Stress-Induced Phenotypic Switching in *Candida albicans*

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Candida albicans is both a common commensal and an opportunistic pathogen, being a prevalent cause of mucosal and systemic infections in humans. Phenotypic switching between white and opaque forms is a reversible transition that influences virulence, mating behavior, and biofilm formation. In this work, we show that a wide range of factors induces high rates of switching from white to opaque. These factors include different forms of environmental stimuli such as genotoxic and oxidative stress, as well as intrinsic factors such as mutations in DNA repair genes. We propose that these factors increase switching to the opaque phase via a common mechanism—inhibition of cell growth. To confirm this hypothesis, growth rates were artificially manipulated by varying expression of the *CLB4* cyclin gene; slowing cell growth by depleting *CLB4* resulted in a concomitant increase in white-opaque switching. Furthermore, two clinical isolates of *C. albicans*, P37005 and L26, were found to naturally exhibit both slow growth and high rates of white-opaque switching. Notably, suppression of the slow growth phenotype suppressed hyperswitching in the P37005 isolate. Based on the sensitivity of the switch to levels of the master regulator *Wor1*, we propose a model for how changes in cellular growth modulate white-opaque switching frequencies.

INTRODUCTION

Phenotypic switching involves reversible and heritable switching between alternative cellular phenotypes, often distinguished by differences in microscopic and macroscopic morphology. In bacteria, these transitions can promote pathogenicity, because traits such as drug resistance are often affected (Dubnau and Losick, 2006). The white-opaque transition in the opportunistic pathogen *Candida albicans* provides an excellent example of phenotypic switching in eukaryotes. In this transition, white cells are round and give rise to smooth, domed colonies, whereas opaque cells are bean shaped and give rise to flatter, translucent colonies (Slutsky *et al.*, 1987). Switching between states occurs infrequently, with an average of one event every 10,000 cell divisions (Rikkerink *et al.*, 1988). More than 450 genes are differentially regulated between the white and the opaque states (Lan *et al.*, 2002; Tsong *et al.*, 2003), and these differences affect *C. albicans* pathogenicity, mating efficiency, and biofilm formation (Bennett and Johnson, 2005). Thus, white cells are more virulent in a mouse tail-vein model of systemic infection, while opaque cells are more efficient at colonization of the skin in a cutaneous model of infection (Kvaal *et al.*, 1997, 1999). Opaque cells are also the mating competent form of *C. albicans*, because they have been shown to undergo mating at least a million times more efficiently than white cells (Miller and Johnson, 2002). Increased mating efficiency is due, at least in part, to increased expression of genes involved in mating signaling (Lan *et al.*, 2002). White and opaque cells have also been shown to differ in their interaction with immune cells. White cells secrete a chemoattractant for leukocytes, whereas opaque cells do not

(Geiger *et al.*, 2004). In addition, white and opaque cells are differentially phagocytosed by macrophages, suggesting that white-opaque switching may be an adaptive mechanism to help *C. albicans* cells escape the attention of the host immune system (Lohse and Johnson, 2008).

Recent studies have begun to elucidate the molecular mechanism underlying white-opaque switching in *C. albicans*. The central factor regulating the white-opaque switch is encoded by the *WOR1* gene (also called *TOS9/EAP2*) (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006). In the absence of *WOR1*, cells are unable to form opaques, whereas overexpression of *WOR1* forces cells to switch to the opaque state. Stable expression of *WOR1* in opaque cells is maintained by positive feedback of *Wor1p* on its own promoter (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006). Genes encoded at the mating type-like (*MTL*) locus provide an additional level of regulation on the white-opaque switch. The white-opaque transition does not occur in *MTLa/α* cells because the *a1/α2* heterodimer inhibits *WOR1* expression, thus locking cells in the white state (Miller and Johnson, 2002; Tsong *et al.*, 2003; Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006). This explains why white-opaque switching is not observed in most clinical isolates of *C. albicans*; only 3–8% of naturally occurring strains are homozygous at the *MTL* locus (Lockhart *et al.*, 2002; Odds *et al.*, 2007).

Other factors shown to be involved in white-opaque switching include *Czf1p*, *Efg1p*, and *Wor2p* (Sonneborn *et al.*, 1999; Vines and Kumamoto, 2007; Zordan *et al.*, 2007). *Czf1p* positively regulates the transition to the opaque state because its absence decreases opaque formation, whereas overexpression increases switching to opaque (Vines and Kumamoto, 2007; Zordan *et al.*, 2007). *Czf1p* regulates switching by inhibiting expression of *EFG1*, a transcription factor promoting formation of the white state (Sonneborn *et al.*, 1999; Srikantha *et al.*, 2000, 2006; Vines and Kumamoto, 2007; Zordan *et al.*, 2007). *Wor2p* is involved in stabilization of the opaque state; deletion of *WOR2* results in a loss of

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opaque formation, although overexpression of *WOR2* does not increase switching to opaque (Zordan *et al.*, 2007).

In this article, we demonstrate that several diverse stimuli increase rates of switching from white to opaque. These stimuli include different forms of cellular stress, including that mediated by genotoxic or oxidative stress. Several distinct mutant forms of *C. albicans* were similarly found to undergo increased white-opaque switching, including mutants defective in DNA repair and nuclear division. Finally, clinical isolates were identified that undergo elevated rates of white-to-opaque switching compared with the standard laboratory strain SC5314. We have investigated these findings and show that, in each case, increased switching to opaque is associated with a decrease in cellular growth rates. We further demonstrate that slowing cell growth artificially, by limiting expression of a cell cyclin gene, also results in increased white-opaque switching. Thus, multiple factors impinge upon rates of switching indirectly through their influence on the rate of cellular growth. To help explain these observations we also demonstrate that switching is extremely sensitive to levels of the master regulator *WOR1*. We therefore propose a model to explain how changes in cell doubling times affect white-opaque switching based on the role of *Wor1p* as the major factor determining formation of the opaque state.

