Candida albicans RFX2 Encodes a DNA Binding Protein Involved in DNA Damage Responses, Morphogenesis, and Virulence

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We previously showed that Candida albicans orf19.4590, which we have renamed RFX2, expresses a protein that is reactive with antibodies in persons with candidiasis. In this study, we demonstrate that C. albicans RFX2 shares some functional redundancy with Saccharomyces cerevisiae RFX1. Complementation of an S. cerevisiae rfx1 mutant with C. albicans RFX2 partially restored UV susceptibility and the repression of DNA damage response genes. DNA damage- and UV-induced genes RAD6 and DDR48 were derepressed in a C. albicans rfx2 null mutant strain under basal conditions, and the mutant was significantly more resistant to UV irradiation, heat shock, and ethanol than wild-type strain SC5314. The rfx2 mutant was hyperfilamentous on solid media and constitutively expressed hypha-specific genes HWP1, ALS3, HYR1, ECE1, and CEK1. The mutant also demonstrated increased invasion of solid agar and significantly increased adherence to human buccal epithelial cells. During hematoanogenously disseminated candidiasis, mice infected with the mutant had a significantly delayed time to death compared to the wild type. During oropharyngeal candidiasis, mice infected with the mutant had significantly lower tissue burdens in the oral cavity and esophagus at 7 days and they were less likely to develop disseminated infections because of mucosal translocation. The data demonstrate that C. albicans Rfx2p regulates DNA damage responses, morphogenesis, and virulence.

Candida albicans is a versatile opportunistic pathogen that causes diverse diseases in humans, ranging from oropharyngeal candidiasis (OPC) and other mucosal infections to life-threatening disseminated bloodstream infections. Indeed, C. albicans is remarkable for its ability to cause invasive diseases of virtually all tissues and major organs. Several properties that contribute to candidal virulence have been characterized; these include adherence to host cells, secretion of hydroltic enzymes, sequestration of iron, phenotypic switching, and the reversible transitioning from single-cell blastospores to forms with extended filaments (morphogenesis) (10). A large number of individual genes involved in these processes have been implicated in virulence through targeted disruption and testing of mutant strains in animal models. Rather than depending upon a dominant virulence factor, C. albicans achieves its success as a pathogen through coordinated expression of multiple genes as it senses and adapts to particular in vivo environments (47). As such, the regulation of biological processes important to the proliferation and survival of C. albicans cells within infected hosts has become an active area of investigation.

As a strategy to identify C. albicans genes that are expressed during the course of candidiasis in humans, we previously used pooled sera from human immunodeficiency virus-infected patients with active oropharyngeal or esophageal candidiasis to screen a C. albicans genomic DNA expression library (12, 36). We identified over 60 C. albicans genes that encoded proteins of diverse function that reacted with antibodies in the pooled sera (12, 36). We implicated several proteins identified by our screening in the regulation of yeast-hyphal morphogenesis and the pathogenesis of mucosal and/or disseminated candidiasis (3, 4, 12–14, 37). Among these previously unstudied regulators of candidal virulence were Not5p, a component of the CCR-NOT transcription-regulatory complex (13), Irs4p, an EH domain-containing protein that interacts with the 5′ phosphatase Inp51p and regulates phosphatidylinositol-(4,5)-bisphosphate levels (3, 4), and Set1p, a histone 3 lysine 4 methyltransferase (37). Interestingly, these proteins, like many identified by our screening, are known or predicted to localize to intracellular compartments, suggesting that they appear at the C. albicans cell surface at some point in the life cycle or that they are released from cells following cell death (13).

One of our previously unstudied genes corresponds to orf19.4590 in the Candida Genome Database (http://www.candidagenome.org). orf19.4590 encodes a protein of 1,112 amino acids that contains an RFX domain of approximately 103 amino acids at positions 427 to 530. This domain has 25.8% amino acid identity to the RFX domain of Saccharomyces cerevisiae Rfx1p (also known as Cr1p). RFX domains are unique winged-helix DNA binding domains that are conserved across eukaryotes (18, 20). Rfx1p, the sole member of the RFX protein family in S. cerevisiae, localizes transcriptional repressors to the promoters of DNA damage response genes like RNR2, -3, and -4 and HOG1 (25, 53). In response to DNA damage, Meclp hyperphosphorylates Rad9p, which activates the protein kinase Rad53p (40, 49, 51). S. cerevisiae Rad53p is required for all transcriptional and cell cycle arrest responses (1, 52), the former of which are mediated, at least in part, by Rfx1p. When Rfx1p is phosphorylated by Rad53p, the transcriptional repressor complex is deactivated and DNA damage
TABLE 1. Candida albicans and Saccharomyces cerevisiae strains used in this study

| Strain            | Genotype or description                  | Reference or source |
|-------------------|-----------------------------------------|---------------------|
| SC5314            | Clinical isolate                        | 23                  |
| RFX2-1            | RFX2pΔf2Δ::S. cerevisiae rfx1::FRT       | This study          |
| RFX2-2            | RFX2pΔf2Δ::FRT                          | This study          |
| RFX2-3            | RFX2pΔf2Δ::FRT                          | This study          |
| RFX2-4 (rfx2 mutant)   | RFX2pΔf2Δ::FRT                          | This study          |
| RFX2-6 (reinsertion)| RFX2pΔf2Δ::FRT                          | This study          |
| BY4743            | Wild-type S. cerevisiae                 | Open Biosystems    |
| Hom14D-1-34125    | S. cerevisiae rfx1 homozygous diploid    | Open Biosystems    |
|                   | mutant                                   |                     |
| Hom14D-1-34125 complemented with C. albicans orf19.4590 | Hom14D-1-34125 | This study |
|                   | [yPB1-ADH1::orf19.4590]                 |                     |
| Hom14D-1-34125 complemented with S. cerevisiae rfx1 | Hom14D-1-34125 | This study |
|                   | [yPB1-ADHP::ScRFX1]                     |                     |

Materials and methods

Strains and growth condition. C. albicans strains used or constructed in this study are described in Table 1. All strains were routinely grown in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 1% Bacto peptone, 2% dextrose) at 30°C unless otherwise noted. To induce hyphal formation in liquid media, strains were grown in YPMal (1% yeast extract, 1% Bacto peptone, 2% maltose) at 30°C. Following growth on solid media, overnight grown cultures were grown on YPD agar plates supplemented with 15 μg/ml nourseothricin, and incubated at 30°C. Colonies were parallel streaked on YPD agar plates supplemented with 15 μg/ml nourseothricin and YPD agar plates supplemented with 15 μg/ml of nourseothricin, and incubated at 30°C. Smaller colonies were selected and confirmed by PCR and Southern blotting. The cassettes were released by digesting with SacI/SacII and SacI restriction sites (underlined). Following amplification, the fragments were ligated sequentially into the plasmid pSFS2, resulting in pSFS2-RFX2. The disruption cassette was released by digesting pSFS2-RFX2 with AapI and SacI, and it was transformed into C. albicans strain SC5314 by electroporation. Nourseothricin-resistant transformants were selected on YPD plates containing 200 μg/ml of nourseothricin at 30°C.

