreated for 2h in YPD alone or YPD + 20 $\mu$ M fluconazole immediately before the growth assay. The growth curves are derived from the mean values of three independent cultures for each time point. The variance between triplicates is <10%.

Fig. 3. Transcriptional control of the *ERG* pathway in response to osmotic stress.

A. Different *ERG* genes of the ergosterol specific conversions from lanosterol to ergosterol respond differently to osmotic stress. A yeast wild type strain was grown in YPD alone or subjected to osmotic stress (0.4M NaCl) for 20 min. The expression of the indicated *ERG* genes was quantified by Northern analysis and normalized to the *ACT1* gene. The fold repression of the transcripts was calculated as the ratio between the mRNA normalized for the *ACT1* messenger at time point 0 and at time point 20min of osmoshock.

B. *ERG2* and *ERG11* expression is rapidly shut off upon osmotic stress dependent of Mot3, Rox1 and Hog1. The indicated wild type and mutant strains were grown in YPD and then

submitted to a brief osmotic shock (0.4M NaCl) for the indicated times. The expression of *ERG2*, *ERG11*, *ERG6*, *GRE2* and *ACT1* (control) was quantified by Northern analysis. The fold repression of the *ERG2*, *ERG11* and *ERG6* transcripts depicted in the graphs was calculated as the ratio between the mRNA normalized for the *ACT1* messenger at time point 0 and at time point 20min of osmoshock. The experiment was repeated twice with similar results.

C. *ERG2* and *ERG11* expression is only minimally affected in salt adapted cells. The indicated yeast wild type and mutant strains were grown to exponential growth phase in YPD medium or YPD medium containing 1M NaCl. The expression of *ERG2*, *ERG11* and *ACT1* (control) was quantified by Northern analysis. The fold repression of *ERG2* and *ERG11* was calculated as in (A) and is depicted below the blots.

Fig. 4. Hog1 both stimulates and represses RNA PolIII density at osmoresponsive loci.

A. Hog1 stimulates Rpb3 occupancy at *STL1* and *GRE2* and represses Rpb3 occupancy at *ERG2* and *ERG11* upon stress. Rpb3-HA occupancy was determined by ChIP in vivo in yeast wild type and *hog1* mutant cells. The cells were grown in YPD and then subjected to a brief osmotic shock (0.4M NaCl) for the indicated times. Rpb3 occupancy was determined at the osmoinducible *STL1* (+100/+212) and *GRE2* (+280/+452) loci, as well as at the osmorepressed *ERG2* (-160/-64) and *ERG11* (-129/-38) loci. The relative occupancy is given as the fold IP efficiency over the *POL1* control region. (B) Hog1 is not stably recruited to the osmorepressed *ERG2* and *ERG11* promoters. Hog1-HA occupancy was determined by ChIP in vivo at the *CTT1* (-486/-329), *GRE2* (-301/-121), *ERG2* (-492/-226), and *ERG11* (-251/-114) promoters in YPD or at the indicated times of salt stress (0.4M NaCl). ChIP was performed from at least two independent chromatin samples and the

standard error is given in the graphs. The numbers in parentheses behind each gene refer to the primer locations used for quantitative PCR.

FIG. 5. The expression of *ECM22* is repressed upon stress dependent on Mot3, Rox1 and Hog1. The expression of *ECM22* and *ACT1* (control) was quantified by Northern analysis. The indicated wild type and mutant strains were grown in YPD and then submitted to a brief osmotic shock (0.4M NaCl) for the indicated times. The fold repression of the *ECM22* transcript depicted in the graph was calculated from three independent experiments as the ratio between the mRNA normalized for the *ACT1* messenger at time point 0 and at time point 20min of osmoshock. The standard error is given in the graph. One representative blot is shown. The fold repression of *ECM22* was different in wt and the *rox1mot3* mutant with  $p < 0.03$  according to the Student's T-test.

FIG. 6. Hog1 stimulates Mot3 abundance and the rapid binding of Mot3 to the *ERG2* promoter.

A. Mot3-myc occupancy was determined by ChIP in vivo in yeast wild type and *hog1* mutant cells. The cells were grown in YPD and then subjected to a brief osmotic shock (0.4M NaCl) for 5 min. Mot3 occupancy was determined at the *ERG2* [*ERG6*] promoter by quantitative PCR covering the -492/-226 [-498/-305] region. The relative occupancy is given as the fold IP efficiency over the *POL1* control region. ChIP was performed from three independent chromatin samples for each strain and condition. The standard error is given in the graph. Binding of Mot3 to *ERG2* was different in wt and the *hog1* mutant with  $p < 0.04$  according to the Student's T-test.