MATERIALS AND METHODS

Media and Reagents

Synthetic complete plus dextrose medium (SCD) and yeast extract peptone plus dextrose medium (YPD) were made as described previously (Guthrie and Fink, 1991). YPD plates containing 200 µg/ml nourseothricin were used for selection of strains that were resistant to nourseothricin (*SAT^R* strains) (Reuss *et al.*, 2004). Plates containing methyl methane sulfonate (MMS; Acros Organics, Fairlawn, NJ), hydroxyurea (HU; MP Biomedicals, Irvine, CA), or hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) were made by adding these agents to SCD medium after autoclaving. For medium deficient in methionine, the recipe for SCD was followed except methionine was excluded from the amino acid mix. Filter-sterilized 100 mM methionine and cysteine amino acid stocks were then added at the appropriate concentrations after autoclaving. Nutritionally deficient medium was generated by mixing autoclaved SCD medium with different amounts of autoclaved 2% agar to achieve the appropriate medium concentration.

Plasmids

Plasmid pRB101 for deletion of the *MTLa* locus was generated by polymerase chain reaction (PCR) amplifying sequences 5' and 3' to *MTLa* by using oligonucleotides (oligos) 1/2 and 3/4, respectively (Supplemental Table S1). The 5' product was digested with *ApaI* and *XhoI*, and the 3' product was digested with *SacII* and *SacI*. The digested fragments were ligated into pSFS2A (Reuss *et al.*, 2004) to create pRB101. Similarly, pRB102 for deletion of the *MTLa* locus was generated by PCR amplifying sequences 5' and 3' to *MTLa* by using oligos 5/6 and 7/8, respectively. The 5' product was digested with *ApaI* and *XhoI*, and the 3' product was digested with *SacII* and *SacI*. Both products were ligated into pSFS2A to create pRB102.

Plasmid pYFP-ARG was generated by PCR amplifying the *YFP* gene from pYFP-HIS (Gerami-Nejad *et al.*, 2001) with oligos 9/10 and the *ARG4* gene from pSN69 (Noble and Johnson, 2005) using oligos 11/12. The products were inserted into a Blunt II Topo vector (Invitrogen, Carlsbad, CA) and digested with *XhoI* and *XbaI*. The *ARG4* gene fragment was then ligated into the *YFP* containing vector to create plasmid pYFP-ARG. Similarly, the *SAT1* maker was PCR amplified from pNIM1 (Park and Morschhauser, 2005) by using oligos 13/14 and ligated into a Blunt II Topo vector. The *SAT1* product was also digested with *XhoI* and *XbaI* and ligated with the *YFP* vector to create pYFP-SAT.

Plasmid pKA1, used for targeting the *WOR1* gene, was generated by cloning the promoter and 3' untranslated region (UTR) of *WOR1* into pSFS2A (Reuss *et al.*, 2004). The promoter was PCR amplified from genomic DNA (SC5314) by using oligos 15/16, purified using Kleen spin columns (Bio-Rad Laboratories, Hercules, CA), digested with *KpnI* and *ApaI*, and ligated into pSFS2A. The 3' UTR was PCR amplified from genomic DNA by using oligos 17/18, purified, digested with *SacII* and *SacI*, and ligated into the vector to create plasmid pKA1.

Plasmid pKA3, used for epitope tagging of the *RAD53* gene, was generated by initially cloning the *RAD53* promoter and open reading frame (ORF) into

pMG1905, containing 13 copies of the myc epitope and the *URA3* gene (a gift from Judith Berman, University of Minnesota, Minneapolis, MN). The *RAD53* sequence was PCR amplified from genomic DNA by using oligos 19/20, purified, digested with *Sall* and *SmaI*, and cloned into pMG1905 to generate plasmid pKA2. Oligos 21/22 were then used to amplify the 3' end of *RAD53* as well as the myc tag from pKA2. The amplified product was then cut with *Apal* and *XhoI* and ligated into pSFS2A to give rise to plasmid pKA3.

Strains

The *C. albicans* strain used to construct *RAD51* and *RAD52* deletion strains, as well as an isogenic control strain, was SN78 (*ura3/ura3 leu2/leu2 his1/his1*) (Noble and Johnson, 2005). One copy of *GAL1* was deleted in SN78 as described previously (Bennett and Johnson, 2003), and the *URA3* gene was counterselected using 5-fluororotic acid (5-FOA). Subsequently, one copy of *ADE2* was deleted using the method described previously (Hull *et al.*, 2000), generating the *URA3⁺* strains RBY1038 and RBY1039, respectively. To obtain α and α derivatives, these strains were grown on sorbose media as described previously (Janbon *et al.*, 1998), producing strains RBY1152 (α/α) and RBY1153 (α/α) (Table 1). RBY1038 and RBY1039 were transformed with a fusion PCR product designed to disrupt either *RAD51* or *RAD52*. The fusion PCR product was created as described previously (Noble and Johnson, 2005). Briefly, oligos 23/24 were used to PCR the 5' homologous flank of *RAD51* and oligos 25/26 used to PCR the 3' homologous flank. Similarly oligos 27/28 and 29/30 were used to PCR the 5' and 3' flanks of *RAD52*, respectively. The *Candida dubliniensis* *HIS1* or *Candida maltosa* *LEU2* markers were PCR amplified from plasmids pSN52 or pSN40, respectively (Noble and Johnson, 2005). Oligos 23/26 and 27/30 were then used to amplify a disruption cassette containing the homologous flanks of *RAD51* or *RAD52* with a selectable marker. The *RAD51* fragment containing the *HIS1* marker was transformed into RBY1039 to create RBY1053/1054. The *RAD52* fragment containing the *HIS1* marker was transformed into RBY1038 to create RBY1055/1056. Integration was confirmed using oligos internal to the *HIS1* marker (Noble and Johnson, 2005) together with oligos 31/32 for *RAD51* and 33/34 for *RAD52*. RBY1053 and RBY1054 were then transformed with the *RAD51* disruption cassette containing *LEU2* to create RBY1047 and RBY1048, respectively. RBY1055/1056 were transformed with the *RAD52* fragment containing *LEU2* to create RBY1051 and RBY1052. Integration was confirmed using oligos 31/32 and 33/34 for *RAD51* and *RAD52*, respectively, together with internal *LEU2* oligos (Noble and Johnson, 2005). Complete disruption of the ORF was confirmed using primers internal to the gene; oligos 35/36 for *RAD51* and 37/38 for *RAD52*. RBY1047 and RBY1048 were grown on sorbose to generate RBY1154 (α/α) and RBY1155 (α/α). RBY1051 and RBY1052 were grown on sorbose to generate the strains RBY1156 (α/α) and RBY1157 (α/α). To reintroduce a copy of *RAD51* or *RAD52* into the deletion strains, oligos 39/40 or 41/42 were used to amplify genomic DNA. PCR reactions were purified using Kleen spin columns (Bio-Rad Laboratories) and cut with restriction enzymes for integration into pSFS2A (Reuss *et al.*, 2004). *BamHI* and *Apal* were used to clone the *RAD51* PCR fragment, whereas *Apal* and *XhoI* were used to clone the *RAD52* PCR fragment. The *RAD51* vector was linearized with *XhoI* and the *RAD52* vector with *BsaI*. The linearized *RAD51* vector was integrated into RBY1154 and RBY1155 to give rise to complemented strains CAY6/7, and CAY8/9, respectively. Integration was checked using oligos 43/44. Similarly, the linearized *RAD52* vector was integrated into RBY1156/1157 to give rise to the partially complemented CAY10/11 (Supplemental Figure S3), as well as the fully complemented KAY11/113 (Supplemental Figure S3). Oligos 45/46 were used to check for integration.