After confirmation of disruption by PCR and Southern analysis, Nmt1 colonies were grown in YPMal (1% yeast extract, 1% Bacto peptone, 2% maltose) at 30°C for 6 h. Thereafter, the culture was diluted, plated on YPD agar plates supplemented with 15 μg/ml nourseothricin, and incubated at 30°C. Smaller colonies were parallel streaked on YPD agar plates supplemented with 15 μg/ml nourseothricin, and YPD agar plates supplemented with 15 μg/ml of nourseothricin, and incubated at 30°C. Smaller colonies were selected and confirmed by PCR and Southern blotting. The cassettes were released by digesting with SacI/SacII and SacI restriction sites (underlined). Following amplification, the fragments were ligated sequentially into the plasmid pSFS2, resulting in pSFS2-RFX2. The disruption cassette was released by digesting pSFS2-RFX2 with AapI and SacI, and it was transformed into C. albicans strain SC5314 by electroporation. Nourseothricin-resistant transformants were selected on YPD plates containing 200 μg/ml of nourseothricin at 30°C.

RT-PCR. RNA was isolated using the Ribopure-Yeast kit (Ambion, Austin, TX). Complementing chromosomal DNA was removed by treatment with DNase I and removal reagents provided in the Ribopure-Yeast kit. cDNA was made using the ImProm-II reverse transcription system (Promega, Madison, WI). PCR was performed on cDNA templates using primers designed from the sequences of the genes of interest (Table 2), under the following conditions: 1 min at 94°C, 1 min at 55°C, and 1 min at 68°C, preceded by denaturation for 5 min at 94°C and followed by a final extension cycle for 7 min at 68°C. The reactions were initially carried out on 3 cycles; for individual genes, additional cycles were performed to verify that expression ratios remained constant until maximal amplification intensity was reached. The absence of genomic DNA contamination was confirmed by primers designed to amplify a region of the housekeeping gene EF1 (elongation factor 1 gene) as an internal mRNA control (12). EF1 contains an intron, such that PCR products from templates of genomic DNA and cDNA yield distinct product sizes of 891 and 526 bp, respectively. PCR products for the genes of interest were sequenced to verify that the desired C. albicans genes were amplified. Measurements of amplified band intensities were made with the ABI Prism 7700 SDS instrument (Applied Biosystems, Foster City, CA) based on relative-intensity measurements normalized against the ATG of RFX2 amplifier. The expression level was normalized to quantify nourseothricin-resistant transformants were selected on YPD plates containing 200 μg/ml of nourseothricin at 30°C.

Based on these observations, we hypothesized that C. albicans orf19.4590 is involved in DNA damage responses and morphogenesis. Given the role of morphogenesis in candidal virulence, we further hypothesized that deletion of orf19.4590 would adversely affect the pathogenesis of mucosal and disseminated candidiasis in mice.

Response genes are derepressed. As is true across eukaryotes (43), DNA checkpoint proteins are conserved in C. albicans. Moreover, they are essential for the filamentation of C. albicans cells in response to genotoxic stress (2, 45). Indeed, deactivation of the C. albicans Mecl-Rad53 pathway through deletion of RAD53 or RAD9 completely abolishes filamentation growth in response to DNA damage, including true hyphal growth (45). Conversely, activation of the DNA checkpoint by deletion of C. albicans RAD52, a gene responsible for the repair of double-stranded DNA breaks by homologous recombination, results in hyphal filamentous growth (2, 11, 15).

Based on these observations, we hypothesized that C. albicans orf19.4590 is involved in DNA damage responses and morphogenesis. Given the role of morphogenesis in candidal virulence, we further hypothesized that deletion of orf19.4590 would adversely affect the pathogenesis of mucosal and disseminated candidiasis in mice.
blot analysis that the transcript levels of EFB1 quantitated against C. albicans ACT1 in the wild-type SCS314, rfs2 null mutant, and heterozygous mutant strains were similar (data not shown). All RT-PCR experiments were performed in triplicate on at least two separate days, and the average and standard deviation of the concentration of each band were calculated. The levels of expression by S. cerevisiae and C. albicans mutant strains were compared with those from the appropriate wild-type strains. The difference in levels of expression was considered significant if the mutant/wild-type ratio of expression was >2 and the difference was statistically significant (P value < 0.05).

**UV irradiation.** To test for susceptibility to UV radiation, overnight-grown yeast cells were diluted with fresh YPD to a concentration of 1,000 CFU/ml; 200 μl of the diluted culture was spread onto YPD agar plates. The plates were then irradiated at 10.0 mJ/cm² (S. cerevisiae) or 15 mJ/cm² (C. albicans) with the UV cross-linker (Spectrolinker XL-1000; Fisher Scientific, Pittsburgh, PA). Nonirradiated yeast cells were plated as controls. The plates were incubated at 30°C for 24 h for colony enumeration.

For RT-PCR experiments, C. albicans cells were grown in individual wells of a six-well plate (Costar) at 30°C with 200 rpm shaking until log phase. The supernatant was removed after centrifugation at 1,200 × g for 10 min. The supernatant was removed after centrifugation at 10.0 mJ/cm² (C. albicans) or 15 mJ/cm² (S. cerevisiae) with the UV cross-linker. Immediately after irradiation, 2 ml of 30°C-rewarmed YPD was added to each well. After 1- and 3-hour incubations at 30°C, the yeast cells were harvested for RNA extraction.