B. Mot3 protein levels are transiently up-regulated upon salt stress in a Hog1 dependent manner. Yeast wild type or *hog1* mutant cells expressing Tap-tagged Mot3 or Rox1 proteins from their chromosomal loci were analyzed in the absence of stress and along a brief salt shock (0.4M NaCl). Mot3-Tap and Rox1-Tap proteins were visualized by immunodetection using anti PAP antibody. Membranes were stained with DB71 to verify equal loading (total).

Fig. 7. Oxidative stress causes a drastic reduction in ergosterol levels and repression of *ERG* gene expression partially dependent on Mot3, Rox1 and Hog1.

A. The impact of menadione treatment on the cellular ergosterol content. Yeast wild type cells (BY4741; wt) and the indicated mutant strains were grown to exponential growth phase in YPD containing or not 75 $\mu$ M of menadione. Total ergosterol levels were determined in duplicate from three independent cultures. The ergosterol concentration of the wild type in normal growth medium was arbitrarily set to 1.

B. *ERG2* and *ERG11* expression is shut off rapidly after menadione treatment only partially dependent on Mot3, Rox1 or Hog1. The expression of *ERG2*, *ERG11*, *CCP1*, and *ACT1* (control) was quantified by Northern analysis. The indicated wild type and mutant strains were grown in YPD and then submitted to oxidative stress (50  $\mu$ M Menadione, 20 min). The fold repression of the *ERG2* and *ERG11* transcripts depicted below the blots was calculated as the ratio between the mRNA normalized for the *ACT1* messenger at time point 0 and at time point 20min of oxidative stress.

C. *mot3* and *hog1* mutants are sensitive to menadione stress. The growth of the indicated wild type (BY4741; wt) and mutant strains was continuously monitored in YPD medium or

YPD medium containing 25  $\mu$ M menadione. The growth curves are derived from the mean values of three independent cultures for each time point. The variance between triplicates is <10%.

Fig. 8. The impact of the *upc2-1* mutation on stress-regulated ergosterol homeostasis and stress resistance.

A. The *upc2-1* mutant hyperaccumulates ergosterol independent of oxidative stress. Yeast wild type cells and the *upc2-1* mutant were continuously grown in YPD containing or not 75  $\mu$ M menadione. Total ergosterol levels were determined in duplicate from three independent cultures. The ergosterol concentration of the wild type in normal growth medium was arbitrarily set to 1.

B. The *upc2-1* mutant is hypersensitive to menadione stress. The growth of the wild type and the *upc2-1* mutant was continuously monitored in YPD medium or YPD medium containing 50  $\mu$ M menadione. The growth curves are derived from the mean values of three independent cultures for each time point.

C. The *upc2-1* mutant is hypersensitive to salt and osmotic stress. Growth of the wild type and the *upc2-1* mutant was monitored on YPD plates with or without the addition of the indicated concentrations of NaCl or KCl.

D. *ERG2* and *ERG11* expression is largely unresponsive to salt stress in the *upc2-1* mutant. The expression of *ERG2*, *ERG11*, and *ACT1* (control) was quantified by Northern analysis. The wild type and the *upc2-1* mutant strains were grown in YPD and then submitted to a brief osmotic shock (0.4M NaCl, 20 min). The fold repression of the *ERG2* and *ERG11* transcripts depicted below the blots was calculated as the ratio between the mRNA



normalized for the *ACT1* messenger at time point 0 and at time point 20min of salt stress.

E. The salt sensitivity of *upc2-1* is alleviated by fluconazole treatment. Yeast wild type cells and the *upc2-1* mutant were treated or not (control) with the indicated concentrations of fluconazole for 2 hours and then spotted onto YPD agar medium with or without 0.4M KCl.

F. The sensitivity of the *upc2-1* mutant to salt and oxidative stress is aggravated by external ergosterol supplementation. The growth of the wild type and the *upc2-1* mutant was continuously monitored in minimal SD medium (control, upper panel), SD medium containing 0.8 M NaCl (middle panel) or SD medium containing 50  $\mu$ M menadione (lower panel) with or without the supplementation of 10  $\mu$ g/ml ergosterol as indicated. The ergosterol content is  $100\pm 3$  for the wild type,  $133\pm 5$  for *upc2-1* without ergosterol feeding and  $240\pm 22$  for *upc2-1* upon ergosterol feeding. The growth curves are derived from the mean values of three independent cultures for each time point.

