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Ascorbic Acid Inhibition of *Candida albicans* Hsp90-Mediated Morphogenesis Occurs via the Transcriptional Regulator Upc2

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Morphogenetic transitions of the opportunistic fungal pathogen *Candida albicans* are influenced by temperature changes, with induction of filamentation upon a shift from 30 to 37°C. Hsp90 was identified as a major repressor of an elongated cell morphology at low temperatures, as treatment with specific inhibitors of Hsp90 results in elongated growth forms at 30°C. Elongated growth resulting from a compromised Hsp90 is considered neither hyphal nor pseudohyphal growth. It has been reported that ascorbic acid (vitamin C) interferes with the yeast-to-hypha transition in *C. albicans*. In the present study, we show that ascorbic acid also antagonizes the morphogenetic change caused by hampered Hsp90 function. Further analysis revealed that Upc2, a transcriptional regulator of genes involved in ergosterol biosynthesis, and Erg11, the target of azole antifungals, whose expression is in turn regulated by Upc2, are required for this antagonism. Ergosterol levels correlate with elongated growth and are reduced in cells treated with the Hsp90 inhibitor geldanamycin (GdA) and restored by cotreatment with ascorbic acid. In addition, we show that Upc2 appears to be required for ascorbic acid-mediated inhibition of the antifungal activity of fluconazole. These results identify Upc2 as a major regulator of ascorbic acid-induced effects in *C. albicans* and suggest an association between ergosterol content and elongated growth upon Hsp90 compromise.

Candida albicans is among the few fungal species that are frequently encountered as clinical pathogens (1). *C. albicans* is a frequent commensal of healthy individuals, but when the host immune system is weakened, it can become a deadly pathogen (2). Its pathogenic nature relies on a multitude of factors, including the ability to grow at 37°C, to produce adhesins and hydrolases, and to change between different morphological forms, including yeast cells, hyphae, pseudohyphae, chlamydospores, opaque cells, and the recently described GUT morphology (3–5). It is generally assumed that the yeast form is crucial for fungal dissemination throughout the body via the bloodstream (6), while hyphae, on the other hand, are required for the production of extracellular enzymes and invasive growth (7).

The yeast-to-hypha transition of *C. albicans* can be triggered by environmental cues, such as pH, serum, elevated temperature, and CO_2 (4, 8). In addition, several small molecules, such as autoregulatory molecules, cell cycle inhibitors, and histone deacetylase inhibitors, are also capable of modulating morphogenetic responses (9, 10). Most environmental triggers and small molecules function through activation of the cyclic AMP (cAMP)-protein kinase A (PKA) or mitogen-activated protein kinase (MAPK) pathway via their downstream transcription factors, Efg1 and Cph1, respectively (4, 11, 12). Over the past few years, several reports have appeared that showed that geldanamycin (GdA), a benzoquinone ansamycin antibiotic, is another small molecule that strongly affects the cell shape of *C. albicans* at 30°C, as its addition results in elongated cells. GdA inhibits the function of Hsp90 by binding to its ADP/ATP binding pocket (10, 13), indicating that Hsp90 prevents an elongated cell shape of *C. albicans* at lower temperatures. This morphogenetic process also involves the Ras1-cAMP-PKA signaling pathway, but it seems to do this independently of the downstream transcription factor Efg1, suggesting the involvement of other transcriptional regulators (13). Screening of a transcription factor (TF) deletion collection for mutants impaired in the Hsp90-mediated elongated-cell-shape phenotype resulted in the identification of Hms1 (14). Two upstream regulators of this TF, the cyclin-dependent kinase Pho85 and the cyclin Pcl1, are also required for elongated cell shape (14). As Hms1 is not a component of the cAMP-PKA pathway, the link between Hsp90-dependent elongated cell shape and the Ras1-PKA signaling pathway remained to be identified. Our aim in this work was to identify transcription factors that upon overexpression affect the Hsp90-dependent elongated cell shape. For this, we screened a TF overexpression library and found that overexpression of Upc2 prevents the elongated-growth phenotype when GdA is added. The Zn_2Cys_6 transcriptional regulator Upc2 is important for the regulation of the ergosterol biosynthetic pathway in response to commonly used antifungals (15). The transcription factor can act as a repressor or an activator depending on its target and on the initiating conditions and performs its function by binding to sterol response elements (SREs) (15–17). Gain-of-function mutations in *UPC2* have been associated with resistance to antifungal treatments (18–21), while strains devoid of Upc2 are hypersusceptible to azole antifungals (22). One of the genes regulated by Upc2 is Erg11, which is a key enzyme in the biosynthesis of ergosterol, and it is the target of the azole antifungal drugs. Overexpression of Erg11 has been shown to cause tolerance for these drugs.

In order to find components linking the GdA-induced elongated-growth phenotype and the block of this phenotype upon overexpression of *UPC2*, we tested a number of compounds that were

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TABLE 1 Strains used in this study

Strain	Description	Genotype	Source
SC5314		Wild type	70
CAI4		*ura3*Δ::*imm434/ura3*Δ::*imm434 iro1*Δ*/iro1*Δ::*imm343*	71
AFA60a	CAI4 transformed with *URA3* vector	*ura3*Δ::*imm434/ura3*Δ::*imm434 iro1*Δ*/iro1*Δ::*imm343 RPS10*::*CIp10*	42
AFA59b	CAI4 carrying extra copy of *CaERG11* under the control of *ACT1* promoter	*ura3*Δ::*imm434/ura3*Δ::*imm434 iro1*Δ*/iro1*Δ::*imm343 RPS10*::*pAFC89b*	42
DSY448	*cdr1*Δ*/cdr1*Δ mutant	*ura3*Δ::*imm434/ura3*Δ::*imm434 iro1*Δ*/iro1*Δ::*imm343 cdr1*Δ::*hisG-URA3-hisG/cdr1*Δ::*hisG*	72
DSY465	*mdr1*Δ*/mdr1*Δ mutant	*ura3*Δ::*imm434/ura3*Δ::*imm434 iro1*Δ*/iro1*Δ::*imm343 ben*Δ::*hisG-URA3-hisG/ben*Δ::*hisG*	72
CMDR1E2A and -B	CAI4 carrying *CaMDR1* under the control of *ADH1* promoter	*ura3*Δ::*imm434/ura3*Δ::*imm434 iro1*Δ*/iro1*Δ::*imm343 ADH1/adh1*::P_{ADH1}-*MDR1-URA3*	39
SN152	Background strain TF wild type and TF077	*arg4*Δ*/arg4*Δ *leu2*Δ*/leu2*Δ *his1*Δ*/his1*Δ *URA3/ura3*Δ::*imm434 IRO1/iro1*Δ::*imm343*	73
TF wild type	Control wild-type strain (paired "wild-type" TF077)	*arg4*Δ*/arg4*Δ *leu2*Δ*/LEU2 his1*Δ*/HIS1 URA3/ura3*Δ::*imm434 IRO1/iro1*Δ::*imm343*	31
TF077	*upc2*Δ*/upc2*Δ mutant	As SN152, but *upc2*Δ::*HIS1/upc2*Δ::*LEU2*	31
BWP17 (TW14901)	Background strain D-6 and EC-7	*ura3*Δ::*imm434/ura3*Δ::*imm434 iro1/iro1*Δ::imm434 *his1*Δ::*hisG/his1*Δ::*hisG arg4*Δ*/arg4*Δ	22
D-6 (TW14903)	*upc2*Δ*/upc2*Δ mutant	As BWP17 but *upc2*Δ::*URA3/upc2*Δ::*ARG4*	22
EC-7 (TW14904)	Reconstituted strain	As D-6 but *upc2*Δ::*URA3/upc2*Δ::*UPC2-HIS1*	22
pTET-ERG11	CAI4 with doxycycline-repressible *ERG11* as the only *ERG11* allele	As CAI4, but *tetO-ERG11/erg11*Δ	41
CaLC1411 (CaLC436)	Strain with doxycycline-repressible *HSP90* as the only *HSP90* allele	*arg4*Δ*/arg4*Δ *his1*Δ*/his1*Δ *URA3/ura3*Δ::*imm434 IRO1/iro1*Δ::*imm434 HIS1/his1*Δ::*TAR-FRT hsp90*Δ::*CdHIS1/his1*Δ::*TAR-FRT*	74
SC2H3	One- and two-hybrid reporter strain in the SN152 background	Like SN152, but 5 × LexAOp-ADH1b/HIS1 5 × LexAOp-ADH1b/lacZ[a]	27
OX control	Strain carrying empty vector under *MET3* promoter	As SC2H3, but *MET3pro*::*lexA*	Stynen et al., unpublished
Upc2 OX	Strain carrying extra copy of *UPC2* under *MET3* promoter	As SC2H3, but *MET3pro*::*lexA*::*UPC2*	Stynen et al., unpublished
Gcn4 OX	Strain carrying extra copy of *GCN4* under *MET3* promoter	As SC2H3, but *MET3pro*::*lexA*::*GCN4*	Stynen et al., unpublished
Cap1 OX	Strain carrying extra copy of *CAP1* under *MET3* promoter	As SC2H3, but *MET3pro*::*lexA*::*CAP1*	Stynen et al., unpublished
Dot6 OX	Strain carrying extra copy of *DOT6* under *MET3* promoter	As SC2H3, but *MET3pro*::*lexA:DOT6*	Stynen et al., unpublished
Rtg3 OX	Strain carrying extra copy of *RTG3* under *MET3* promoter	As SC2H3, but *MET3pro*::lexA::*RTG3*	Stynen et al., unpublished