**Susceptibility testing.** Log phase C. albicans cells were tested against hydroxyurea (10, 20, 30, 40, and 60 mg/ml), methyl methanesulfonate (MMS; twofold dilution from 12 mM to 0.325 mM), bleomycin (twofold dilution from 16 μg/ml to 0.5 μg/ml), streptonigrin (twofold dilution from 1.6 μg/ml to 0.05 μg/ml), ethyl methanesulfonate (twofold dilution from 120 mM to 1.67 mM), H₂O₂ (17.6, 13.2, 8.8, 6.6, and 4.4 mM), NaCl (0.7 mM), and ethanol (10%). For heat shock experiments, log phase C. albicans cells were diluted 10-fold in YPD and placed in 1-ml Eppendorf tubes. The tubes were placed in a 48°C water bath for 3 min, and 100 μl of the samples was plated onto Sabouraud dextrose agar (SDA) plates for colony enumeration.

**Agar invasion assay.** Overnight-grown Candida cells in YPD at 30°C were resuspended in fresh YPD to achieve a concentration of 10⁶ CFU/ml. Two microliters of this inoculum was spotted onto the surfaces of YPD agar plates, which were then incubated for 48 h at 30°C. Cells that had not invaded the agar were washed away by rubbing the plate with a gloved finger while rinsing under running water. Cross-sectional slices of the agar-invasive growth were photographed.

**Adherence to BECs.** Adherence to buccal epithelial cells (BECs) was measured as previously described (12, 39). Briefly, BECs pooled from three investigators were dispensed into 10 ml of phosphate-buffered saline (PBS), washed, and adjusted to a final concentration of 1 × 10⁶ cells/ml PBS. Then, 0.5 ml of the epithelial cells was incubated in a glass tube with 0.5 ml of C. albicans cells at a concentration of 1 × 10⁶ cells/ml in a shaking incubator at 35°C for 1 h. Following incubation, the cells were vacuum filtered through prewet 20-mm-diameter polycarbonate filters with 10-μm pore size (Millipore, Billerica, MA) mounted on a filter manifold (Millipore, Bedford, MA). Each filter was washed 10 times with PBS to remove unattached Candida cells. The washed filters were then removed and pressed gently onto glass slides. The slides were air dried, heat fixed for 1 min, Gram stained, and examined by light microscopy. The Candida cells attached to 100 BECs were counted as controls.

**Murine model of OPC.** A murine model of OPC was developed as previously described (13) with some modifications. Seven-week-old male ICR mice (Harlan Sprague) with an approximate weight of 20 g were immunosuppressed with 200 mg/kg of body weight of cortisone acetate (Sigma Aldrich, St. Louis, MO) in saline with 0.1% Tween 80 administered subcutaneously on the day before inoculation and day 1 and day 4 after inoculation. Mice received tetracycline hydrochloride in their drinking water (0.5 mg/ml), starting the day before inoculation. For infection, the mice were first anesthetized by intraperitoneal injec-

**TABLE 2. Primers used for RT-PCR**

| Species and gene | Primers | Source or reference |
|------------------|---------|---------------------|
| S. cerevisiae    |         |                     |
| RFX1             | 5'-ACTTCAAGGCGAAGTTTGCA-3', 5'-GTCAAAGGCGATGTGTGG-3' | This study |
| HUG1             | 5'-GCTTAAAACAAAGCATT-3', 5'-TCTTACAAATGTCAGAAC-3' | This study |
| RNR3             | 5'-ATTGCCATGAGGATGACTCTT-3', 5'-TGAAACTACAGGAATGCTG-3' | This study |
| EFB1             | 5'-CCTTGCGTGCTCAGTCCATGCATTG-3', 5'-ATAGCACATATCGTAGTG-3' | This study |
| C. albicans      |         |                     |
| RFX2             | 5'-GAGTACGCGCACCACCATATA-3', 5'-GCATTCAAGAGAAGAC-3' | This study |
| HWP1             | 5'-ATGACTCCAGCTGGTT-3', 5'-TTAGATCAAGAATGCG-3' | 12 |
| ECE1             | 5'-ACCCTACGTTCCTGACCTCA-3', 5'-CCGACAGTTTCATGCTTTT-3' | This study |
| ALS3             | 5'-CACAATCCCACATGATT-3', 5'-TGATCAAACCATACAAACA-3' | This study |
| HYR1             | 5'-TTCTGGTTCTGGCTCACA-3', 5'-CCACAGTAACATTAGTGA-3' | This study |
| RNR21            | 5'-GAGATGAAAGTTTACACCCG-3', 5'-AAAGGAAAAGCAGCGG-3' | This study |
| RNR1             | 5'-GGATTTGGGATCATATTAAAAC-3', 5'-AGGACATCGGATACCTTGG-3' | This study |
| RAD6             | 5'-GACCTTCGATACACATTTT-3', 5'-TTCTCCCTCCTCATCATA-3' | This study |
| DDR48            | 5'-CGTAAAGAGACGACGACA-3', 5'-CAGAAGATCGATCGTGAC-3' | This study |
| MEC1             | 5'-GTGGAATTTGATGGAACAA-3', 5'-CGATAAATCCATACACTA-3' | This study |
| EFB1             | 5'-ATGTAACAGAATCTGCTGCCA-3', 5'-CATCTTCTTCACAGCGTCTG-3' | 12 |
| ACT1             | 5'-ACTCTCTCTGTGATAACTACCC-3', 5'-ACTTTCATAGAAGTGGAGA-3' | This study |
tions with 140 mg/kg of pentobarbital sodium (Nembutal) solution (Abbott Laboratories, North Chicago, IL); this dose kept the mice well sedated for at least 3 h. After the mice were fully sedated, cotton wool balls (diameter, 3 mm) saturated with 50 μl of 1 × 10^7 CFU/ml of C. albicans were placed sublingually in the oral cavity for 2 h. Fifteen to 17 mice per group were used. The mice were euthanized by CO2 asphyxiation followed by cervical dislocation at 6, 24, and 72 h and on day 7 after infection. The esophagus, mandibular soft tissue, and tongue were dissected free of teeth and bone. The tissue was homogenized in saline, plated onto SDA containing ampicillin (100 μg/ml) and amikacin (60 μg/ml), and incubated at 30°C for 48 h. Tissue burden was enumerated by colony count. Interquartile values were used in the data analysis, and tissue burdens for mice infected with each strain were presented as mean log_{10} CFU/g tissue ± standard deviation. The difference in tissue burden between mice infected with the wild-type or mutant strains was determined by Wilcoxon's test. P values of ≤0.05 were considered significant. For histopathology study, the tissues were fixed with formalin and embedded in paraffin, after which thin sections were prepared and stained with Gomori methenamine silver stain.