FIG. 9. Model: Transcriptional control of *ERG* genes in response to different stimuli. Under normal growth conditions, *ERG* gene expression is maintained by the transcriptional activators Ecm22 and Upc2 (Vik & Rine, 2001). Sterol depletion is compensated by enhanced *ERG* gene transcription mainly via activation of Upc2 (Davies *et al.*, 2005). The shutdown of *ERG* gene expression in the absence of oxygen is assured by the Mot3 transcription factor (Hongay *et al.*, 2002). The signaling events upstream of Ecm22, Upc2 or Mot3 upon hypoxia or sterol depletion are largely unknown. Hyperosmotic stress activates the HOG MAP kinase pathway, which triggers *ERG* gene repression by several mechanisms: (a) the Hog1 MAP kinase targets *MOT3* and *ROX1* via the Sko1 transcription

factor, but mainly regulates *MOT3* expression upon osmostress (Proft *et al.*, 2005), Hog1 regulates Mot3 protein levels (Figure 6B); (b) Mot3 and Rox1 downregulate the expression of specific *ERG* genes in response to osmotic stress (Figures 3 and 6), (c) Mot3 and Rox1 shut down *ECM22* expression upon osmostress (Figure 5). The *ERG* genes encoding the enzymes of the last sterol specific reactions of the ergosterol biosynthesis pathway depicted on the right.

Table 1. Strains used in this study.

Strain	Genotype	Source
BY4741	<i>MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0</i>	EUROSCARF
BY4741 <i>mot3</i>	BY4741 <i>mot3::KanMX4</i>	EUROSCARF
BY4741 <i>rox1</i>	BY4741 <i>rox1::KanMX4</i>	EUROSCARF
MAP93	BY4741 <i>rox1::KanMX4 mot3::his5+ (S. pombe)</i>	This study
W303-1A	<i>MATa ade2-1 ura3-1 his3-11,-15 leu2,3-112 trp1</i>	R. Rothstein
W3Rpb3HA	W303-1A <i>RPB3-3HA</i>	P. Mason
MAP109	W303-1A <i>RPB3-3HA hog1::KanMX4</i>	This study
FY1339	<i>MATa ura3Δ0 his3Δ200 trp1Δ63</i>	F. Winston
FY2081	FY1339 <i>MOT3-18myc::TRP1</i>	F. Winston
MAP95	FY1339 <i>MOT3-18myc::TRP1 hog1::his5+ (S. pombe)</i>	This study
WPY361	<i>MATa upc2-1 ura3-1 his3-11,-15 leu2,3-112 trp1</i>	W. Prinz
BYMOT3TAP	BY4741 <i>MOT3-TAP::HIS3MX6</i>	(Ghaemmaghani <i>et al.</i> , 2003)
BYROXITAP	BY4741 <i>ROX1-TAP::HIS3MX6</i>	(Ghaemmaghani <i>et al.</i> , 2003)
MAP113	BY4741 <i>MOT3-TAP::HIS3MX6 hog1::KanMX4</i>	This study
MAP114	BY4741 <i>ROX1-TAP::HIS3MX6 hog1::KanMX4</i>	This study

Table 2. Comparison of the expression levels of the ERG pathway in wild type and *mot3rox1* mutant cells under osmotic stress.

**A Genes encoding the ergosterol specific enzymes of the ERG pathway:**

Gene	Expression Wt +/- SD	Expression <i>mot3rox1</i> +/- SD	Ratio <i>mot3rox1</i> /wt	P-value
<i>ERG25 (YGR060W)</i>	8345+/-644	12900+/-169	1.55	2E-04
<i>ERG28 (YER044C)</i>	3937+/-184	5980+/-89	1.52	6E-05
<i>ERG11 (YHR007C)</i>	8042+/-530	10895+/-195	1.35	2E-03
<i>ERG1 (YGR175C)</i>	7913+/-322	10703+/-356	1.35	1E-03
<i>ERG27 (YLR100W)</i>	4317+/-140	5762+/-163	1.33	1E-03
<i>ERG26 (YGL001C)</i>	4158+/-22	5549+/-77	1.33	2E-04
<i>ERG2 (YMR202W)</i>	8862+/-325	11366+/-112	1.28	2E-03
<i>ERG7 (YHR072W)</i>	1987+/-90	2230+/-55	1.12	0.01
<i>ERG3 (YLR056W)</i>	3353+/-437	3721+/-57	1.12	0.15
<i>ERG6 (YML008C)</i>	6208+/-421	6968+/-64	1.12	0.02
<i>ERG9 (YHR190W)</i>	9591+/-110	9921+/-318	1.03	0.20
<i>ERG24 (YNL280C)</i>	4826+/-103	4945+/-12	1.02	0.37
<i>ERG5 (YMR015C)</i>	2684+/-299	2192+/-60	0.82	0.02
<i>ERG4 (YGL012W)</i>	8200+/-13	6754+/-219	0.82	4E-04