[a] This is SN152, but it contains two reporter genes (HIS1 and LacZ), and the expression of these reporter genes is controlled by a promoter element consisting of 5 times the LexA operator sequence and the ADH1 basic promoter sequence.

shown to mediate morphogenesis in *C. albicans*. Ascorbic acid (vitamin C) was previously shown to play a role in morphogenesis, as it blocks serum-initiated hypha formation, a process mediated by adenylate cyclase (23, 24). How ascorbic acid affects morphogenesis is not clear, and we propose that it may function by modulating the role of Hsp90 in cell shape formation. In this paper, we describe a previously unreported negative effect of ascorbic acid on the Hsp90-dependent cell shape. Focusing on the mode of action of ascorbic acid, we provide evidence that it requires Upc2 and Erg11 to perform its function. We further demonstrate that intracellular ergosterol levels play a role in the ascorbic acid-mediated effect on cell shape. In addition, we show that Upc2 is also required for the antagonistic effect of ascorbic acid on fluconazole toxicity. Together, these results show that ascorbic acid inhibits Hsp90-mediated cell shape transition via the transcriptional regulator Upc2.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. *C. albicans* strains were grown overnight in 3 ml of YPD medium (1% yeast extract, 2% bacteriological peptone, and 2% glucose) at 30°C. The cells were subsequently diluted to an optical density at 600 nm (OD_{600}) of 0.2 and cultured for the indicated times at 30°C with the indicated treatments (4 μM or 10 μM geldanamycin, 2.5 mM ascorbic acid, L-cysteine, D-cysteine, glutathione, dithiothreitol [DTT], or 0.1 μg/ml doxycycline). When ascorbic acid was added, the medium was buffered to pH 7. All chemicals were purchased from Sigma-Aldrich.

Transcription factor overexpression strains were grown in complete supplement mixture minus methionine (CSM−Met medium) [0.073% CSM−Met−Ura, 0.17% yeast nitrogen base (YNB) without amino acids and $(NH_4)_2SO_4$, 0.5% $(NH_4)_2SO_4$, pH 5.5] supplemented with 0.1% uridine and the indicated treatment (10 μM GdA or 2.5 or 10 mM methionine).

Microscopy. Imaging of the cells was done by differential interference contrast microscopy using the Zeiss Axioplan 2 microscope. Images were

obtained with a Zeiss Axiocam MRc5 camera using Axiovision software 3.0 (Carl Zeiss, Inc., NY).

Cell sedimentation assay. The cell sedimentation rate was quantified using the assay described by Eboigbodin and Biggs (25) and Fu et al. (26). Cells were grown in YPD liquid medium with or without 0.1 μg/ml doxycycline (for transcriptional repression) and 2.5 mM L-cysteine or ascorbic acid. The OD_{600} of the cells in the upper part of the glass tube was determined at the indicated time points, as sediments settle to the bottom of the glass tube. The formula $(OD_I - OD_t/OD_I) \times 100$, where OD_I is the initial OD taken at time zero and OD_t is the OD taken at the indicated time point, was utilized to calculate the percentage of sedimented cells.

Upc2 overexpression strain: transcription factor library. The *UPC2* overexpression strain used in this study is part of a transcription factor overexpression library constructed in our laboratory on the basis of the previously developed *C. albicans* two-hybrid system (27). Briefly, the nuclear localization sequence (NLS) was removed from the one-hybrid plasmid pC2HB, resulting in pC1H. Subsequently, genes encoding known and putative transcription factors were successfully cloned in fusion with the DNA-binding domain of LexA in the plasmid to create the pC1H-PTF library. The resulting plasmids were integrated into the two-hybrid reporter strain S2CH3 (27) between *XOG1* and *HOL1* loci on chromosome 1 after linearization at the NotI restriction site. Selection was performed on CSM−Leu medium. The complete library consists of ~200 strains, each carrying an ectopically expressed protein under the control of an inducible *MET3* promoter (B. Stynen and P. Van Dijck, unpublished data). The use of the inducible *MET3* promoter allows conditional expression, as it is repressed in the presence of methionine and cysteine (28).

Ergosterol extraction and quantification. Ergosterol extraction and quantification were performed as reported by Arthington-Skaggs et al. (29), with minor modifications. Briefly, cells were grown overnight in 4 ml YPD medium at 30°C. The cells were diluted to an OD_{600} of 0.2 and grown in YPD medium supplemented with 10 μM GdA, 2.5 mM ascorbic acid, or a combination of both. After the indicated time, 220 ODs of cells at 600 nm was used in the heptane extraction. The same procedure was used for all experimental conditions. Cells were harvested by centrifugation at 3,000 rpm for 5 min and washed once with 10 ml sterile water. Three milliliters of 25% alcoholic potassium hydroxide solution (25 g KOH and 35 ml sterile H_2O brought to 100 ml with 100% ethanol) was added to each pellet and vortexed for 1 min. The cell suspensions were heated in an 85°C water bath for 1 h and then allowed to cool down to room temperature. Sterols were extracted with a mixture of 1 ml of sterile water and 3 ml of *n*-heptane, followed by mixing for 3 min. The organic layer was transferred to a clean glass tube and stored at −20°C for 24 h. Prior to the spectrophotometrical scan at between 230 and 300 nm using a Shimadzu UV-1650PC spectrophotometer, the sterol extracts were diluted 5-fold in 100% ethanol. A dilution of heptane and ethanol was used as the blank. Ergosterol levels were subsequently calculated as a percentage of the wet weight, as described previously (29).

Quantitative real-time PCR. Overnight cultures of *C. albicans* strains were diluted to an OD_{600} of 0.2, and cultures were grown at 30°C for the indicated times in the presence and absence of either doxycycline (0.1 μg/ml), ascorbic acid (2.5 mM), or GdA (10 μM) before RNA extraction. RNA was DNase treated prior to cDNA synthesis with the Promega A3500 reverse transcription kit. Quantitative real-time PCR was performed using the GoScript Reverse Transcription System (Promega) on a StepOne Plus real-time PCR system (Applied Biosystems). Reactions were performed in triplicate, with oligonucleotides CaERG11up and CaERG11down, UPC2_fw and UPC2_rv, HSP90_fw and HSP90_rv, and TEF1a-fw and TEF1a-rv as the primer pairs (Table 2). The degree of regulation was determined by the $\Delta\Delta C_T$ method, using expression of *TEF1* to normalize the data.

Checkerboard assay. A checkerboard assay was performed on 96-well plates by combining different concentrations of ascorbic acid and fluconazole, and the fractional inhibitory concentration index (FICI) was determined. The assay was performed with both the wild type and the *UPC2* deletion mutant to verify whether the effect of ascorbic acid on fluconazole susceptibility is exerted via Upc2.