Murine model of disseminated candidiasis (DC). Seven-week-old, male ICR mice (Harlan Sprague) were inoculated by intravenous injection of the lateral tail vein with 5 × 10^6 CFU of C. albicans strains in 0.2 ml of normal saline solution. Ten to 12 mice per group were used. Mice were followed until they were moribund, at which point they were sacrificed, or for 30 days. For tissue burden and histopathologic study, 12 mice per group were sacrificed at 6, 24, and 72 h postinfection, respectively, and the kidneys, livers, and spleens were obtained. For the tissue burden study, the kidneys, livers, and spleens were removed, weighed, and then homogenized in normal saline solution and plated on SDA containing ampicillin (100 μg/ml) and amikacin (60 μg/ml). Survival curves were calculated according to the Kaplan-Meier method using the PRISM program (GraphPad Software) and compared using the Newman-Keuls analysis. A P value of ≤0.05 was considered significant. As in OPC experiments, interquartile values were used in the data analysis. The tissue burdens were logarithmically transformed, and data were presented as mean log_{10} CFU/g tissue ± standard deviations; the differences in tissue burden between strains were calculated using Wilcoxon's test.

RESULTS

C. albicans orf19.4590 encodes an RFX domain-containing protein that complements an S. cerevisiae rfx1 null mutant strain. In response to genotoxic insults, eukaryotes activate a conserved DNA damage response pathway that regulates the expression of genes such as those encoding the subunits of RNase reductase (RNR) (17, 56, 57). RNR catalyzes the rate-limiting step in the synthesis of deoxyribonucleotide triphosphates, which are necessary for DNA replication and repair. S. cerevisiae RNR2, -3, and -4 and HUG1 (a gene of unknown function that is induced by hydroxyurea/UV light) are repressed under nonstressful conditions by S. cerevisiae Rfx1p, which eliminates its repressor activity. The induction of RFX1 expression upon UV exposure is believed to ensure that ample nonphosphorylated Rfx1p is available to rapidly repress target gene transmission upon deactivation of the damage response pathway (33, 52). As such, RNR genes and HUG1 are derepressed in an S. cerevisiae strain in which RFX1 is deleted (53).

To determine if the protein encoded by C. albicans orf19.4590 is functionally related to S. cerevisiae Rfx1p, we measured expression of RNR3 and HUG1 in a diploid S. cerevisiae rfx1 null mutant strain (strain HOM14D-1-34152) transformed with a vector containing orf19.4590 and its flanking regions (Fig. 1). The S. cerevisiae rfx1 null mutant transformed with an S. cerevisiae RFX1 plasmid and the same mutant transformed with the vector alone served as positive and negative controls, respectively. In YPD medium at 30°C, the derepression of RNR3 and HUG1 in the null mutant was fully reversed in the transformants complemented with S. cerevisiae RFX1 (Fig. 1). In transformants complemented with orf19.4590, RNR3 and HUG1 derepression was partially reversed (Fig. 1). As anticipated, RNR3 and HUG1 derepression was unaffected in the S. cerevisiae rfx1 null mutant transformed with the vector alone. Experiments were repeated with independently created S. cerevisiae transformants with similar results. These findings suggest that orf19.4590 has some functional redundancy with S. cerevisiae RFX1.

We next exposed the strains to UV radiation, which is known to induce S. cerevisiae RFX1 expression (33). In the presence of genotoxic insults, the S. cerevisiae DNA damage response pathway phosphorylates Rfx1p, which eliminates its repressor activity. The induction of RFX1 expression upon UV exposure is believed to ensure that ample nonphosphorylated Rfx1p is available to rapidly repress target gene transmission upon deactivation of the damage response pathway (33, 52). We exposed S. cerevisiae strains to 15 ml/cm² of UV radiation, followed by incubation in YPD at 30°C. As anticipated, S. cerevisiae RFX1 was upregulated by 13.1-fold ± 1.0-fold (data not shown). Deletion of RFX1 rendered S. cerevisiae significantly more resistant to UV killing (Fig. 2A). UV susceptibility was restored to levels consistent with the wild type in null mutant strains complemented with either orf19.4590 or S. cerevisiae RFX1 (Fig. 2A). The S. cerevisiae rfx1 null mutant transformed with the vector alone was indistinguishable from the null mutant.

Taken together, the data suggest that C. albicans orf19.4590 can perform at least some of the functions of S. cerevisiae RFX1. Of note, a search of the C. albicans genome (http://www.candidagenome.org and http://genolist.pasteur.fr/CandidaDB) revealed two open reading frames that encode RFX domain-containing proteins (orf19.4590 and orf19.3865). In fact, the protein encoded by orf19.3865 more closely resembles S. cerevisiae RFX1p in the DNA binding domain and over its full sequence (45.7% and 48.9% amino acid identity, respectively) than does the protein encoded by orf19.4590 (25.8% and 44.2% amino acid identity, respectively). For this reason, we will refer to orf19.4590 as C. albicans RFX2 hereafter, and we propose that the name C. albicans RFX1 be reserved for orf19.3865.
Deletion of *C. albicans* RFX2 results in increased resistance to UV killing, as well as the derepression of various stress-related genes. To further study *C. albicans* RFX2, we independently constructed two sets of isogenic mutant strains in which one or both copies of the gene were disrupted and reinsertion strains in which a copy of the gene was reintroduced to the null mutant at the native locus. After verifying the desired disruptions and reinsertions by PCR amplifications and Southern analyses, we demonstrated that the mutant and reinsertion strains had growth rates similar to that of wild-type strain SC5314 in both liquid YPD and minimal media at 35°C (data not shown).