To construct strains expressing *WOR1-YFP*, oligos 47/48 were used to PCR amplify *YFP* together with either *SAT1* or *ARG4* selectable markers from plasmids pYFP-SAT or pYFP-ARG, respectively. The amplified construct containing *ARG4* was transformed into RBY1175 to yield strain KAY4, and integration checked using oligos 49/50. The *SAT1* version of the construct was transformed into RBY1175 giving rise to KAY17/19. Integration was checked using oligos 49/50. The *SAT1* construct was also used to transform the *RAD52* heterozygote RBY1056, giving rise to KAY21/24. A *LEU2* marked PCR fusion product was used to replace the remaining copy of *RAD52*, and subsequent sorbose selection gave rise to *WOR1-YFP* Δ /*rad52* strains KAY41/42.

RAD51 and *RAD52* heterozygotes were created in *MTLa* and *MTLa* backgrounds. RBY1053 and RBY1054 were transformed with pRB101 and pRB102 cassettes to delete *MTLa* and *MTLa*, respectively. Integration was checked by PCR using oligos 51–54. *SAT^S* colonies were generated by culture in YPD medium without nourseothricin to allow excision of the *SAT1* cassette and plated on low nourseothricin plates (25 µg/ml) to identify colonies that had lost *SAT1* by the smaller size of the colonies (Reuss *et al.*, 2004). Loss of *SAT1* was confirmed by PCR using oligos 51–54. By this method, RBY1053 gave rise to KAY35 (α/Δ) and KAY37 (α/Δ) and RBY1054 generated KAY36 (α/Δ) and KAY38 (α/Δ). Similarly, RBY1055 and RBY1056 were transformed with pRB101 and pRB102 cassettes, integrants were checked with oligos 51–54, and *SAT^S* colonies were selected. KAY56/57 (α/Δ) strains were derived from RBY1055, whereas KAY58/59 (α/Δ) strains derived from RBY1056. Additional wild-type strains were also created by transforming SC5314 with pRB101 and pRB102. Transformation of SC5314 with pRB101 and subsequent