TABLE 2 Primers used in this study

Primer	Sequence	Source
CaERG11up	TTACCTCATTATTGGAGACGTGATG	42
CaERG11down	CACGTTCTCTTCTCAGTTTAATTTCTTTC	42
TEF1a_fw	CCACTGAAGTCAAGTCCGTTGA	42
TEF1a-rv	CACCTTCAGCCAATTGTTCGT	42
UPC2_fw	GGATGCTCGACATGCAAAAAG	This study
UPC2_rv	TGCCACATACAGGTCTCTGTTCA	This study
HSP90_fw	CCATCTGATATCACTCAAGATG	This study
HSP90_rv	AGTGATAAACACTCTACGGACG	This study

The concentrations of ascorbic acid ranged from 0.078 mM to 5 mM for both the wild type and the *UPC2* deletion mutant. The concentration of fluconazole ranged from 0.004 μg/ml to 2 μg/ml for the *UPC2* deletion mutant and 0.125 μg/ml to 64 μg/ml for the wild type. The FICI was calculated according to the following formula: $FIC_{index} = FIC_A + FIC_B = (MIC_{A\ comb}/MIC_{A\ alone}) + (MIC_{B\ comb}/MIC_{B\ alone})$, where $MIC_{A\ alone}$ and $MIC_{B\ alone}$ are the MICs of ascorbic acid and fluconazole alone and $MIC_{A\ comb}$ and $MIC_{B\ comb}$ are the MICs of ascorbic acid and fluconazole in combination. Interactions were referred to as synergistic when the FIC_{index} value was ≤0.5 and antagonistic when the FIC_{index} value was >4. FIC_{index} values that were >0.5 or ≤4 indicated no interaction between ascorbic acid and fluconazole (30). There was no difference in FIC indexes observed between 24 and 48 h.

Oxidative-stress sensitivity assay. The wild type and the two independent *UPC2* deletion mutants of the Homann collection (31) were grown to mid-log phase in liquid YPD medium. The cells were diluted to an OD_{600} of 1, and 10-fold serial dilutions were spotted on YPD plates containing the indicated concentrations of H_2O_2. The plates were photographed after 24-h incubation at 37°C.

Propidium iodide staining. Viability staining was performed by incubating a subset of cells (taken at the indicated time points of the experiments) for 20 min in the dark with the fluorescent stain propidium iodide (PI) (46 mM; Sigma-Aldrich). Prior to PI fluorescence analysis (excitation and emission maxima, 535 and 617 nm), the cells were washed and resuspended in phosphate-buffered saline (PBS).

Statistical analysis. Statistical significance was calculated using a Student *t* test analysis. All experiments were performed with at least three biological repeats.

RESULTS

Ascorbic acid interferes with the morphogenetic process governed by Hsp90. The transition of *C. albicans* from yeast to hyphae can be induced or repressed by a wide variety of triggers (32). Partial inhibition of Hsp90, obtained either by addition of GdA or by decreased transcription of the corresponding *HSP90* gene, results in elongated cells, which are considered neither hyphae nor pseudohyphae (13). In order to understand the mechanism by which Hsp90 affects morphogenesis, we tested a number of compounds known to inhibit filamentation for interaction with GdA and determined the effect of ascorbic acid on the GdA-induced elongated growth. Addition of ascorbic acid, which was previously reported to interfere with the yeast-to-hypha transition (24), resulted in a strong reduction of the GdA-induced elongated cell shape (Fig. 1A). Similar results were obtained using radicicol, an Hsp90 inhibitor structurally distinct from GdA (reference 33 and data not shown). Since Hsp90 is an essential chaperone, cell viability was assessed with PI, a dye that is excluded from viable cells. As shown in Fig. 1B, the viability of GdA-treated cells is similar to

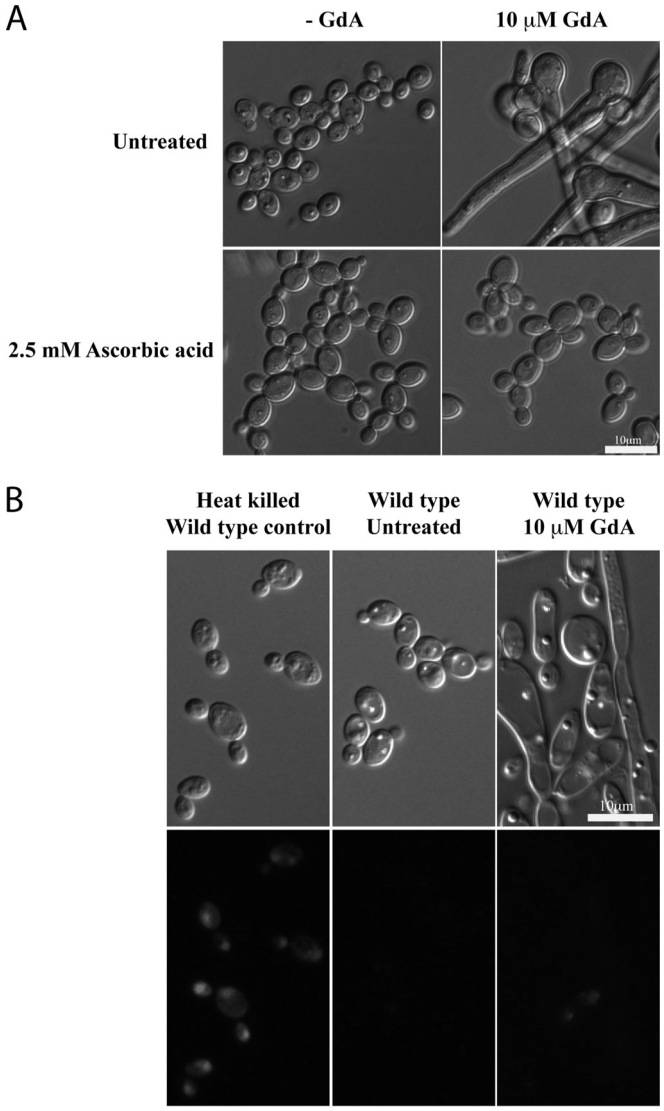

FIG 1 Ascorbic acid abrogates GdA-induced elongated growth. (A) Inhibiting effect of ascorbic acid on elongated growth induced by 10 μM GdA. Wild-type SC5314 cells were grown at 30°C in liquid rich medium containing GdA (10 μM) with 2.5 mM ascorbic acid. The images were taken after 8 h of growth. (B) GdA-mediated pharmacological inhibition of Hsp90 for extended periods is not lethal. Propidium iodide-stained heat-killed wild-type cells (15 min at 80°C) clearly illustrate a complete lethal phenotype, as indicated by the staining (which is excluded from viable cells), while the GdA-treated cells closely resemble the untreated wild-type cells. Top, DIC images; bottom, propidium iodide fluorescence images. The images were taken after 24 h.

that of untreated cells, ruling out possible effects due to elevated cell mortality.

To test whether the observed effect of ascorbic acid was dependent on Hsp90 or the result of chemical-chemical interference with GdA, similar experiments were conducted using a strain whose Hsp90 content can be modulated via the use of a tetracycline-repressible promoter (13). As expected, lowering the expression of *HSP90* with doxycycline in this strain resulted in elongated growth (Fig. 2A). Cell viability tests were also performed and indicated that the cells were alive (Fig. 2B). Addition of ascorbic acid to cells genetically depleted of Hsp90 greatly increased the percentage of yeast cells (75%) compared to the control conditions without the addition of ascorbic acid (32%) (Fig. 2C). The effect of ascorbic acid was also determined using a sedimentation assay. Addition of ascorbic acid to cells in a liquid culture resulted in a lower sedimentation rate, indicating a higher proportion of yeast cells in these cultures than in non-ascorbic-acid-treated cells (Fig. 2D). Since transcription of *HSP90* in the *tetO-HSP90* strain is reduced in the presence of doxycycline, the possibility of a doxycycline-ascorbic acid chemical interaction was ruled out in experiments with wild-type control cells. Here, the concentration of doxycycline used in our experiments was demonstrated not to interfere with the effect of ascorbic acid on the GdA-dependent elongated cell shape described above (see Fig. S1A in the supplemental material). We also confirmed that doxycycline-mediated repression of Hsp90 was not significantly altered in the presence of ascorbic acid (see Fig. S1B in the supplemental material). Taken together, these results indicate that ascorbic acid has a negative effect on elongated cell shape formation resulting from lower Hsp90 activity or expression.