**B Transcriptional activators of the ERG pathway:**

Gene	Expression Wt +/- SD	Expression <i>mot3rox1</i> +/- SD	Ratio <i>mot3rox1</i> /wt	P-value
<i>ECM22 (YLR228C)</i>	227+/-13	537+/-15	2.37	3E-07
<i>UPC2 (YDR213W)</i>	233+/-5	419+/-15	1.79	1E-06

The global gene expression profiles of yeast wild type (BY4741) and the *mot3rox1* mutant were determined upon osmotic stress (0.4M NaCl, 20 min) by microarray analysis. A. The expression levels of all genes representing the ergosterol specific enzymatic steps were compared between the *mot3rox1* and wt strains. Absolute expression levels are given including the standard deviation (SD). Genes are listed with decreasing expression ratios. B. Expression ratios for the two known transcriptional activators of the ERG pathway, *ECM22* and *UPC2*.

Figure 1

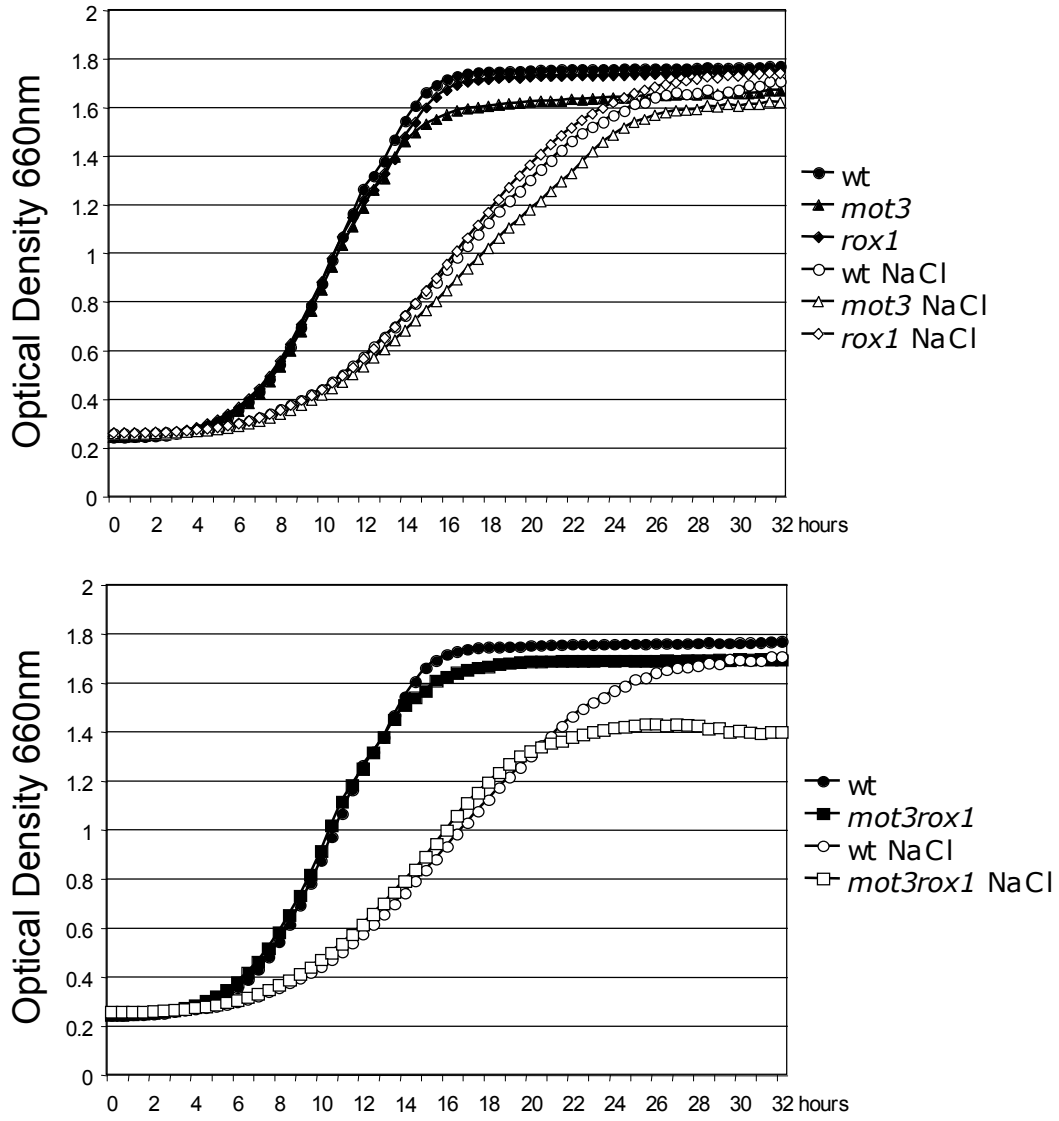


Figure 2

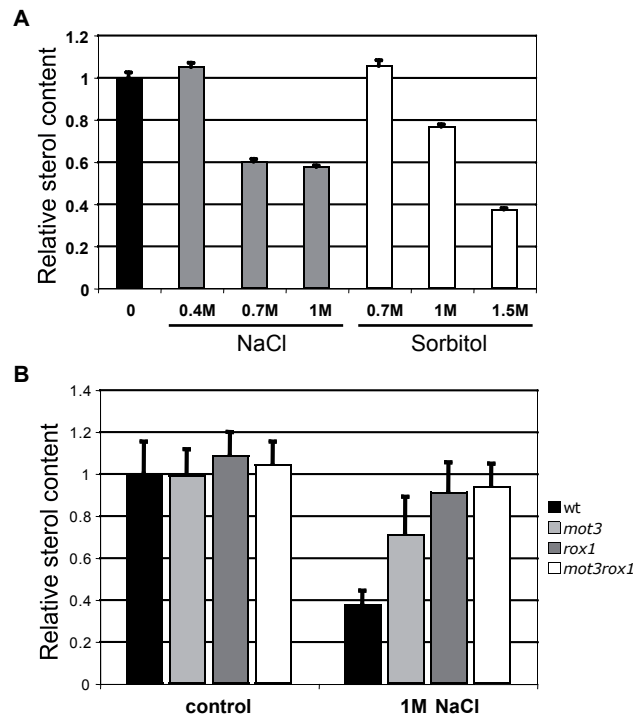


Figure 3

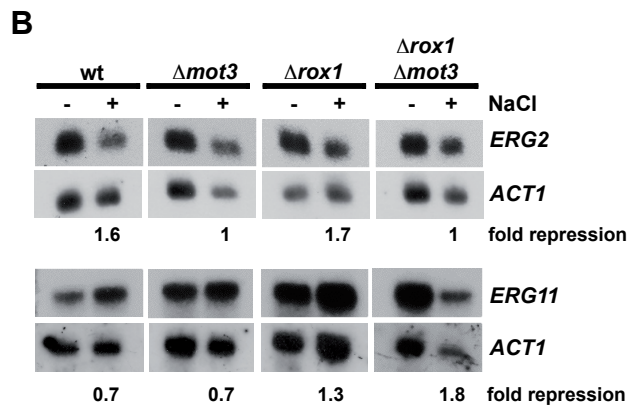
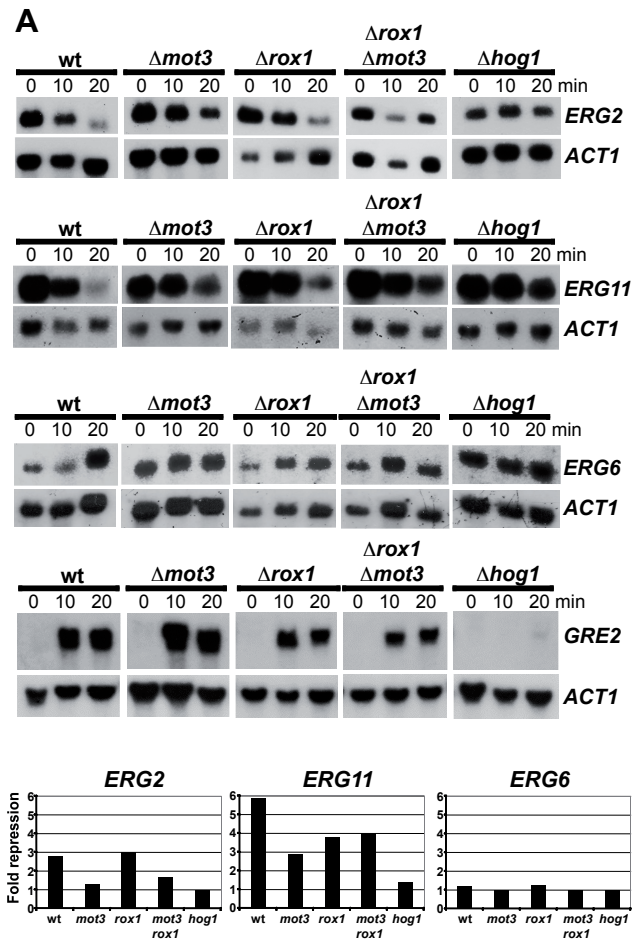