Table 1. Strains used in this study

Name	Genotype	MTL	Reference
CAY6, CAY7	<i>gal1::URA3 ade2::URA3 rad51::HIS1/rad51::leu2/RAD51::SAT1</i>	a/a	This study
CAY8, CAY9	<i>gal1::URA3 ade2::URA3 rad51::HIS1/rad51::leu2/RAD51::SAT1</i>	α/α	This study
CAY10, CAY11	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2/RAD52::SAT1*</i>	a/a	This study
CAY70, CAY71	BWP17 <i>clb4::ARG4/clb4::HIS1 ura3/ura3</i>	a/a	This study
CHY439	<i>MTLa/mtlα1::HisG mtlα2::HisG ura3/ura3</i>	a/ $\Delta\alpha$	Miller and Johnson (2002)
DSY246	SC5314 <i>MTLa::SAT1^S</i>	$\alpha/\Delta a$	This study
DSY247	SC5314 <i>MTLalpha::SAT1^S</i>	a/ $\Delta\alpha$	This study
KAY4	WOR1::WOR1-YFP:ARG	a/a	This study
KAY17	WOR1::WOR1-YFP:SAT1	a/a	This study
KAY19			
KAY36	<i>gal1::URA3 ade2::URA3 rad51::HIS1 MTLalpha::SAT1^S leu2/leu2</i>	a/ $\Delta\alpha$	This study
KAY38	<i>gal1::URA3 ade2::URA3 rad51::HIS1 MTLa::SAT1^S leu2/leu2</i>	$\alpha/\Delta a$	This study
KAY41	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2 WOR1::WOR1-YFP:SAT1</i>	α/α	This study
KAY42	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2 WOR1::WOR1-YFP:SAT1</i>	a/a	This study
KAY55	BWP17 <i>clb4::ARG4/P_{MET3}-CLB4:URA3::clb4 MTLalpha::SAT1</i>	a/ $\Delta\alpha$	This study
KAY56	<i>gal1::URA3 ade2::URA3 rad52::HIS1 MTLalpha::SAT1^S leu2/leu2</i>	a/ $\Delta\alpha$	This study
KAY58	<i>gal1::URA3 ade2::URA3 rad52::HIS1 MTLa::SAT1^S leu2/leu2</i>	$\alpha/\Delta a$	This study
KAY60, KAY61	BWP17 <i>clb4::ARG4/P_{MET3}-CLB4:URA3::clb4 MTLalpha::SAT1^S</i>	a/ $\Delta\alpha$	This study
KAY111	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2/RAD52::SAT1</i>	a/a	This study
KAY113	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2/RAD52::SAT1</i>	α/α	This study
KAY220	<i>gal1::URA3 ade2::URA3 RAD53::RAD53-MYC:SAT1 leu2/leu2 his1/his1</i>	a/a	This study
KAY234, KAY235	<i>gal1::URA3 ade2::URA3 rad51::HIS1/rad51::leu2 wor1::SAT1/wor1::SAT1</i>	a/a	This study
KAY240, KAY241	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2 wor1::SAT1/wor1::SAT1</i>	α/α	This study
KAY245	<i>gal1::URA3 ade2::URA3 rad51::HIS1/rad51::leu2 RAD53::RAD53-MYC:SAT1</i>	a/a	This study
KAY250	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2 RAD53::RAD53-MYC:SAT1</i>	α/α	This study
KAY287, KAY289	RZ7 <i>RPS10::pACT1-GFP:URA3</i>	α/α	This study
KAY286, KAY292	RZ10 <i>RPS10::pACT1-GFP:URA3</i>	a/a	This study
KAY308, KAY309	<i>MTLa/mtlα1::HisG mtlα2::HisG ura3/ura3 wor1::SAT1</i>	a/ $\Delta\alpha$	This study
KAY314, KAY315	<i>MTLa/mtlα1::HisG mtlα2::HisG ura3/ura3 ACT1::pWOR1-3:SAT1</i>	a/ $\Delta\alpha$	This study
KAY326-KAY334	Passaged P37005 Isolates	a/a	This study
L26	Clinical Isolate	a/a	Lockhart <i>et al.</i> (2002)
P37005	Clinical Isolate	a/a	Lockhart <i>et al.</i> (2002)
RBV1153	<i>gal1::URA3 ade2::URA3 leu2/leu2 his1/his1</i>	a/a	This study
RBV1154	<i>gal1::URA3 ade2::URA3 rad51::HIS1/rad51::leu2</i>	a/a	This study
RBV1155	<i>gal1::URA3 ade2::URA3 rad51::HIS1/rad51::leu2</i>	α/α	This study
RBV1156	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2</i>	a/a	This study
RBV1157	<i>gal1::URA3 ade2::URA3 rad52::HIS1/rad52::leu2</i>	α/α	This study
RBV1175	<i>arg/arg</i>	a/a	This study
RSY12, RSY14	<i>kar3::LEU2/kar3::HIS1 arg4/arg4</i>	a/a	Sherwood and Bennett (2008)
RSY153, RSY155	RBV1175 <i>TUB2::TUB2-GFP:SAT1</i>	a/a	Sherwood and Bennett (2008)
RSY240	<i>H2B::H2B-RFP:ARG4</i>	a/a	Sherwood and Bennett (2008)
RSY247	RBV1175 <i>H2B::H2B-CFP:SAT1</i>	a/a	Sherwood and Bennett (2008)
	<i>TUB2::TUB2-YFP:ARG4</i>	α/α	Sherwood and Bennett (2008)
RZ7	<i>TUB2::TUB2-YFP:SAT1</i>		
RZ10	CAI4	α/α	Bennett <i>et al.</i> (2005)
RZY219	CAI4	a/a	Bennett <i>et al.</i> (2005)
SN78	<i>wor1::LEU2/wor1::HIS1</i>	a/a	Zordan <i>et al.</i> (2006)
YJB6407	<i>ura3/ura3 leu2/leu2 his1/his1</i>	a/ α	Noble and Johnson (2005)
YJB6767	BWP17 <i>clb4::ARG4/P_{MET3}-CLB4:URA3::clb4</i>	a/ α	Bensen <i>et al.</i> (2005)
	BWP17 <i>clb4::ARG4/clb4::HIS1 ura3/ura3</i>	a/ α	Bensen <i>et al.</i> (2005)

SAT^s selection gave rise to DSY246, whereas transformation with pRB102 gave rise to DSY247.

Plasmid pKA1 was cut with KpnI/SacI and transformed into strains KAY35/KAY58 to generate *WOR1* heterozygous strains KAY138 and KAY142/143, respectively. Integrations were checked using oligo pairs 52/55 and 54/56. These strains gave SAT^s derivatives KAY187, KAY188, and KAY190, respectively. The latter strains were transformed again with pKA1 to construct *WOR1* homozygous deletions, confirmed using oligos 57/58, with representative strains being KAY209, KAY212, and KAY214.

Plasmid pKA3 was linearized by partial digestion with KpnI and transformed into wild-type (RBY1153), *RAD51* heterozygous (KAY35), and *RAD52* heterozygous (KAY58) strains. Transformation of RBY1153 yielded KAY220, whereas transformation of KAY35 and subsequent disruption of the remaining *RAD51* allele by using a *LEU2* marker yielded KAY245. Transformation into KAY58 and subsequent *RAD52* disruption yielded strain KAY250. Integration was confirmed using oligos 59/60.

CHY439 (Miller and Johnson, 2002) was transformed with the KpnI/SacI fragment of pKA1 to yield *WOR1* heterozygous strains KAY308-10. Integration was checked using oligo pairs 52/55 and 54/56. CHY439 also was transformed with an ApeI/SacII fragment from pWOR1-3, a plasmid containing the *WOR1* gene as well as ~9 kb of the *WOR1* promoter (Ramirez-Zavala *et al.*, 2008), a gift from Joachim Morschhauser (University of Würzburg, Würzburg, Germany). This yielded strains KAY314-17 containing three copies of the *WOR1* gene, and integration was checked using oligos 61/62.

To generate the green fluorescent protein (GFP)-expressing strains KAY287/289, as well as KAY290/292, strains BZ7 and BZ10 (Bennett *et al.*, 2005) were transformed with a BglII-linearized pACT-GFP (a gift from Alistair Brown, University of Aberdeen, Aberdeen, United Kingdom) (Barelle *et al.*, 2004).

The strain YJB6407 expressing *P_{MET3}-CLB4* (Bensen *et al.*, 2005) was a gift from Judith Berman (University of Minnesota). This strain was transformed with the SacI/SacII fragment from pRB102 to delete the *MTL α* locus, producing strain KAY55. KAY55 was grown in YPD medium to recycle the SAT1 marker as described previously (Reuss *et al.*, 2004). Two additional SAT^s strains, KAY60/61, also were derived from KAY55 by this method. The Δ *Clb4* strain YJB6767 was grown on sorbose medium as described previously (Janbon *et al.*, 1998) to generate the *a/a* strains CAY70/71.