Upc2 is required for the ascorbic acid-mediated effect. A transcription factor deletion collection was previously used to characterize the regulatory system mediating Hsp90-regulated elongated growth (14). In a complementary approach, we screened a transcription factor overexpression library to identify TFs that upon overexpression prevent GdA-induced morphogenesis. The library consists of ~200 strains, each carrying an ectopically expressed TF under the control of the inducible *MET3* promoter (Stynen et al., unpublished). Methionine only was used to repress transcription, as cysteine was shown to interfere with elongation even in a wild-type strain due to its antioxidant properties (see Discussion and Fig. S2 in the supplemental material). Several transcriptional regulators whose overexpression resulted in blocked or decreased elongated growth in the presence of GdA were identified (Table 3). We continued with the strain overexpressing *UPC2* (Fig. 3A), as it showed the strongest effect, based on semiquantitative observations. Expression of *UPC2* under inducing conditions was ~4.5-fold higher than that of an isogenic control strain carrying an empty plasmid (Fig. 3A). As expected, the characteristic elongated growth form was visible when the *MET3* promoter was repressed by the addition of methionine, confirming that an increased dosage of Upc2 is responsible for the loss of elongation in the presence of inactive Hsp90 (Fig. 3B).

To confirm the role of Upc2 in the Hsp90-mediated inhibition of elongated growth forms, we tested two independent strains in which the endogenous *UPC2* genes were deleted (22, 31), as such strains are expected to be hypersusceptible to GdA. Treatment of the wild-type strain with a low concentration of GdA (4 μM) has no effect on morphogenesis of wild-type cells. However, the same concentration was sufficient to induce elongated growth in the *upc2*Δ/*upc2*Δ strains, confirming their hypersusceptibility (strain D-6 is shown in Fig. 4A). In the complemented strain, elongation was impaired (Fig. 4A). Taken together, these observations indicate that Upc2 plays a role in the GdA-associated elongation process and confirm the previously observed genetic interaction between Upc2 and Hsp90 (34).

The obvious question, then, was whether Upc2 is required for the effect that ascorbic acid has on Hsp90-mediated cell elongation. Addition of ascorbic acid to wild-type cells prevents GdA-induced cell elongation. In the absence of Upc2 (in both independent deletion strains D-6 and TF077), GdA-induced cell elongation was not af-

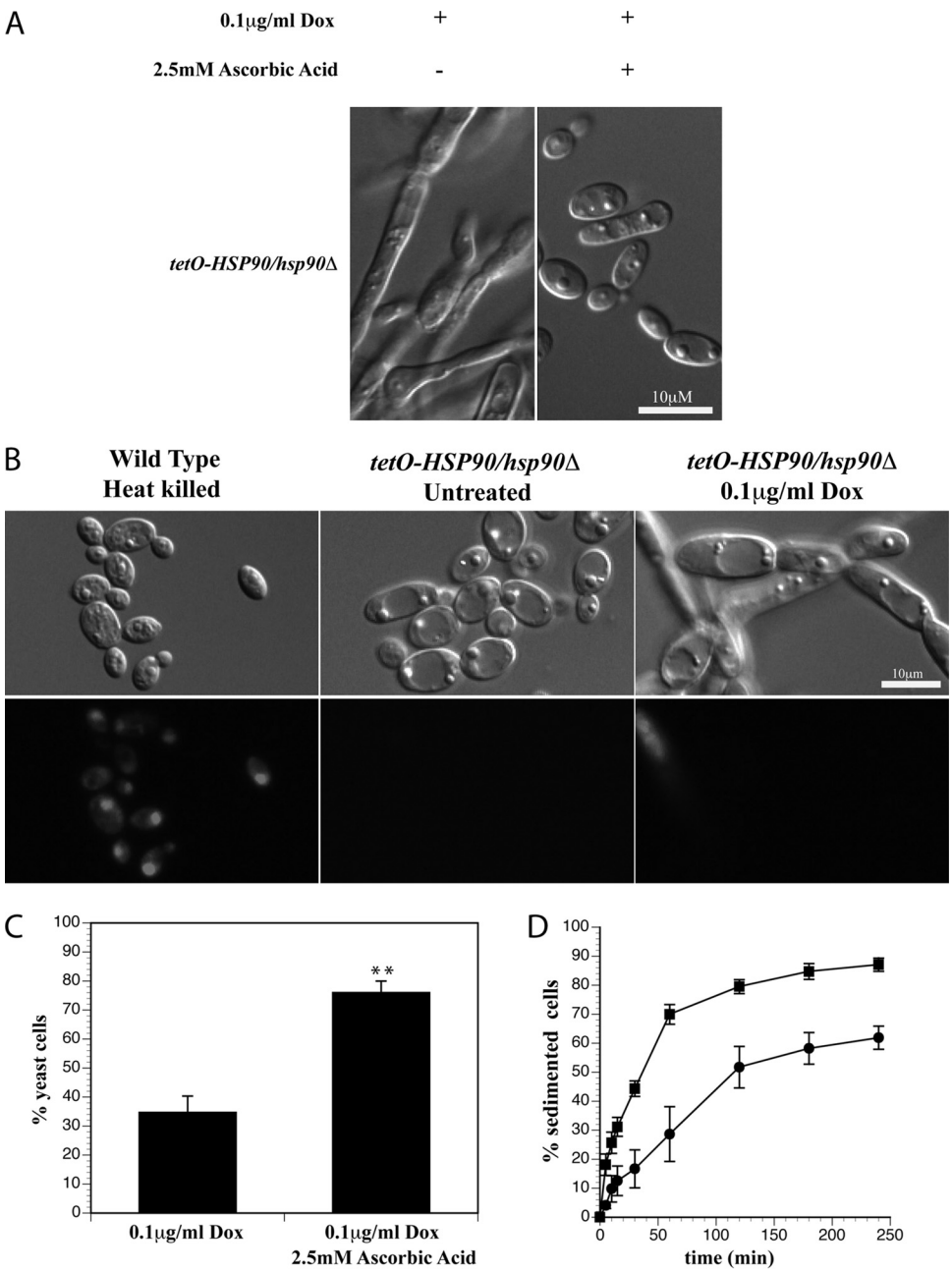

FIG 2 Ascorbic acid influences phenotypes caused by Hsp90 genetic depletion. (A) Ascorbic acid (2.5 mM) counteracts elongated growth upon genetic depletion of Hsp90 (0.1 μg/ml doxycycline [Dox]). The images were taken after 24 h. (B) Genetic depletion of Hsp90 for extended periods in the presence of 0.1 μg/ml doxycycline is not completely lethal. Propidium iodide-stained heat-killed wild-type cells (15 min at 80°C) clearly illustrate a complete lethal phenotype, as indicated by the staining, while cells genetically depleted of Hsp90 closely resemble the untreated wild-type cells. The images were taken at 24 h. (C) The percentage of yeast cells grown for 24 h in the presence of ascorbic acid was significantly (**, $P < 0.001$) higher than under the untreated conditions. (D) The sedimentation rate of cultures of a *Candida* strain in which the remaining *Hsp90* was under the control of a tetracycline-repressible promoter was monitored in liquid YPD rich medium supplemented with 0.1 μg/ml Dox (■) and 0.1 μg/ml Dox plus 2.5 mM ascorbic acid (●). The error bars indicate standard deviations.

fected, whereas the *UPC2* reconstituted strain EC-7 resulted in a phenotype similar to that of the wild-type strain (Fig. 4B). These results indicate that the effect of ascorbic acid on the morphological response upon inhibition of Hsp90 is Upc2 dependent.