Figure 4

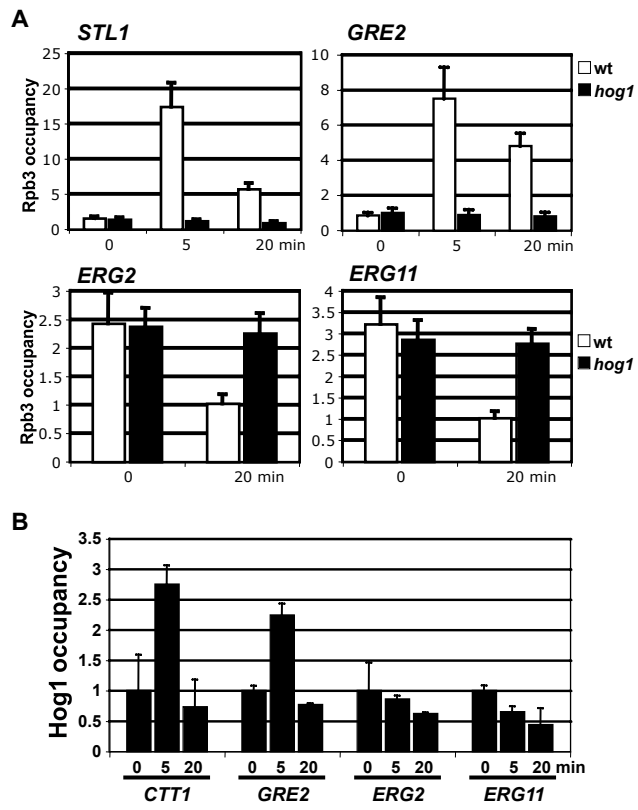




Figure 5

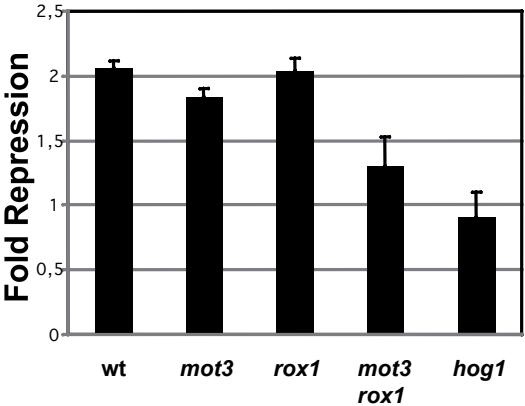
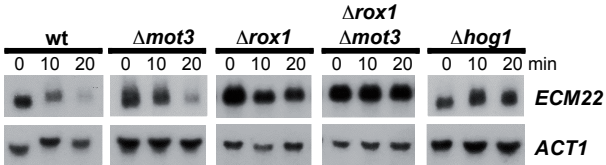