Switching Assays

White phase cells were inoculated into liquid YPD medium and incubated overnight at room temperature. Cultures were checked for the purity (>99%) of white cell forms (round cells), diluted in H₂O, and plated onto solid SCD medium (unless stated otherwise) at a concentration of ~100 colonies per plate. Colonies were scored for opaque sectors after growth of colonies at room temperature for 7 d.

Doubling Time Analysis

White cells were inoculated into liquid YPD medium and grown overnight at room temperature. Cultures were diluted to 10⁷ cells/ml in fresh YPD medium (unless otherwise stated) and grown at room temperature. OD₆₀₀ readings were taken every 75 min for 6–8 h. OD readings were then plotted in Excel (Microsoft, Redmond, WA) as a function of time and analyzed by exponential regression to deduce the cell doubling time. In each experiment, doubling times were normalized to the percentage of change from the control.

For determination of the doubling time of cells grown on SCD medium containing hydrogen peroxide, single cells were visualized using time-lapse microscopy and growth rates were measured as described previously (Sherwood and Bennett, 2008).

Colony Size Determination

Colonies were imaged after 3 d of growth at room temperature by using a Stemi 2000-C stereoscope (Carl Zeiss, Thornwood, NY) with an Infinity 1 camera. Images were analyzed and colony size determined using ImageJ software (National Institutes of Health, Bethesda, MD) (Abramoff *et al.*, 2004). Briefly, images were converted to black and white, and the color threshold was adjusted so colonies were distinct from surrounding media. Colony size was then determined using the Analyze Particle feature and compared with control strains.

Selection of Faster Growing Suppressor Strains

P37005 was grown in 3-ml cultures of YPD medium at 30°C. Every day for 1 wk 5 μ l of an overnight culture was diluted back into fresh YPD medium. After this time, cells were plated onto YPD plates for single colonies and grown overnight at 30°C. Nine larger colonies representative of faster growing cells were selected. Each of these nine colonies (derived from three independent experiments) was then subjected to doubling time analysis and white-opaque switching assays as described above.

RNA Extraction

RNA was isolated from cells grown to logarithmic phase at room temperature in YPD medium. An equal amount of cells (3.0 OD₆₀₀) was used for total RNA

isolation by using the RiboPure-Yeast kit (Ambion, Austin, TX). RNA was quantified using a spectrophotometer (260 nm). In each experiment, total RNA was normalized to that isolated from wild-type SC5314 cells.

ACT1-GFP Expression

Metabolic activity was monitored by measuring the fluorescence intensity of cells from culture using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA). Cells were grown overnight at 30°C in varying concentrations of SCD media and diluted back into the same media concentration the following morning. After 6 h of growth cells were analyzed by flow cytometry for expression of GFP.

Statistical Analysis

Statistical analyses were performed using two-sample *t* tests in Excel. All *p* values are two-tailed and compared with control unless otherwise stated.

RESULTS

Genotoxic Stress Induces White-Opaque Switching in *C. albicans*

Previous studies have shown that exogenous factors, such as UV irradiation, increase switching from white to opaque in *C. albicans* (Morrow *et al.*, 1989; Kolotila and Diamond, 1990). We hypothesized that white-opaque switching may be induced as part of a general response to cell stress in white cells; therefore, we examined whether other types of genotoxic stress could induce switching to opaque. MMS is a DNA-alkylating agent that triggers the DNA damage checkpoint in *C. albicans* (Shi *et al.*, 2007), whereas treatment of cells with HU induces the DNA replication checkpoint (Zhao *et al.*, 1998; Shi *et al.*, 2007). To examine whether treatment of cells with either of these agents induces white-to-opaque switching, white phase cells were plated on SCD medium containing increasing amounts of MMS or HU. Plates were grown for 7 d at room temperature before being analyzed for switching events. Because white-to-opaque switching is heritable, opaque cells are stable after a switching event; thus, the presence of opaque sectors within white colonies allows for macroscopic evaluation of the switching frequency (Supplemental Figure S1, top left) (Slutsky *et al.*, 1987).

In control experiments, white phase *a/a* or α/α cells derived from the laboratory strain SC5314 (see *Materials and Methods*) were plated on SCD medium and allowed to grow for 7 d. White colonies grew up on these plates in which 1–2% of colonies contained opaque sectors (Figure 1). Cells plated on SCD medium supplemented with MMS, however, showed a dose-dependent increase in the number of white colonies containing opaque sectors. For example, 28% of colonies grown on medium containing 0.020% MMS showed opaque sectors, an increase in switching of nearly 20-fold over background levels (*p* < 0.0001). Similarly, when cells were plated on medium containing 25 mM HU, 44% of colonies developed opaque sectors (*p* < 0.0001). Significantly, both MMS- and HU-induced switching was dependent on the master regulator of white-opaque switching, *WOR1*, because performing the same assays in Δ *wor1* strains failed to cause any observable switching to the opaque state (25 mM HU and 0.020% MMS tested; Supplemental Table S2). These results demonstrate that chemical agents that induce genotoxic stress increase switching from white to opaque in a *WOR1*-dependent manner.

Genotoxic Stress-Induced Switching Is Not Due to Genomic Changes

Treatment of cells with MMS is known to cause DNA damage, raising the possibility that increased switching in MMS is due to genomic changes. Similarly, hydroxyurea has been reported to cause DNA damage under some conditions

Figure 1. Genotoxic stress increases white-to-opaque switching. White cells from wild-type SC5314 *a/a* or *α/α* strains (RBY1153 or DSY246/247) were plated on SCD medium containing increasing amounts of genotoxic agents and grown at room temperature for 7 d. Colonies were then counted and scored for opaque sectors. Data are combined from a minimum of two independent experiments for each strain. NT, no treatment. Error bars represent SE. **p* < 0.01 compared with no treatment control. ***p* < 0.0001 compared with the control.