Hsp90 inhibition or depletion results in reduced intracellular ergosterol levels. To determine the mechanism by which ascorbic acid and Upc2 affect elongated cell growth, we tested the roles of genes whose expression is regulated by Upc2, such as the drug efflux pump genes *CDR1* and *MDR1* (17, 35). First, we hypothesized that ascorbic acid could increase the efflux of GdA, which was reported to be a substrate of the human ATP-binding cassette (ABC) transporter efflux pump, Mdr1 (36, 37). If this hypothesis were valid in *C. albicans*, one would expect mutants lacking Cdr1, a fungal ABC multidrug transporter (38), to elongate in the presence of both GdA and ascorbic acid. In addition, one would expect a strain devoid of this efflux pump to

TABLE 3 Transcription factors that upon overexpression block GdA-induced elongated growth

TF	Description[a]
Upc2	Zn_2-Cys_6 transcriptional regulator of ergosterol biosynthetic genes and sterol uptake
Cap1	AP-1 family bZIP transcription factor involved in drug resistance and oxidative-stress regulation
Dot6	Uncharacterized ORF encoding a protein with a predicted role in telomeric gene silencing and filamentation
Rtg3	Uncharacterized ORF encoding a putative transcription factor with a bZIP DNA-binding motif
Gcn4	bZIP transcription factor involved in amino acid control response

[a] ORF, open reading frame.

be hypersusceptible to GdA. However, neither hypersusceptibility to GdA nor impairment of the morphogenetic effect of ascorbic acid was observed for the *cdr1*Δ/*cdr1*Δ mutant (data not shown).

We also investigated Mdr1, a member of the multidrug resistance 1 (MDR1) major facilitator family. The ascorbic acid-mediated effect was not affected in strains overexpressing *MDR1* (39) or with *MDR1* deleted, and they were not hypersusceptible to GdA (data not shown). Together, these results rule out the hypothesis that an ascorbic acid-mediated increased efflux of GdA causes the ascorbic acid-mediated phenotype.

In addition to *CDR1* and *MDR1*, expression of *ERG11*, a key gene in the ergosterol biosynthesis pathway, is also under the control of Upc2 (15, 22, 40). We therefore reasoned that levels of Erg11 might play an instrumental role in the morphogenetic transition regulated by Hsp90. The role of Erg11 in ascorbic-acid-induced inhibition was investigated using an engineered strain in which the only *ERG11* allele is under the control of a tetracycline-repressible promoter (41). Cells in which the transcription of *ERG11* was repressed by the addition of doxycycline but that were still viable (see Fig. S3 in the supplemental material) were tested for GdA-induced elongation in the presence of ascorbic acid. As shown in Fig. 5A (compare images a, c, g, and h), Erg11 expression is required for the ascorbic acid-induced inhibition of cell elongation upon GdA treatment. We also tested an *ERG11* overexpression strain (~3.5-fold higher expression than the control strain) (42), but as shown in Fig. 5B, such increased dosage of *ERG11* was not able to suppress the elongated phenotype upon Hsp90 inhibition, demonstrating that increased dosage of Erg11 is not sufficient to block GdA-induced elongation. We also investigated whether ascorbic acid induces *ERG11* expression in the presence and absence of Hsp90 inhibitors. In the wild-type strain, there is a significant drop in *ERG11* expression upon GdA treatment, while simultaneous addition of ascorbic acid to the treatment leads to increased transcription of *ERG11* (Fig. 5C). This increase is absent in a *upc2*Δ/*upc2*Δ mutant, suggesting a role for Upc2 as an important regulator.

Erg11's eminent role in the regulation of sterol biosynthesis (15, 22, 43) and ascorbic acid-mediated effects on the Hsp90 morphogenetic circuitry (Fig. 1A and 2A) suggest a possible relationship between the ergosterol content and ascorbic acid-induced inhibition of cell elongation in the presence of GdA. To verify the impact of impaired Hsp90 function on ergosterol levels, we determined ergosterol levels in the wild-type and *upc2*Δ/*upc2*Δ strains upon treatment with GdA (10 μM) and/or ascorbic acid (2.5 mM). As shown in Fig. 6A, a significant decrease in ergosterol levels can be observed after addition of GdA to the wild-type strain ($P < 0.05$) and the *upc2*Δ/*upc2*Δ mutant ($P < 0.001$) compared to the corresponding untreated control strains. Whereas ergosterol levels are restored when ascorbic acid (2.5 mM) is added together with GdA to the wild-type strain, addition of both compounds to *upc2*Δ/*upc2*Δ cells failed to show a similar restoration. The difference in ergosterol levels compared to the untreated *upc2*Δ/*upc2*Δ mutant remained significant ($P < 0.05$). As can be seen, there was

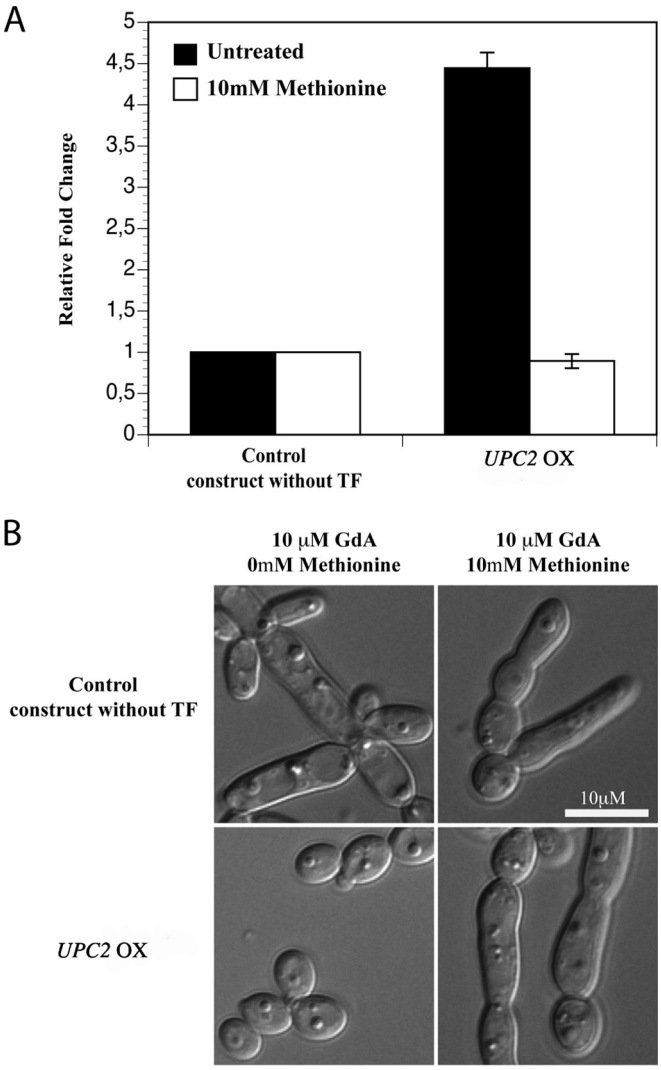

FIG 3 Overexpression of *UPC2* represses GdA-induced elongated growth. (A) Quantitative real-time PCR of *UPC2* expression under the control of the inducible *MET3* promoter in the *UPC2* overexpression (OX) strain and the control strain carrying an identical construct with the exception of the transcription factor. Promoter-repressing conditions in the presence of 10 mM methionine are represented by the white bars, while the black bars represent inducing conditions. The graph shows mean values with standard deviations from two independent experiments. (B) The inducible *MET3* promoter allows regulated expression. Overexpression of *UPC2* in the presence of GdA (10 μM) blocks the rise of the characteristic elongated growth form. The yeast form is reversed to the elongated structures under promoter-repressing conditions (represented by addition of 10 mM methionine). The control strain, carrying an identical expression construct except for the transcriptional regulator, displays a filamentous growth form under both promoter-repressing and -inducing conditions. The images were taken after 8 h of growth at 30°C in CSM−Met medium.