Figure 6

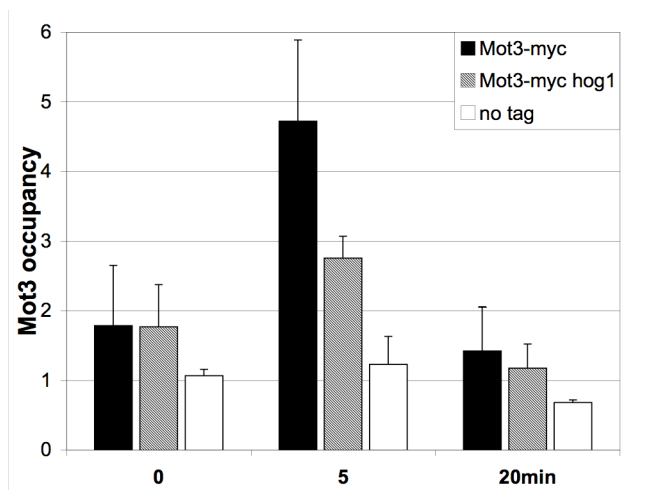


Figure 7

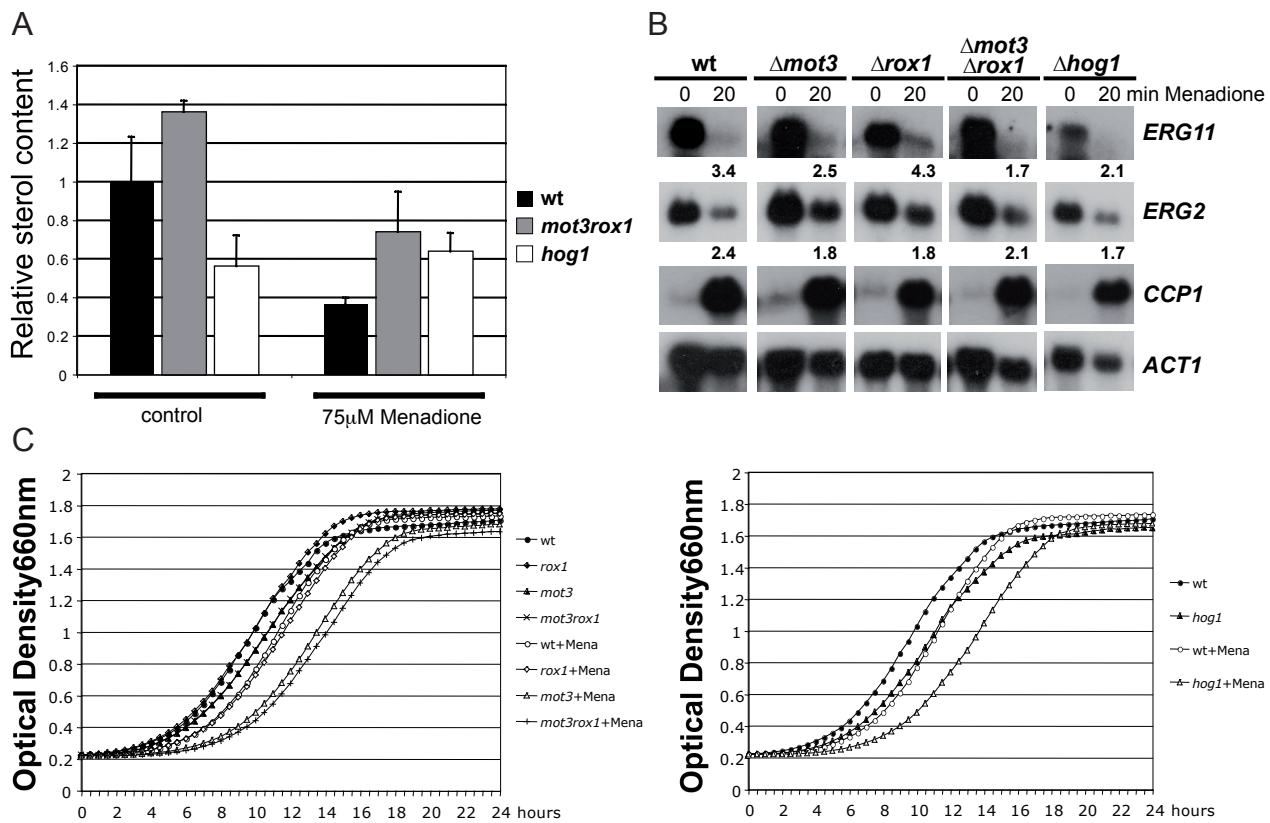


Figure 8

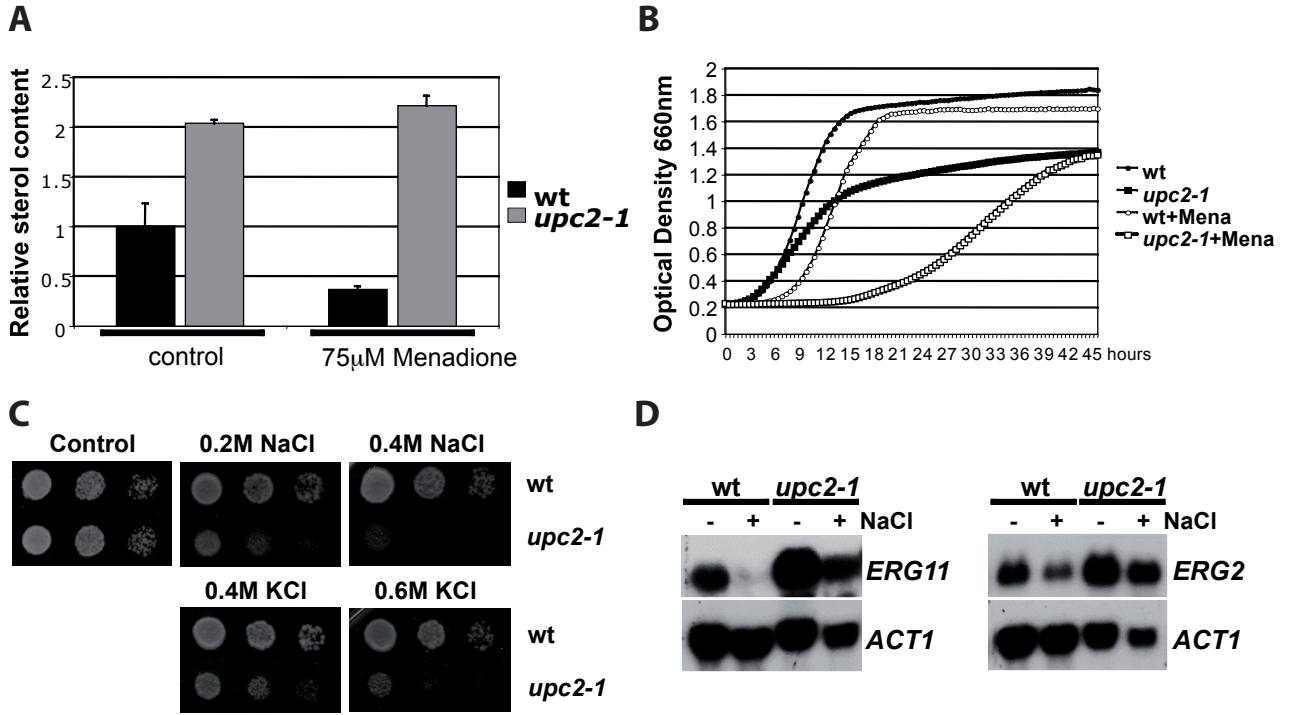