(Sakano *et al.*, 2001). To determine whether MMS or HU increased switching by introducing changes at the genomic level, opaque sectors from plates containing these agents were restreaked onto SCD medium and grown at 37°C for 4 d, because growth at this temperature in the laboratory promotes mass conversion of opaques back to white cells (Slutsky *et al.*, 1987). White cells, derived from opaque sectors, were then reassayed for switching on control SCD medium. If increased switching was caused by genomic changes, then cells should still exhibit elevated switching frequencies. However, if increased switching is dependent on the presence of MMS or HU in the medium, then cells should exhibit normal levels of switching (~1.5%) when restreaked on SCD medium lacking these agents. The latter was observed in these experiments, because even at the highest levels of MMS and HU tested, rates of switching in restreaked cells were 2.7 and 3.9% respectively, levels not significantly different (*p* = 0.24 and *p* = 0.16, respectively) to that of control experiments (Table 2). These results demonstrate that genotoxic stress increases white-to-opaque switching but does so without genomic changes.

Deletion of RAD51 or RAD52 Results in Increased White-Opaque Switching

In *C. albicans*, as in many fungi, DNA repair is often mediated through homologous recombination (Ciudad *et al.*, 2004; Krogh and Symington, 2004). Two conserved proteins integral to homologous recombination in *Saccharomyces cerevisiae* and higher eukaryotes are Rad51p and Rad52p (Krogh and Symington, 2004). *C. albicans* Rad52p has also been shown to be critical for survival in the presence of DNA damaging agents such as MMS (Ciudad *et al.*, 2004). Based on its homology to *S. cerevisiae* RAD51, orf19.3752 has been designated *C. albicans* RAD51, with a putative role in homologous recombination (candidagenome.org). Because genotoxic stress had a major impact on the frequency of white-opaque switching, mutants in RAD51 and RAD52 were tested in switching assays to see whether the loss of DNA repair functions influenced rates of switching.

Table 2. Switching frequency of strains after treatment with MMS or HU

Initial treatment	Switching frequency posttreatment	p value vs. no treatment
None	1.78 ± 0.40	n/a
0.005% MMS	0.78 ± 0.13	=0.10
0.010% MMS	1.54 ± 0.52	=0.72
0.020% MMS	2.73 ± 0.74	=0.24
12.5 mM HU	2.52 ± 0.72	=0.36
25 mM HU	3.92 ± 1.78	=0.16

Opaque sectors from colonies on plates containing MMS and HU were restreaked onto SCD medium and grown at 37°C for conversion to the white phase (Slutsky *et al.*, 1987). Cells were then plated on SCD medium and analyzed for the presence of opaque sectors. Approximately 10 sectors were reanalyzed for each condition. The switching frequency is listed as the mean frequency ± SE. The *p* values were determined using a two-tailed, unpaired *t* test compared with control.

We first note that strains lacking RAD52 produced irregular colonies and additional experiments were necessary to determine the white/opaque status of cells in these colonies. Loss of RAD52 produced colonies that were opaque and wrinkled, whereas many were also “fuzzy” due to the presence of multiple aerial filaments (Supplemental Figure S1). Microscopically cells from both wrinkled colonies and fuzzy colonies had an elongated cell phenotype characteristic of the opaque state (Slutsky *et al.*, 1987). To determine whether colonies contained white or opaque cells, a *WOR1-YFP* construct was introduced into these strains. This construct allows white and opaque cells to be easily distinguished because only opaque cells express detectable levels of Wor1 protein (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006). Notably, only cells taken from the fuzzy portions of colonies showed Wor1 expression, indicating that these were opaque cells, and allowing accurate quantification of switching frequencies in Δ/Δ rad52 strains (Supplemental Figure S1).

As shown in Figure 2, when either RAD51 or RAD52 was deleted switching frequencies from white to opaque increased over 40-fold, to 68 and 62%, respectively (*p* < 0.0001). These increases were due to loss of the targeted gene as complementation with a wild-type copy of the gene added back into the mutant reduced switching frequencies to those seen in wild-type strains (Figure 2). To examine the dependence of switching in Δ/Δ rad51 and Δ/Δ rad52 mutants on *WOR1*, double mutants Δ/Δ wor1 Δ/Δ rad51 and Δ/Δ wor1 Δ/Δ rad52 were constructed. Both double mutants showed undetectable levels of switching to the opaque state (Supplemental Table S2). Thus, as was the case for switching in response to MMS and HU, switching in Δ/Δ rad51 and Δ/Δ rad52 mutants was dependent on *WOR1*. Together, these results indicate unsuspected links between DNA damage, genotoxic stress, and white-opaque switching.

Activation of the S Phase Checkpoint Is Not Required for Increased White-Opaque Switching

We first speculated that increased white-to-opaque switching observed in Δ/Δ rad51 and Δ/Δ rad52 mutants, as well as that induced by MMS and HU, could be due to activation of cell cycle checkpoints. In *C. albicans*, DNA damage by MMS is known to activate the DNA-damage checkpoint, whereas treatment with HU activates the DNA replication check-

Figure 2. Deletion of *RAD51* or *RAD52* results in increased white-to-opaque switching. White cells from wild-type (WT, RBY1153), $\Delta/\Delta rad51$ (RBY1154/55), $\Delta/\Delta rad52$ (RBY1156/57), or reconstituted strains (CAY6/7 and KAY111) were plated on SCD medium and grown at room temperature for 7 d. Colonies were counted and scored for opaque sectors. Opaque sectors for $\Delta/\Delta rad52$ colonies were confirmed using fluorescence microscopy for Wor1-YFP (see Supplemental Figure S1). Data are combined from three independent experiments for each strain. Error bars represent SE. ** $p < 0.0001$ compared with wild-type cells.

