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 ofazole 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.

*Ascorbic acid inhibition of Candida albicans* Hsp90-mediated morphogenesis occurs via the transcriptional regulator Upc2.
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 adenylyl 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, 1% cysteine, 0.5% cysteine, glutathione, diethiothreitol [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.

Transmission 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})_{2}SO_{4}, 0.5% (NH_{4})_{2}SO_{4}, pH 5.5] supplemented with 0.1% uridine and the indicated treatment (10 μM geldanamycin). Microscopy. Imaging of the cells was done by differential interference contrast microscopy using the Zeiss Axioplan 2 microscope. Images were taken using the Zeiss Axiocam MRc.

#### Table 1: Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>SC5314</td>
<td>CAI4</td>
<td>Wild type</td>
<td>70</td>
</tr>
<tr>
<td>AFA60a</td>
<td>CAI4 transformed with URA3 vector</td>
<td>una3Δ::imm434/ura3Δ::imm434 iro1Δ::iro1Δ::imm434</td>
<td>71</td>
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<tr>
<td>AFA59b</td>
<td>CAI4 carrying extra copy of CaERG11 under the control of ACT1 promoter</td>
<td>una5Δ::imm434/ura3Δ::imm434 iro1Δ::iro1Δ::imm434</td>
<td>42</td>
</tr>
<tr>
<td>DSY448</td>
<td>cdr1Δ/cdr1Δ mutant</td>
<td>cde1Δ::hisG-URA3-hisG/cde1Δ::hisG</td>
<td>72</td>
</tr>
<tr>
<td>DSY465</td>
<td>mdr1Δ/mdr1Δ mutant</td>
<td>ben1Δ::hisG-URA3-ben1Δ::hisG</td>
<td>72</td>
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<tr>
<td>CMDR1E2A and -B</td>
<td>CAI4 carrying CaMDR1 under the control of ADH1 promoter</td>
<td>arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434</td>
<td>39</td>
</tr>
<tr>
<td>SN152</td>
<td>Background strain TF wild type and TF077</td>
<td>arg4Δ/arg4Δ leu2Δ/LEU2 his1Δ/HIS1 URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434</td>
<td>73</td>
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<tr>
<td>TF wild type</td>
<td>Control wild-type strain (paired “wild-type” TF077)</td>
<td>arg4Δ/arg4Δ leu2Δ/LEU2 his1Δ/HIS1 URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434</td>
<td>31</td>
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<td>TF077</td>
<td>upc2Δ/upc2Δ mutant</td>
<td>As SN152, but upc2Δ::HIS1/upc2Δ::LEU2</td>
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<td>BWP17 (TW14901)</td>
<td>Background strain D-6 and EC-7</td>
<td>una3Δ::imm434/ura3Δ::imm434 iro1Δ::iro1Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ/arg4Δ</td>
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<td>D-6 (TW14903)</td>
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<td>As BWP17 but upc2Δ::URA3/upc2Δ::ARG4</td>
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<td>EC-7 (TW14904)</td>
<td>Reconstituted strain</td>
<td>As D-6 but upc2Δ::URA3/upc2Δ::UPC2::HIS1</td>
<td>22</td>
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<td>pTET-ERG11</td>
<td>CAI4 with doxycycline-repressible ERG11 as the only ERG11 allele</td>
<td>arg4Δ/arg4Δ his1Δ/his1Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434 HIS1/his1Δ::P_{TET}–ERG11/erg11</td>
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<td>CaLC1411 (CaLC436)</td>
<td>Strain with doxycycline-repressible HSP90 as the only HSP90 allele</td>
<td>arg4Δ/arg4Δ his1Δ/his1Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm443 HIS1/his1Δ::P_{TET}–HSP90/erg11</td>
<td>74</td>
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<td>SC2H3</td>
<td>One- and two-hybrid reporter strain in the SN152 background</td>
<td>Like SN152, but 5 × LexAop-ADH1b/HIS1 5 × LexAop-ADH1b/lacZ</td>
<td>27</td>
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<td>OX control</td>
<td>Strain carrying empty vector under MET3 promoter</td>
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<td>Stynen et al., unpublished</td>
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<td>Upc2OX</td>
<td>Strain carrying extra copy of UPC2 under MET3 promoter</td>
<td>As SC2H3, but MET3::pre::lecA::UPC2</td>
<td>Stynen et al., unpublished</td>
</tr>
<tr>
<td>Gcn4OX</td>
<td>Strain carrying extra copy of GCN4 under MET3 promoter</td>
<td>As SC2H3, but MET3::pre::lecA::GCN4</td>
<td>Stynen et al., unpublished</td>
</tr>
<tr>
<td>Cap1OX</td>
<td>Strain carrying extra copy of CAP1 under MET3 promoter</td>
<td>As SC2H3, but MET3::pre::lecA::CAP1</td>
<td>Stynen et al., unpublished</td>
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<tr>
<td>Dot6OX</td>
<td>Strain carrying extra copy of DOT6 under MET3 promoter</td>
<td>As SC2H3, but MET3::pre::lecA::DOT6</td>
<td>Stynen et al., unpublished</td>
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<td>Rtg3OX</td>
<td>Strain carrying extra copy of RTG3 under MET3 promoter</td>
<td>As SC2H3, but MET3::pre::lecA::RTG3</td>
<td>Stynen et al., unpublished</td>
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* 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.
obtained with a Zeiss Axioscam MRc5 camera using Axiosview 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 t-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_{1} − OD_{OD}) × 100, where OD_{1} is the initial OD taken at time zero and OD_{OD} 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 pCIH. 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 pCIH-PTF library. The resulting plasmids were integrated into the two-hybrid reporter strain S2CH3 (27) between XOG1 and HOLLI loci on chromosome I 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 μg/Mda, 2.5 mM ascorbic acid, or a combination of both. After the indicated time, 220 ODs of cells at 60 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_{2}O 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 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 CaERG1 up and CaERG1 down, UPC2 fw and UPC2 rv, HSP90 fw and HSP90 rv, and TEF1α-fw and TEF1α-rv as the primer pairs (Table 2). The degree of regulation was determined by the ΔΔCT 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.