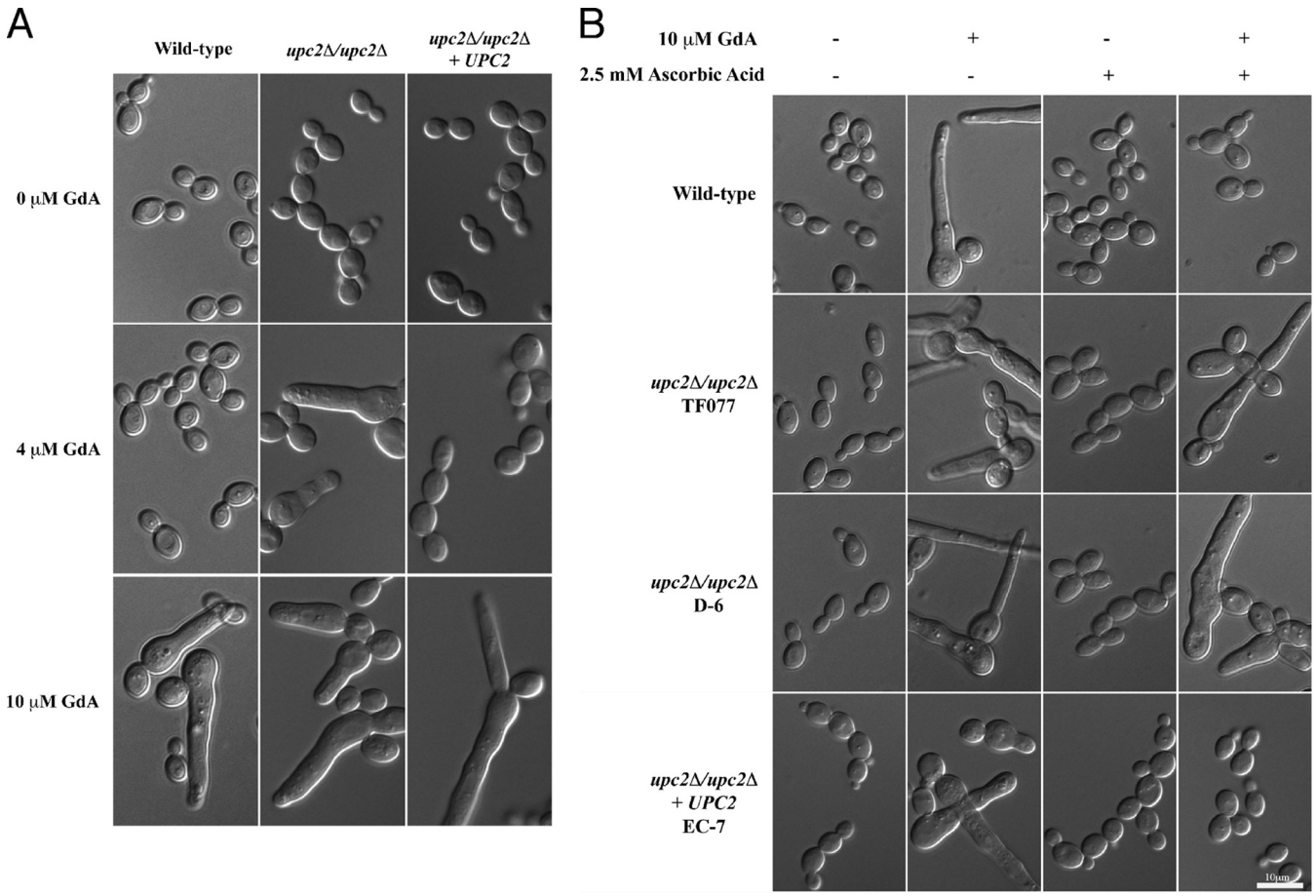

FIG 4 The *UPC2* deletion strain is hypersusceptible to GdA and impairs ascorbic acid-associated blockage of the elongated cell shape. (A) The *UPC2* deletion strain is hypersusceptible to GdA. The *upc2*Δ/*upc2*Δ (D-6) strain was tested for its hypersusceptibility to GdA by treating it with 4 μM GdA, a concentration that is unable to induce elongated growth in the corresponding wild type. The *UPC2* reintegrant strain (EC-7) (*upc2*Δ/*upc2*Δ + *UPC2*) displays a phenotype similar to that of the wild type. The images were taken after 8 h of growth at 30°C in YPD medium. (B) Deletion of *UPC2* impairs ascorbic acid-associated blockage of the elongated cell shape. Deletion strains were grown at 30°C in YPD rich medium supplemented with either 10 μM GdA, 2.5 mM L-ascorbic acid, or a combination of the two. While ascorbic acid blocks the GdA-induced filamentation of the wild-type strain, it is unable to do so in the *upc2*Δ/*upc2*Δ mutants D-6 and TF077, while the reconstitution of *UPC2* restores the yeast growth response. The images were taken after 8 h.

also a detectable decrease in ergosterol levels when ascorbic acid alone was administered, but this reduction was not significant ($P > 0.05$). These results point to a correlation between ergosterol levels and Hsp90-governed elongated growth. To determine whether reduced ergosterol levels were the result of oxidative stress caused by the absence of Upc2, as was previously claimed (44), we tested the two independent *upc2* mutant strains from the Homann collection (31) that we used throughout our study for their susceptibility to oxidative stress. Figure S4 in the supplemental material shows that the tolerance for H_2O_2 of the mutants is absolutely comparable to that of the corresponding isogenic wild-type strain, thus ruling out the hypothesis that deletion of *UPC2* could lower the anti-oxidative-stress potential of the cells. Different response of other *upc2* mutants to oxidative stress may have different causes, such as a different genetic background (44).

The results obtained with pharmacological inhibition of Hsp90 were confirmed using genetic depletion of Hsp90. The *tetO-HSP90/hsp90*Δ strain was grown in the presence and absence of doxycycline, and ergosterol levels were determined. As shown in Fig. 6B, depletion of Hsp90 results in a significant decrease in ergosterol levels ($P < 0.001$). Similar to pharmacological inhibition, addition of ascorbic acid results in restoration of ergosterol levels, with higher ergosterol levels in the presence of higher ascorbic acid levels. To rule out a more indirect effect of ascorbic acid on ergosterol levels via its antioxidant effect, we analyzed reactive oxygen species (ROS) production by way of rhodamine fluorescence in the wild-type strain and in the *Tet0-HSP90/hsp90*Δ strain in the presence or absence of doxycycline and with or without ascorbic acid. The fluorescence of doxycycline-treated cells, as well as of doxycycline- and ascorbic acid-treated cells, was comparable to that of untreated cells. H_2O_2-treated cells were used as the positive control (data not shown).

These results suggest that decreased ergosterol levels caused by Hsp90 inhibition (Fig. 6A and B) promote elongated growth. However, Fig. 5A (image d) shows that a reduction of ergosterol levels (by downregulating *ERG11* expression) was not sufficient to induce elongation, since elongated growth forms still required Hsp90 inhibition.

To our knowledge, this is the first time that a clear correlation between impaired/decreased Hsp90 expression and ergos-