point (Shi *et al.*, 2007). Rad53 is a protein kinase that acts downstream of both S phase checkpoint pathways and is required for arrest of cell growth in response to both MMS and HU (Shi *et al.*, 2007). Activation of Rad53p can be monitored by following protein phosphorylation, because Rad53p is hyperphosphorylated during checkpoint signaling (Shi *et al.*, 2007). To test whether Rad53p activation was important for increased switching from white to opaque, strains carrying myc-tagged versions of Rad53p were constructed. Previous studies have shown that treatment of *C. albicans* cells with MMS or HU induced hyperphosphorylation of Rad53p that could be visualized by a retarded electrophoretic mobility in Western blots (Shi *et al.*, 2007). We confirmed that MMS treatment of cells led to hyperphosphorylation of Rad53, as shown in Supplemental Figure S2 (lane 7). We next tested whether loss of *RAD51* or *RAD52* also led to activation of the Rad53-mediated cell cycle checkpoint. Wild-type, $\Delta/\Delta rad51$, and $\Delta/\Delta rad52$ strains carrying myc-tagged Rad53p were grown in logarithmic phase, and extracts were analyzed by Western blotting. In contrast to cells responding to MMS treatment, $\Delta/\Delta rad51$ and $\Delta/\Delta rad52$ cells did not show any hyperphosphorylation of Rad53p (Supplemental Figure S2, lanes 3 and 5). These results indicate that activation of the Rad53-mediated checkpoint is not required for the hyperswitching observed in $\Delta/\Delta rad51$ or $\Delta/\Delta rad52$ mutants of *C. albicans* and that another mechanism must therefore be responsible for the increased switching.

A Connection between White-Opaque Switching and Cellular Growth Rates

During the course of these experiments, we noted that many of the conditions that promote high rates of white-opaque switching also caused slower growth of the strains. Cells treated with the genotoxic agents MMS and HU, as well as $\Delta/\Delta rad51$ and $\Delta/\Delta rad52$ mutant strains, all exhibited significantly increased generation times. For example, $\Delta/\Delta rad51$ and $\Delta/\Delta rad52$ strains grew at rates that were 19 and 42% slower, respectively, than

Table 3. Growth rate analysis of *C. albicans* strains

Strain	% Increase in generation time compared with wild-type	p value vs. wild-type
$\Delta/\Delta rad51$	18.82 ± 1.55	<0.0001
$\Delta/\Delta rad51 + RAD51$	1.22 ± 1.90	$=0.57$
$\Delta/\Delta rad52$	41.66 ± 5.92	<0.0005
$\Delta/\Delta rad52 + RAD52$ (CAY11)	12.98 ± 2.55	<0.005
$\Delta/\Delta rad52 + RAD52$ (KAY111)	2.85 ± 0.9	$=0.03$
P37005	16.47 ± 1.82	<0.0001
L26	8.23 ± 1.12	<0.0001
H2B/Tub2 fluorescent fusion proteins	10.02 ± 1.01	<0.0001

White cells were grown overnight in YPD at room temperature, diluted to 1×10^7 cells/ml and grown for 6–8 h. The OD_{600} was measured every 75 min, and the resulting points were analyzed using exponential regression to determine doubling time of the population. Doubling times were normalized to a wild-type SC5314 control strain (RBY1153/DSY247) and are listed as percentage of increase over wild type $\pm$ SE. RBY1154/1155 were used as $\Delta/\Delta rad51$ strains with a representative reconstituted strain being CAY6. RBY1156/1157 were used as $\Delta/\Delta rad52$ strains with CAY11 representing a partially complemented mutant, and KAY111 a completely complemented mutant (see Supplemental Figure S3). Data is combined from a minimum of three experiments for each strain. The p values were determined using a two-tailed, unpaired *t* test compared with control.

that of the parental strains (Table 3). This led to the hypothesis that increased rates of white-opaque switching were a direct consequence of longer generation times. We subsequently examined several other slow growing strains in the laboratory, including a strain lacking the molecular motor *KAR3*, as well as a strain expressing fluorescently labeled histone (H2B) and tubulin (Tub2) proteins (Sherwood and Bennett, 2008). Both of these strains exhibit a significant delay in cell generation times, growing at least 10% slower than SC5314 (see Sherwood and Bennett, 2008; Table 3). In addition, we found that white colonies from $\Delta/\Delta kar3$ and H2B/Tub2-labeled strains also switched to opaque at high frequency (25 and 22%, respectively; Table 4), thereby supporting a connection between cell growth and switching.

Table 4. White-to-opaque switching analysis of slow growing strains of *C. albicans*

Strain/genotype	Switching frequency (%)	p value vs. wild-type
$\Delta/\Delta kar3$	25.11 ± 2.40	<0.0001
H2B/Tub2 fluorescent fusion proteins	28.42 ± 3.38	<0.0001
Parasexual isolate P6	21.78 ± 2.89	<0.0001
Parasexual isolate Ss10	13.39 ± 2.08	<0.0001

White cells were grown overnight, plated on SCD medium, and analyzed for the presence of opaque sectors after 7 days of growth at room temperature. Switching values are listed as mean switching frequency $\pm$ SE and represent data from at least three independent experiments. The p values were determined using a two-tailed, unpaired *t* test compared with control.

Figure 3. Titrating *CLB4* cyclin expression results in altered white-opaque switching frequencies. (A) Size of $P_{\text{MET3}}\text{-CLB4}$ colonies. White cells expressing $P_{\text{MET3}}\text{-CLB4}$ were plated on synthetic medium containing increasing amounts of methionine (Met) and cysteine (Cys) and grown at room temperature for 3 d. Colonies were subsequently analyzed using ImageJ software to determine relative colony size. Approximately 100 total colonies from three independent strains (KAY55/60/61) were analyzed at each concentration. Single cell time-lapse microscopy confirmed that smaller colonies exhibited slower generation times; cells grown on 50 μM Met/Cys grew 53% slower than cells grown on medium without Met/Cys. (B) Switching frequency of $P_{\text{MET3}}\text{-CLB4}$ strains. White phase $P_{\text{MET3}}\text{-CLB4}$ cells were plated on synthetic medium containing varying amounts of methionine and cysteine and grown at room temperature for 7 d. Colonies were analyzed for opaque sectors. Data are combined for three experiments using three strains (KAY55/60/61). Error bars represent SE. * $p < 0.0001$ compared with plates/cultures containing no Met/Cys. # $p < 0.01$ compared with plates containing 25 μM Met/Cys. (C) Total RNA concentrations in switching and nonswitching strains of *C. albicans*. Total RNA was isolated from wild-type (RBY1153/DSY247), Δclb4 (CAY70/71), and H2B/TUB2 FP-expressing (RSY240/247) strain. The Δclb4 strains and H2B/TUB2 fluorescent strain are both slow growing but the Δclb4 strain does not exhibit increased rates of white-to-opaque switching due to limited RNA expression (see text for details). Error bars represent SE. ** $p < 0.0002$ compared with SC5314.