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: FICI_{index} = FICA + FICI_{GdA} = (MIC_{A-comb}/MIC_{A-alone}) + (MIC_{GdA-comb}/MIC_{GdA-alone}), where MIC_{A-alone} and MIC_{GdA-alone} are the MICs of ascorbic acid and fluconazole alone and MIC_{A-comb} and MIC_{GdA-comb} are the MICs of ascorbic acid and fluconazole in combination. Interactions were referred to as synergistic when the FICI_{index} value was ≤0.5 and antagonistic when the FICI_{index} value was >4. FICI_{index} values that were >0.5 or ≤4 indicated no interaction between ascorbic acid and fluconazole (30). There was no difference in FICI 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_{2}O_{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...
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.

**Umc2 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 upc2Δ/upc2Δ 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-

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**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.
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

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**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.
be hypersusceptible to GdA. However, neither hypersusceptibility to GdA nor impairment of the morphogenetic effect of ascorbic acid was observed for the cdr1Δ/cdr1Δ 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 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 upc2Δ/upc2Δ 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 upc2Δ/upc2Δ 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 upc2Δ/upc2Δ 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 upc2Δ/upc2Δ cells failed to show a similar restoration. The difference in ergosterol levels compared to the untreated upc2Δ/upc2Δ mutant remained significant (P < 0.05). As can be seen, there was

<table>
<thead>
<tr>
<th>TF</th>
<th>Description</th>
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<tr>
<td>Upc2</td>
<td>Zn2-Cys6, transcriptional regulator of ergosterol biosynthetic genes and sterol uptake</td>
</tr>
<tr>
<td>Cap1</td>
<td>AP-1 family bZIP transcription factor involved in drug resistance and oxidative-stress regulation</td>
</tr>
<tr>
<td>Dot6</td>
<td>Uncharacterized ORF encoding a protein with a predicted role in telomeric gene silencing and filamentation</td>
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<td>Rtg3</td>
<td>Uncharacterized ORF encoding a putative transcription factor with a bZIP DNA-binding motif</td>
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<tr>
<td>Gcn4</td>
<td>bZIP transcription factor involved in amino acid control response</td>
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A ORF, open reading frame.
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\(_2\)O\(_2\) 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 \(tetrO-H\_SP90/hsp90\Delta\) 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 \(Tet0-H\_SP90/hsp90\Delta\) 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\(_2\)O\(_2\)-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-
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 \( \text{upc2}\)/\( \text{H9004} \)/\( \text{upc2}\)/\( \text{H9004} \) 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 \( \text{upc2}\)/\( \text{H9004} \)/\( \text{upc2}\)/\( \text{H9004} \) 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 \( \text{C. albicans} \) 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-
the presence of reactive oxygen species, but also inhibited H$_2$O$_2$ and addition of ascorbic acid not only lowered the intracellular concentration of ergosterol in the wild-type strain. This could be explained by earlier reports describing that thiol-containing antioxidants such as GdA and radicicol, and render them inactive as a consequence of the thiol-mediated interaction (52–57). Nasution and colleagues (23) 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 \textit{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 \textit{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 \textit{MET3} promoter.

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 \textit{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$_2$O$_2$ 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 \textit{C. albicans}.

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$_2$O$_2$-mediated activation of Cap1 was a prerequisite for yeast-to-hypha transition.

### TABLE 4 Antagonistic effect of ascorbic acid on fluconazole

| Strain        | MIC\textsubscript{FLC} (µg/ml) | MIC\textsubscript{FLC/AA} (µg/ml) | MIC\textsubscript{AA} (µg/ml) | MIC\textsubscript{AA/FLC} (µg/ml) | FIC index
|---------------|-------------------------------|----------------------------------|------------------------------|----------------------------------|-----------
| Wild type     | 2                             | >64                              | 1761.2 (10 mM)               | 1761.2 (10 mM)                  | 33        |
| \textit{upc2Δ/upc2Δ} | 0.25                          | 0.125                            | 1761.2 (10 mM)               | 440.3 (2.5 mM)                  | 0.75      |

\textsuperscript{a}The antagonistic effect of ascorbic acid on fluconazole is not present in a \textit{upc2Δ/upc2Δ} mutant. MIC\textsubscript{FLC} and MIC\textsubscript{AA} are the MICs of fluconazole and ascorbic alone, while MIC\textsubscript{FLC/AA} and MIC\textsubscript{AA/FLC} are the MICs of ascorbic acid and fluconazole in combination.

\textsuperscript{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,Cys, 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 impaired Hsp90 function. 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 upc2Δ/upc2Δ 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.

ACKNOWLEDGMENTS

We are grateful to L. Cowen, O. Homann, J. Morschhäuser, D. Sanglard, T. White, and T. Roemer for providing strains. We especially thank Ilse Palmons for excellent technical assistance.

This work was supported by a grant from the Flemish Institute for Science and Technology (IWT) to F.V.H. and by the Fund for Scientific Research Flanders (FWO) and the Research Fund of KU Leuven.

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October 2014 Volume 13 Number 10 eca...