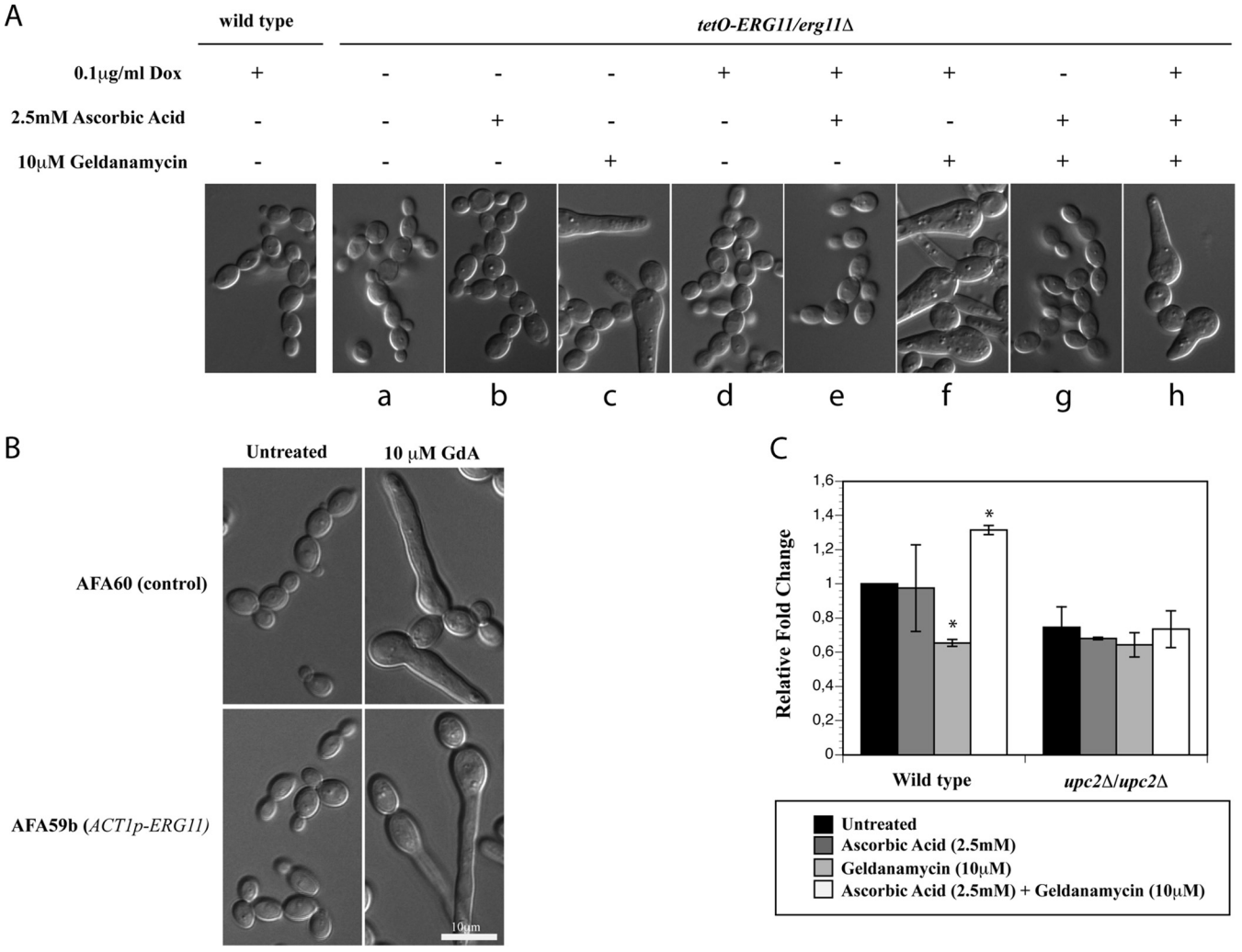

FIG 5 *ERG11* is required for the ascorbic acid-mediated effect. (A) A *tetO-ERG11/erg11*Δ strain was grown at 30°C in YPD rich medium supplemented with either 10 μM GdA, 2.5 mM L-ascorbic acid, 0.1 μg/ml doxycycline, or a combination of these substances. While ascorbic acid blocks elongation upon GdA treatment of the *tetO-ERG11/erg11*Δ strain in the absence of doxycycline, compromising *ERG11* expression is able to overcome this ascorbic acid-associated blockage. The images were taken after 8 h. (B) Overexpression of *ERG11* is not sufficient to block elongated growth caused by Hsp90 inhibition. *C. albicans* strains AFA60 (control) and AFA59b (*ACT1p-CaERG11*) were grown at 30°C in YPD rich medium supplemented with 10 μM GdA. GdA-induced elongation of both the control and the *ACT1p-CaERG11* strains shows that an increased dosage of Erg11 cannot suppress elongation. The images were taken after 8 h. (C) There is a significant drop (*, $P < 0.05$) in *ERG11* expression upon GdA treatment in the wild type, while simultaneous addition of ascorbic acid during this treatment leads to an increase. Interestingly, the latter observation is absent in the *upc2*Δ/*upc2*Δ mutant, suggesting a role for Upc2. The data are the means ± standard deviations of triplicate experiments.

terol levels has been observed. Moreover, addition of ascorbic acid restores ergosterol levels to wild-type levels, and this is clearer upon pharmacological inhibition than upon genetic depletion of Hsp90.

Upc2 is required for other ascorbic acid-mediated effects. It has been reported in previous studies that ascorbic acid reduces the antifungal effect of fluconazole, possibly via its antioxidant properties (45). The authors suggested that the antioxidant properties of ascorbic acid counteract fluconazole-induced reactive oxygen species, resulting in an effect similar to that obtained with other antioxidants, such as glutathione (45). In order to establish a general role for Upc2 in orchestrating ascorbic acid-mediated phenomena, we verified a possible relationship between ascorbic acid and fluconazole in a *upc2*Δ/*upc2*Δ strain by means of checkerboard assays, two-dimensional tests designed to measure drug-drug interactions. Fluconazole and ascorbic acid display antagonistic activities, as determined via the calculation of the FICI (FICI, 33) (Table 4). This kind of interaction is no longer detectable when the checkerboard is performed using cells of the *upc2*Δ/*upc2*Δ strain (FICI, 0.75) (Table 4). Thus, the combination of ascorbic acid with fluconazole no longer displayed antagonistic activity in cells lacking the Upc2 transcription factor.

DISCUSSION

A decreased dosage of Hsp90, as well as its pharmacologic depletion, results in a morphology change of *C. albicans* at low temperature from yeast to elongated cells (13). The molecular mechanism of this morphogenetic transition has been studied extensively over the last few years (46, 47). We contribute to this characterization by showing that reduced levels of ergosterol re-

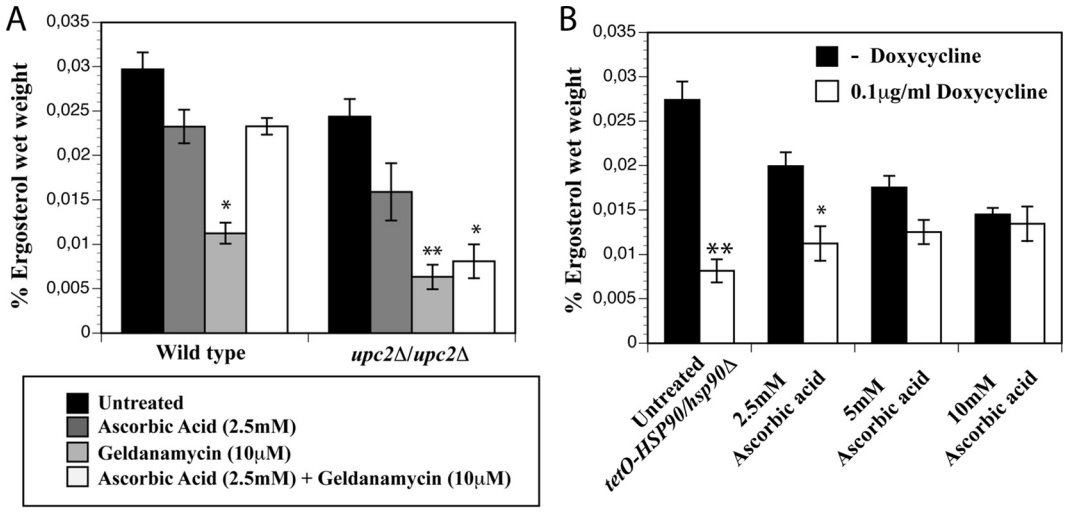

FIG 6 Hsp90 inhibition or depletion results in reduced intracellular ergosterol levels. (A) Ergosterol measurements in the wild type and the *upc2*Δ/*upc2*Δ mutant show a significant decrease in ergosterol content after pharmacological inhibition of Hsp90 in comparison with the representative untreated control (*, $P < 0.05$; **, $P < 0.001$). Addition of ascorbic acid under these conditions was reflected by an increase of ergosterol content only in the wild type. The results are means ± standard deviations of at least three independent measurements. (B) Ergosterol measurements in the *tetO-HSP90/hsp90*Δ strain show a similar significant decrease in ergosterol content in the presence of doxycycline (0.1 μg/ml) compared to the untreated control (*, $P < 0.05$; **, $P < 0.001$). This drop in ergosterol levels has great similarities to pharmacological inhibition by GdA. Addition of ascorbic acid is reflected by an increase in ergosterol content in the presence of doxycycline, while a general decrease is noticeable in its absence. The data are means ± standard deviations of triplicate experiments.

sult from GdA-mediated impairment of Hsp90 and that these changes correlate with elongated growth. The effect of GdA can be suppressed by addition of ascorbic acid, which again increased levels of ergosterol to normal and resulted in cell growth in the yeast form. Ascorbic acid suppression did not depend on its antioxidant activity but on the transcriptional regulator Upc2, as in a *upc2*Δ/*upc2*Δ mutant ascorbic acid cannot restore ergosterol levels to normal upon GdA treatment. In summary, ascorbic acid can block GdA-dependent elongated growth by restoring normal ergosterol levels in a Upc2-dependent fashion.