Figure 9

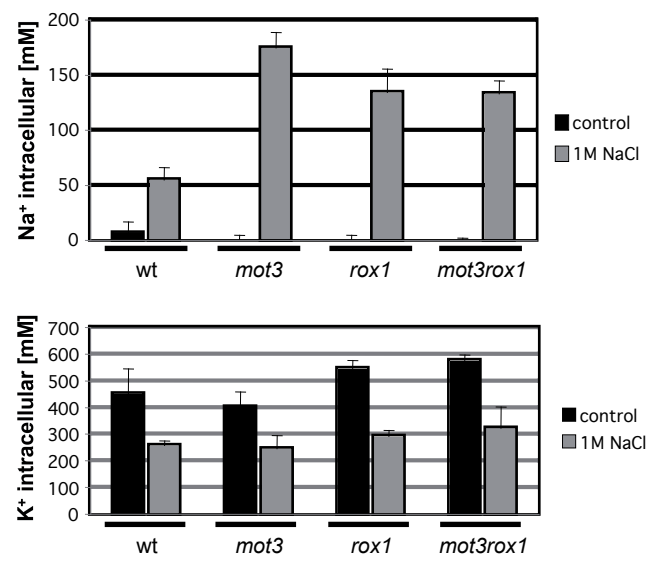


Figure 10

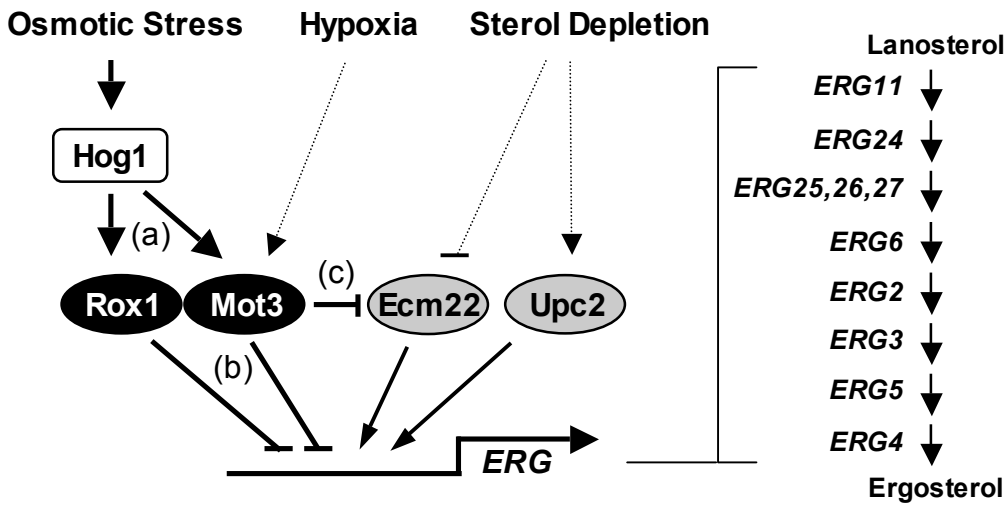


Figure 11