Next, we tested for susceptibility to ionizing radiation, hydroxyurea, and several radiomimetic drugs (bleomycin, MMS, streptonigrin, and ethyl methanesulfonate). Similar to the *S. cerevisiae* rfx1 null mutant and wild-type strains, the *C. albicans* rfx2 null mutant was more resistant to UV killing than SC5314 (Fig. 2B). The rfx2 heterozygous mutant and RFX2 reinsertion strains did not significantly differ from SC5314. We found no differences in the sensitivities of the rfx2 null mutant and SC5314 to hydroxyurea, bleomycin, MMS, streptonigrin, or ethyl methanesulfonate (data not shown). We also tested the susceptibility of the *C. albicans* strains to heat shock at 48°C and oxidative (ethanol, H$_2$O$_2$) and osmotic (NaCl) stresses. The rfx2 null mutant was significantly more resistant than SC5314 to heat shock and 10% ethanol (Fig. 3A and B) but equally susceptible to 0.7 mM NaCl and 13.2 mM H$_2$O$_2$ (data not shown). The rfx2 heterozygous mutant and RFX2 reinsertion strains were more resistant than SC5314 but more susceptible than the rfx2 null mutant to heat shock and 10% ethanol, results reflecting possible haploinsufficiency. As for each of the in vitro phenotypes presented below, independently created heterozygous mutant, null mutant, and reinsertion strains yielded similar results.

In *S. cerevisiae*, various stress-associated genes are upregulated in response to UV radiation (26, 27). Based on these reports, we examined the expression of *C. albicans* RFX2,
RNRI, RNR2, RAD6 (a gene induced by and required for resistance to UV radiation) (5, 31), and DDR48 (a gene encoding a Hog1p-induced immunogenic-stress-related protein and involved in DNA repair) (16). S. cerevisiae RNRI and RNRI, -3, and -4 encode large and small subunits of RNR, respectively. In the C. albicans genome database, the corresponding genes are RNR1 and RNR21 (also referred to as RNR2). In YPD at 30°C, C. albicans RAD6 and DDR48 were significantly derepressed in the rf2 null mutant compared to their expression by strain SC5314 (29.9- and 8.6-fold, respectively) (Table 3). RNR21 and RNRI were derepressed only 1.9- and 1.3-fold, respectively, in the null mutant, differences of uncertain biological significance. The levels of RAD6 and DDR48 expression in the rf2 heterozygous mutant and RFX2 reinsertion strains were intermediate to the levels in the null mutant and SC5314 (Table 3). The findings suggest that the expression of RAD6 and DDR48 is directly or indirectly repressed by RFX2.

Following exposure to UV radiation, C. albicans RFX2 was upregulated by 10.12-fold ± 0.25-fold in strain SC5314 (data not shown), a finding that was consistent with our earlier data for S. cerevisiae RFX1. RAD6 and DDR48 were also significantly upregulated in SC5314 after UV exposure (Table 3). Unlike SC5314, the rf2 mutant was not able to significantly increase the expression of any of the genes in response to UV exposure, suggesting that the genes were already maximally derepressed.

S. cerevisiae MEC1 encodes a phosphoinositide kinase that functions upstream of Rad53p and Rfx1p as the major checkpoint in the DNA damage response pathway (25, 41). We demonstrated that, as anticipated, C. albicans MEC1 was induced in response to UV exposure (Table 3). The expression pattern was similar for the C. albicans rf2 null mutant strain, demonstrating that RFX2 does not repress MEC1 and likely functions downstream in the damage response pathway.

The C. albicans rf2 null mutant displays hyperfilamentous growth and overexpresses hypha-specific genes. Having confirmed our hypothesis that RFX2 would repress the expression of DNA damage response genes, we evaluated the effects of gene deletion on morphogenesis. Colonies of SC5314, rf2 heterozygous mutant, and RFX2 reinsertion strains were smooth under non-hypha-inducing conditions on solid agar (YPD at 30°C for 72 h). The colonies of the rf2 null mutant, on the other hand, were wrinkled (data not shown). Examination of the colony margins revealed extensive filaments for the null mutant and no or minimal filaments for SC5314, heterozygous mutant, and reinsertion strains (Fig. 4). Similar results were obtained following growth on SDA medium at 30°C (Fig. 4). Under hypha-inducing conditions on solid agar (YPD medium supplemented with 5% FCS, medium 199, and Spider medium at 37°C), all strains demonstrated filamentous growth (data not shown). In YPD liquid medium incubated at 30°C, the strains were all in the yeast morphology and indistinguishable from one another. Under hypha-inducing conditions in liquid medium (YPD supplemented with 5% FCS at 37°C), the strains formed extensive hyphae, although those of the null mutant were 20 to 50% longer than those of the SC5314, rf2 heterozygous mutant, and RFX2 reinsertion strains at the same time points (data not shown). Moreover, the majority of SC5314, heterozygous mutant, and reinsertion cells reverted to yeast forms following overnight growth in YPD plus 5% serum at 37°C, whereas the majority of null mutant cells remained in hyphal and pseudohyphal morphologies (Fig. 5). In addition to a hyperfilamentous phenotype, the rf2 null mutant strain showed enhanced invasion into solid agar (Fig. 6). The invasive growth of rf2 heterozygous mutant and RFX2 reinsertion strains was intermediate to that of SC5314 and that of the rf2 null mutant.

As a follow-up to the morphogenesis experiments, we evaluated the expression of hypha-specific genes using quantitative RT-PCR. Under conditions that typically promote growth as yeast (YPD liquid medium at 30°C), HWP1, ECE1, ALS3, HYR1, and CEK1 mRNA transcript levels in strain SC5314 were predictably low. mRNA transcript levels in the rf2 null mutant strain were higher by 4.2-fold to 16.8-fold (Table 4), suggesting that Rfx2p either directly or indirectly repressed the hypha-specific genes under non-hypha-inducing conditions. Reintroducing RFX2 into a null mutant background reduced mRNA transcript levels of each gene to those observed for the rf2 heterozygous mutant. Following exposure to UV radiation, HWP1, HYR1, and ECE1 were significantly induced in SC5314 (14.5-fold, 11.0-fold, and 4.9-fold, respectively), ALS3 was slightly induced (2.3-fold), and CEK1 was not significantly induced (1.8-fold) (Table 4). After UV exposure, the rf2 null mutant was able to induce HWP1 and ECE1 above their derepressed basal levels but was not able to further induce ALS3, HYR1, and CEK1.