In the course of our work, we found that several antioxidants interfered with the activity of GdA. Apart from its known inhibition of Hsp90, GdA is also involved in the generation of superoxide radicals, which is attributed to the presence of its quinone group (48). The presence of molecules with antioxidant properties alleviates the detrimental effects of oxidative stress imposed by free radicals. A variety of molecules, such as reduced glutathione (GSH) and its derivatives, cysteine, dithiothreitol, and ascorbic acid, are able to fulfill these protective requirements (49). It is possible that the superoxide radicals produced in the presence of GdA could affect elongated cell shape formation, and addition of ascorbic acid would then block this through its antioxidant activities. This would fit with data obtained by Patterson et al. (50), who showed that H_2O_2-mediated activation of Cap1 was a prerequisite for yeast-to-hypha transition.

However, the fact that the antagonistic effect of ascorbic acid on morphology is observed on cells genetically depleted of Hsp90, as well as on cells whose Hsp90 is inhibited by GdA, suggests that ascorbic acid acts directly or indirectly on Hsp90 or Hsp90-mediated processes. Other authors have reported an influence of ascorbic acid or its analogues on morphogenetic transitions in *C. albicans* (23, 24, 51). Nasution and colleagues (23) reported that addition of ascorbic acid not only lowered the intracellular concentration of reactive oxygen species, but also inhibited H_2O_2 and serum-initiated hyphal differentiation. Ojha and coworkers (24) similarly demonstrated inhibition of serum-induced hyphal formation by ascorbic acid and suggested a mode of action by interruption of the hyphal formation signal of *C. albicans*.

During the TF overexpression screening, we observed that cysteine directly affected the activity of GdA, as no elongated growth could be observed in the wild-type strain. This could be explained by earlier reports describing that thiol-containing antioxidants (e.g., cysteine) could physically interact with Hsp90 inhibitors, such as GdA and radicicol, and render them inactive as a consequence of the thiol-mediated interaction (52–57). This means that one has to be careful when using the *MET3* promoter in combination with GdA or radicicol, as in this case cysteine, which is used to repress the promoter, will affect the activity of GdA and radicicol. This is the reason why in our experiments we used only methionine to repress the *MET3* promoter.

TABLE 4 Antagonistic effect of ascorbic acid on fluconazole[a]

Strain	MIC_{FLC} (μg/ml)	$MIC_{FLC/AA}$ (μg/ml)	MIC_{AA} (μg/ml)	$MIC_{AA/FLC}$ (μg/ml)	FIC index[b]
Wild type	2	>64	1761.2 (10 mM)	1761.2 (10 mM)	33
*upc2*Δ/*upc2*Δ	0.25	0.125	1761.2 (10 mM)	440.3 (2.5 mM)	0.75

[a] The antagonistic effect of ascorbic acid on fluconazole is not present in a *upc2*Δ/*upc2*Δ mutant. MIC_{FLC} and MIC_{AA} are the MICs of fluconazole and ascorbic alone, while $MIC_{FLC/AA}$ and $MIC_{AA/FLC}$ are the MICs of ascorbic acid and fluconazole in combination.
[b] Interpretation of the FIC index was performed as previously described (30).

One of the TFs that inhibited GdA-induced morphogenesis was Upc2, a Zn_2Cys_6 transcription factor, a key regulator of drug efflux pump expression and ergosterol biosynthesis (15, 35). Here, we show that Upc2 is required for ascorbic acid inhibition of cell elongation upon compromised Hsp90 function. Previously, it was shown that Upc2 is a key regulator of the ascorbic acid-mediated effect on fluconazole, as it decreases the antifungal effect of fluconazole, thus displaying a protective role in fungi (45). Together, these data show that Upc2 plays a central role as the transcriptional regulator in ascorbic acid-associated phenomena. Ascorbic acid is very important for human life because of its antioxidant properties, protecting cells from oxidative stress (58). The use of ascorbic acid is even clinically relevant for the treatment of a variety of diseases, such as respiratory tract infections (59, 60).

One of the genes that is regulated by Upc2 is *ERG11*, a gene involved in ergosterol biosynthesis and the molecular target of the azole antifungals (15, 22, 43, 61, 62). Previous work demonstrated that lower ergosterol levels, caused by ergosterol biosynthesis inhibitors, such as azoles, have an inhibitory effect on hypha formation (63, 64). However, elongated growth forms resulting from impaired Hsp90 function are not considered true hyphae (14, 65, 66). Consistent with the relationship between ergosterol depletion and *C. albicans* morphogenesis mentioned by Victoria et al. (67), we report a correlation between ergosterol content and elongated growth caused by impairment of Hsp90 function. The morphogenetic switch to elongated growth forms by either pharmacological inhibition or genetic depletion is accompanied with a significant decrease in ergosterol content. One could question whether this drop in ergosterol is really the cause or rather a consequence of the morphogenetic transition upon Hsp90 inhibition. The formation of elongated growth structures under low ergosterol levels could be interpreted as a cellular stress response caused by the disruption of sterol homeostasis. However, this seems unlikely, since our results argue against an elongation-inducing role for low ergosterol levels. First, we demonstrated that reduced levels of Erg11 are not sufficient to promote elongation (Fig. 5A). In addition, it was found that increasing concentrations of ascorbic acid reduced ergosterol content without initiating elongated growth (Fig. 6A and B). The latter data are consistent with previous observations by Singh et al. (68), who reported low ergosterol levels in *C. albicans* cells when they were grown in the presence of ascorbic acid.

On the other hand, the increase of ergosterol to wild-type levels in the presence of GdA upon addition of ascorbic acid is absent in a *upc2*Δ/*upc2*Δ mutant, suggesting the importance of Upc2 as a transcriptional regulator of Erg11 to overcome the drop in ergosterol levels caused by impaired Hsp90 (Fig. 6A). As mentioned above, we showed that an increased dosage of *ERG11* alone was not sufficient to overcome the induction of elongated growth upon pharmacological inhibition of Hsp90. This could indicate that ergosterol biosynthesis is not the most important Upc2-mediated effect in response to ascorbic acid or that the expression of other *ERG* genes could be regulated by Upc2. We are currently identifying other Upc2-regulated genes by performing chromatin immunoprecipitation-sequencing (ChIP-seq) and RNA-seq analyses. We cannot exclude a possible connection with the previously established pathways in the Hsp90-dependent morphogenetic circuitry, such as the cAMP-PKA and cell cycle pathways or the Pho85-Pcl1-Hms1 module (13, 14, 46, 65, 69) or a novel, yet-to-be-determined pathway. Ascorbic acid or Upc2 may be required for the modulation of an inhibitor of these Hsp90 signaling modules.

In conclusion, our study elucidates the molecular circuitry through which ascorbic acid influences Hsp90-dependent *C. albicans* morphogenesis, involving the transcriptional regulator Upc2. We found that ascorbic acid can block GdA-dependent elongated growth by restoring normal ergosterol levels in a Upc2-dependent fashion. We suggest that influencing ergosterol biosynthesis via Erg11 is not the primary mode of action of Upc2-mediated effects in response to ascorbic acid, as overexpression of *ERG11* did not block elongation upon GdA treatment. Further research is required to explore the interesting relationship between Hsp90, Upc2, Erg11, and the morphogenetic machinery.

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