The rf2 null mutant demonstrates increased adherence to epithelial cells in vitro but decreased virulence during murine DC and OPC. To assess the potential role of RFX2 in candidal

### Table 3. Effects of C. albicans RFX2 on the expression of DNA damage genes

| Gene      | In YPD at 30°C | After UV radiation |
|-----------|----------------|-------------------|
|           | SC5314         | rf2 heterozygous mutant | rf2 null mutant | rf2 reinsertion strain |
| RNR21     | 1.00 ± 0.02    | 1.37 ± 0.03        | 1.86 ± 0.01    | 1.32 ± 0.02            |
| RRNI      | 1.00 ± 0.01    | 2.21 ± 0.04        | 1.30 ± 0.01    | 1.10 ± 0.01            |
| RAD6      | 1.00 ± 0.02    | 2.89 ± 0.37*       | 29.92 ± 4.18*  | 3.34 ± 0.81*           |
| DDR48     | 1.00 ± 0.04    | 3.63 ± 0.03*       | 8.61 ± 0.30*   | 3.41 ± 0.02*           |
| MEC1      | 1.02 ± 0.02    | 1.15 ± 0.01        | 1.01 ± 0.09    | 1.01 ± 0.02            |
|           | 2.19 ± 0.01*   | 2.29 ± 0.02*       | 2.43 ± 0.02*   | 2.33 ± 0.03*           |
|           | 1.07 ± 0.01    | 1.37 ± 0.00        | 1.78 ± 0.01    | 1.43 ± 0.01            |
|           | 4.52 ± 0.62*   | 5.68 ± 0.48*       | 27.51 ± 0.17*  | 5.72 ± 0.70*           |
|           | 4.93 ± 0.04*   | 4.61 ± 0.30*       | 9.38 ± 0.14*   | 4.96 ± 0.06*           |
|           | 4.28 ± 0.11*   | 3.66 ± 0.04*       | 3.84 ± 0.10*   | 3.72 ± 0.18*           |

* Asterisks denote biological (increase of more than twofold) and statistical (P < 0.05) significance of the level of expression by the respective strains compared with the level of expression by SC5314.
virulence, we first measured the adherence of *C. albicans* strains to BECs in vitro. The \textit{rfx2} null mutant was significantly more adherent to BECs than SC5314, the \textit{rfx2} heterozygous mutant strain, or the \textit{RFX2} reinsertion strain (Fig. 7). The results were not ascribed to differences in cell morphology, as all strains were found to be blastoconidia by Gram staining (data not shown).

We next tested the strains in murine models of DC and OPC. For DC, groups of 10 to 12 ICR mice were infected via the lateral tail vein with $5 \times 10^5$ CFU/mouse. There was a
significant delay in the time to death among mice infected with the rfx2 null mutant compared to those infected with SC5314 (mean of 17.5 ± 8.7 versus 6.3 ± 2.4 days, \( P = 0.002 \)) (Fig. 8). All of the mice infected with SC5314 died by day 9, whereas 25% of mice infected with the null mutant were still alive on day 30. Results with the RFX2 reinsertion strain were similar to those with SC5314. In separate experiments, we measured candidal tissue burdens in the kidneys, livers, and spleens at 6, 24, and 72 h after intravenous infection with SC5314 or the rfx2 null mutant (12 mice per strain per time point) (Table 5). At 6 h, concentrations of the null mutant were slightly higher than those of SC5314 within the kidneys and liver (0.52 and 0.77...
log_{10} CFU/g tissue, respectively). Concentrations within the spleen did not differ. At 24 h, there were no significant differences in tissue burdens within any of the organs. By 72 h, concentrations of SC5314 in the kidneys were 1.11 log_{10} CFU/g tissue higher than those of the rfx2 null mutant (P < 0.0001), while concentrations within the liver and spleen did not differ. Histopathologic examination of the kidneys at 72 h showed that both strains grew as mixtures of yeasts and hyphae (data not shown).

In the OPC model, mice were immunosuppressed with cortisone acetate and infected sublingually for 2 h with 5 \times 10^6 CFU (15 to 17 mice per strain per time point). The candidal burdens within the tongues, buccal mucosa, and esophagi of mice infected with SC5314 at 6 and 24 h postinfection (3.45 ± 0.62 and 5.06 ± 0.40 log_{10} CFU/g tissue, respectively) were not different from the burdens due to the rfx2 null mutant (3.48 ± 0.45 and 5.03 ± 0.29 log_{10} CFU/g tissue, respectively). On the sixth day following infection, three mice infected with SC5314 died, with evidence of DC. For this reason, we sacrificed the remaining mice on day 7 and assessed the presence of C. albicans in the kidneys, livers, spleens, and preputial glands (the preputial glands, genital organs that secrete pheromones, often harbor significant concentrations of C. albicans during DC [S. Cheng, C. J. Clancy and M. H. Nguyen, unpublished data]). We found that 82.4% (14/17) of mice infected with SC5314 had evidence of DC (defined as the presence of C. albicans in any organ) on day 7, compared to only 26.7% (4/15) of mice infected with the rfx2 null mutant (P = 0.004). We then repeated the analysis of the OPC model by applying the same inoculum for 1 hour, including the RFX2 reinsertion strain in addition to SC5314 and the rfx2 null mutant (15 mice per strain per time point). The mice were observed for 7 days; no deaths were recorded. At 7 days postinfection, the mice infected with SC5314 had significantly higher tissue burdens within the tongue, buccal mucosa, and esophagus than mice infected with the null mutant (4.8 ± 0.5 versus 2.6 ± 0.8 log_{10} CFU/g tissue, respectively; P < 0.0001). There was no significant difference in tissue burdens of mice infected with SC5314 and the RFX2 reinsertion strain (data not shown). A histopathology study showed that the tongues and esophagi of mice infected with the rfx2 null mutant had minimal chronic inflammatory reaction compared with those of mice infected with SC5314. Both yeast and hyphal morphologies were observed in the tissues from mice infected with SC5314 (Fig. 9). A histopathology study of the tongues from mice infected with the rfx2 null mutant showed only rare hyphal elements, and no fungal elements were visualized on stains of the esophagus.

### DISCUSSION

Microbes suffer DNA damage due to a variety of stresses in the course of their interactions with infected hosts. As such, the regulation of DNA damage responses is an important determinant of successful adaptation to in vivo environments (19). In this study, we show that C. albicans RFX2 encodes an RFX domain-containing protein that represses the transcription of DNA damage response genes under nonstressful conditions. A C. albicans rfx2 null mutant exhibits hypofilamentous and hyperinvasive growth, constitutive

![FIG. 7. Adherence by C. albicans strains to BECs in vitro. C. albicans cells were coincubated with BECs for 60 min, as described in Materials and Methods. Percent adherence is defined as the mean number of C. albicans cells/100 BECs. Data are presented as means of all experiments ± standard deviations.](image)

![FIG. 8. Effects of RFX2 on the survival of mice with HDC. Seven-week-old, male ICR mice (Harlan Sprague) were inoculated by intravenous injection of the lateral tail vein with 5 × 10^5 CFU of C. albicans strains and followed for 30 days.](image)
The data demonstrate that Rfx2p plays crucial roles in the regulation of DNA damage responses, morphogenesis, and virulence. The attenuated virulence of the rfx2 null mutant is consistent with reports of other hyperfilamentous C. albicans strains, including null mutants in which negative transcriptional regulators encoded by C. albicans TUP1, NRG1, RFG1, and SPT3 were disrupted (6–8, 28, 30, 34, 35). We showed that Rfx2p contributes to pathogenesis by assessing two distinct models of murine candidiasis. During hematoogenously disseminated candidiasis (HDC), the rfx2 mutant caused significantly less overall mortality. The null mutant was also less likely than SC5314 to cause DC by mucosal translocation, as demonstrated in cortisone-treated mice that received 2-hour sublingual inoculations of C. albicans. When the inoculation time in these mice was shortened to 1 hour, the null mutant caused lower tissue burdens of infection and less inflammation within oral and esophageal mucosa than SC5314 after 7 days. It is not possible to discern from our data whether the attenuated virulence is a consequence of impaired DNA damage responses, dysregulated morphogenesis, some other process, or a combination of mechanisms. In general, it is unclear whether morphogenesis per se contributes to the pathogenesis of candidiasis rather than morphology-associated patterns of gene expression (24, 46, 48). In fact, pathways regulating C. albicans morphogenesis converge to influence the expression of genes encoding diverse virulence factors (29, 32). Nevertheless, it is notable that the rfx2 mutant not only exhibited derepressed filamentation but also failed to revert back to yeast morphology following overnight growth in liquid medium. To the extent that morphogenesis is a virulence determinant, therefore, the ability to switch back and forth between blastoconidial and filamentous growth might be more important to survival in different environments in vivo than merely the ability to form filaments.

It is interesting that the rfx2 null mutant was significantly more adherent than SC5314 to BECs in vitro and more invasive into solid agar. Although it has long been established that C. albicans hyphae are generally more adherent to host cells than conidia (39), our results in vitro cannot be attributed to the hyperfilamentous growth of the mutant since both strains were tested as yeasts. Indeed, Gram stains confirmed that SC5314 and the null mutant were morphologically similar during the in vitro assay. Rather, Rfx2p is likely to influence adhesion through its role in transcriptional repression. The derepression of HWP1 and ALS3, hypha-specific genes encoding adhesins (46, 55), under basal conditions associated with growth in the yeast morphology supports this hypothesis. The equivalent tissue burdens of SC5314 and the rfx2 null mutant after 6 h of murine OPC; however, highlight several important points: (i) adherence and penetration are not the sole determinants of pathogenicity, even at relatively early time points; (ii) in vitro assays cannot completely replicate complex in vivo systems; and (iii) mechanisms of pathogenesis differ at unique tissue sites.

The precise mechanisms by which Rfx2p contributes to the regulation of the interrelated processes of morphogenesis, virulence, and adhesin expression by C. albicans have eluded investigators for some time. However, with the discovery of functions of RFX2 in both yeast and filamentous growth, it is likely that Rfx2p plays a central role in the regulation of hypha-specific gene expression. The observations that Rfx2p is required for the transcriptional repression of several hypha-specific genes during both in vivo and in vitro experiments are consistent with a model in which RFX2 is an evolutionarily conserved transcriptional repressor that functions in the regulation of hypha-specific gene expression.

### Table 5. C. albicans tissue burdens among mice with HDC*  

| Organ and strain | Mean log₁₀ CFU/g of tissue± SD at: |
|------------------|---------------------------------|
|                  | 6 h | 24 h | 72 h |
| Kidney           |     |      |      |
| SC5314           | 4.22 ± 0.12 | 5.17 ± 0.27 | 5.59 ± 0.09 |
| rfx2 mutant      | 4.74 ± 0.10 (0.03) | 5.11 ± 0.12 (NS) | 4.48 ± 0.14 (NS) |
| Liver            |     |      |      |
| SC5314           | 3.23 ± 0.05 | 3.03 ± 0.13 | 2.38 ± 0.31 |
| rfx2 mutant      | 4.00 ± 0.04 (<0.0001) | 3.08 ± 0.14 (NS) | 2.56 ± 0.26 (NS) |
| Spleen           |     |      |      |
| SC5314           | 3.94 ± 0.15 | 3.41 ± 0.66 | 3.06 ± 0.48 |
| rfx2 mutant      | 4.11 ± 0.31 (NS) | 3.79 ± 0.32 (NS) | 2.92 ± 0.37 (NS) |

* Mice were infected via lateral tail vein injection with 5 × 10⁵ CFU/mouse. They were sacrificed at 6, 24, and 72 h. The kidneys, spleens, and livers were removed for CFU enumeration.  

* Significance (P values or NS [not significant]) for the wild type versus mutants is indicated in parentheses.

expression of hypha-specific genes, and attenuation of virulence during disseminated and mucosal candidiasis in mice. The data demonstrate that Rfx2p plays crucial roles in the regulation of DNA damage responses, morphogenesis, and virulence.

C. albicans RFX2 is one of two genes encoding RFX domain-containing proteins in the genome sequence database. The other is C. albicans orf19.3865, which encodes a protein that more closely resembles the sole RFX domain-containing protein of S. cerevisiae, Rfx1p. Given the sequence homology, we propose that orf19.3865 be named C. albicans RFX1, and we have named our gene RFX2. In this study, we demonstrate that C. albicans RFX2 has at least partial function redundancy with S. cerevisiae RFX1. In S. cerevisiae rfx1 null mutants, DNA damage response genes like NNR3 and HUG1 are derepressed and cells are rendered more resistant to UV killing. Transformation of an S. cerevisiae rfx1 mutant with a plasmid expressing C. albicans RFX2 significantly reduced expression of RNR3 and HUG1 and restored wild-type UV susceptibility. C. albicans wild-type strain SC5314 responded to UV exposure by inducing RFX2, RAD6 (a gene induced by and required for UV resistance), and DDR48 (a gene encoding a Hog1p-induced stress protein). Deletion of RFX2 resulted in significant derepression of RAD6 and DDR48 under nonstressful conditions. Moreover, the C. albicans rfx2 null mutant was more resistant than SC5314 to UV killing, 48°C heat shock, and 10% ethanol, suggesting that the basal derepression of DNA damage and UV-induced genes was protective. Taken together, the data indicate that C. albicans RFX2 can perform at least some of the functions of S. cerevisiae RFX1 and that the genes contribute to similar phenotypes.

UV exposure and other genotoxic insults to C. albicans result in filamentous growth (45). We demonstrate that Rfx2p is a crucial link between DNA damage responses and morphogenesis. As anticipated, exposure of wild-type C. albicans SC5314 to UV radiation induced the expression of hypha-specific genes HWP1, HYR1, ECE1, and, to a lesser extent, ALS3. Deletion of RFX2 resulted in the constitutive overexpression of these genes as well as CEKI. In addition, the rfx2 null mutant demonstrated hyperfilamentous growth on solid agar under non-hypha-inducing conditions. Along these lines, it is notable that genotoxic stress-induced filamentous growth by C. albicans is dependent upon intact DNA damage checkpoints (2, 45). Similar to S. cerevisiae Rfx1p, C. albicans Rfx2p is likely to function as a downstream effector of the highly conserved and well-characterized Mec1-Rad53 DNA checkpoint pathway.

The attenuated virulence of the rfx2 null mutant is consistent with reports of other hyperfilamentous C. albicans strains, including null mutants in which negative transcriptional regulators encoded by C. albicans TUP1, NRG1, RFG1, and SPT3 were disrupted (6–8, 28, 30, 34, 35). We showed that Rfx2p contributes to pathogenesis by assessing two distinct models of murine candidiasis. During hematoogenously disseminated candidiasis (HDC), the rfx2 mutant caused significantly less overall mortality. The null mutant was also less likely than SC5314 to cause DC by mucosal translocation, as demonstrated in cortisone-treated mice that received 2-hour sublingual inoculations of C. albicans. When the inoculation time in these mice was shortened to 1 hour, the null mutant caused lower tissue burdens of infection and less inflammation within oral and esophageal mucosa than SC5314 after 7 days. It is not possible to discern from our data whether the attenuated virulence is a consequence of impaired DNA damage responses, dysregulated morphogenesis, some other process, or a combination of mechanisms. In general, it is unclear whether morphogenesis per se contributes to the pathogenesis of candidiasis rather than morphology-associated patterns of gene expression (24, 46, 48). In fact, pathways regulating C. albicans morphogenesis converge to influence the expression of genes encoding diverse virulence factors (29, 32). Nevertheless, it is notable that the rfx2 mutant not only exhibited derepressed filamentation but also failed to revert back to yeast morphology following overnight growth in liquid medium. To the extent that morphogenesis is a virulence determinant, therefore, the ability to switch back and forth between blastoconidial and filamentous growth might be more important to survival in different environments in vivo than merely the ability to form filaments.

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| Spleen           |     |      |      |
| SC5314           | 3.94 ± 0.15 | 3.41 ± 0.66 | 3.06 ± 0.48 |
| rfx2 mutant      | 4.11 ± 0.31 (NS) | 3.79 ± 0.32 (NS) | 2.92 ± 0.37 (NS) |

* Mice were infected via lateral tail vein injection with 5 × 10⁵ CFU/mouse. They were sacrificed at 6, 24, and 72 h. The kidneys, spleens, and livers were removed for CFU enumeration.  

* Significance (P values or NS [not significant]) for the wild type versus mutants is indicated in parentheses.
Fig. 9. Histopathology of the tongues (A and B) and esophagi (C and D) of mice infected with C. albicans strains. Mice were infected sublingually with C. albicans SC5314 (A and C) or the rfx2 null mutant (B and D), as described in Materials and Methods. Organs were harvested after 7 days, processed, and stained with hematoxylin-eosin (left) and Gomori methenamine silver (right). In the tongues of mice infected with C. albicans SC5314 (A), an intense intraepithelial lymphocyte response is noted in the squamous epithelium, with associated hyperkeratosis and neutrophils. Fungal elements are extensive and comprise roughly equal measures of hyphae and yeasts. In the tongues of mice infected with the rfx2 null mutant (B), the epithelium has a mild degree of intraepithelial lymphocytosis and only focal keratosis. Fungal elements are less common and predominantly hyphae. In the esophagi of mice infected with C. albicans SC5314 (C), there is a moderate lymphocyte response. As in the tongue, there are extensive yeasts and hyphae. In the esophagi of mice infected with the rfx2 null mutant (D), no inflammation or fungal elements were detected.
ulence, and adherence will need to be elucidated in future studies. At least some of the cellular functions of Rfx2p are likely to be mediated through the transcriptional repressor Tup1p. Tup1p forms an evolutionarily conserved corepressor complex with Snf6p, which is targeted to specific promoters through interactions with a variety of DNA binding proteins. S. cerevisiae Rfx1p targets Tup1p-Snf6p to DNA damage response genes. As alluded to earlier, deletion of C. albicans TUP1 results in hyperfilamentous and invasive growth, derepression of hypha-specific genes, and attenuated virulence during murine candidiasis, similar to our observations with the TUP1 C. albicans cerevisiae complex with Ssn6p, which is targeted to specific promoters likely to be mediated through the transcriptional repressor TUP1.

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ilarities, there are morphological differences between Florida. Reported by the VA Medical Research Service and the University of Health System and the University of Pittsburgh. We thank Joachim and C. J. Clancy at the North Georgia/South Florida Veterans

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