To further analyze a potential relationship between growth rates and white-opaque switching, we tested progeny from the parasexual cycle of *C. albicans* for their doubling times and switching frequencies. Sexual reproduction in *C. albicans* occurs between opaque α and α cells and is completed by an unusual mechanism of chromosome loss in tetraploid strains, generating recombinant diploid and aneuploid products (Bennett and Johnson, 2003; Forche *et al.*, 2008). Previous studies demonstrated that many of the progeny derived from the parasexual cycle were slower growing than parental SC5314 strains, even in progeny with a euploid complement of chromosomes (Forche *et al.*, 2008). Analysis of euploid progeny identified several slow-growing diploid strains that also underwent switching at high frequency (e.g., isolates P6 and Ss10; Table 4). These experiments provide additional support for a connection between the rate of growth and white-opaque switching in *C. albicans*.

To establish the hypothesis that switching frequencies are influenced by rates of cell growth, cell doubling times were artificially manipulated by varying expression of a cell cyclin gene. *C. albicans* has two B-type cyclins, *CLB2* and *CLB4*, and although *CLB2* is essential, loss of *CLB4* produces viable cells that exhibit delayed progression through G2/M of the cell cycle (Bensen *et al.*, 2005). Previously, a strain expressing *CLB4* under control of the *MET3* promoter was created to allow titratable expression of the only copy of *CLB4* in this strain (Care *et al.*, 1999; Bensen *et al.*, 2005). With the *MET3*

promoter the target gene is highly expressed when levels of methionine and cysteine in the medium are low. As the concentrations of these amino acids are increased, the gene is gradually repressed, and at high levels of methionine/cysteine the gene is essentially shut off. To demonstrate the effect of titrating *CLB4* expression on cell growth, a strain expressing the $P_{\text{MET3}}\text{-CLB4}$ construct was plated onto medium containing varying amounts of methionine and cysteine. At very low levels of methionine and cysteine, colonies were the same size as those plated on control medium (containing no methionine or cysteine) (Figure 3A). As the concentration of methionine/cysteine was increased, the average colony size decreased, although at methionine/cysteine concentrations above 500 μM colony sizes were small but remained constant (Figure 3A). To confirm that smaller colonies were a result of slower cellular growth, time-lapse microscopy was used to measure cell generation times. This analysis revealed that cells grown on medium containing 50 μM methionine/cysteine grew 53% slower than cells grown on medium lacking methionine and cysteine.

White-opaque switching analysis was performed on the $P_{\text{MET3}}\text{-CLB4}$ strain expressing varying amounts of *CLB4*. Notably, colonies that showed intermediate rates of growth exhibited the highest rates of white-opaque switching. As shown in Figure 3B, addition of low amounts (5 μM) of methionine/cysteine to the medium did not alter the frequency of switching from white to opaque. At 25 μM methionine/

cysteine, switching frequencies peaked, with ~18% of colonies containing opaque sectors. At higher concentrations of methionine/cysteine, switching frequencies declined once again, returning to baseline levels (Figure 3B). Thus, a significant increase in switching was observed at intermediate concentrations of methionine/cysteine compared with conditions both lacking methionine/cysteine as well as high concentrations of these amino acids ($p < 0.0001$). We note that the decrease in switching seen at high levels of methionine/cysteine is not due to a direct effect of these amino acids on white-opaque switching. A strain exhibiting hyperswitching (one in which the only copy of *RAD52* was under control of the *MET3* promoter) showed high levels of switching that were maintained even at high concentrations of methionine and cysteine (data not shown).

These experiments demonstrate that changes in the generation times of *C. albicans* strains can directly influence rates of white-opaque switching. The studies with *CLB4* also establish that a window exists for optimal white-opaque switching with regard to cellular growth. At high levels of *CLB4* expression, or when *CLB4* expression is repressed, basal rates of white-opaque switching occur. However, at intermediate levels of *CLB4* expression high rates of switching are promoted. The lack of switching in slow growing strains (lacking *CLB4* expression) is addressed below.

Dependence of White-Opaque Switching on Cell Metabolism

Our results suggest that while delaying the cell cycle can stimulate white-opaque switching, very slow growing strains undergo only limited rates of switching. We speculated that the latter could be due to a general decrease in protein expression under these growth conditions, thereby limiting the production of protein factors necessary for switching to opaque. To test this possibility, we carried out two sets of experiments to address the role of cell metabolism (specifically RNA and protein synthesis) in white-opaque switching.

First, we examined overall levels of RNA production in cells growing at normal rates and those in very slow-growing cells. Total levels of RNA in wild-type SC5314 cells were compared with those in $\Delta\Delta clb4$ mutants that grow slowly (47% slower than SC5314) yet do not exhibit high switching frequencies (<1%). Cultures of wild-type and $\Delta\Delta clb4$ cells were grown to logarithmic phase in YPD medium at room temperature, cells were harvested, and total RNA was extracted (see *Materials and Methods*). We found that $\Delta\Delta clb4$ cultures showed a significant decrease in total RNA levels, containing approximately half the amount of RNA of wild-type cultures ($p < 0.0002$; Figure 3C). These results are consistent with studies in *S. cerevisiae* that found reduced RNA expression in slow-growing strains (Grummt and Ladurner, 2008). In contrast, analysis of a strain that grows

Figure 4. Nutrient levels affect white-to-opaque switching frequencies. (A) White-to-opaque switching on dilute media. White cells were plated on SCD medium (100% medium) or on diluted SCD medium (25–75%) and grown at room temperature for 7 d. Colonies were counted and analyzed for opaque sectors. Black bars represent average switching frequencies of wild-type strains (RBY1153 and DSY246/247). Gray and white bars represent switching frequencies of two hyperswitching strains (*rad52* mutant strains) CAY10 and CAY11, respectively. (B) Opaque-to-white switching on dilute media. Opaque phase cells from wild-type strain RBY1153 were plated on different concentrations of SCD medium and grown at room temperature for 7 d. Colonies were counted and analyzed for presence of white phase cells. (C) Cellular metabolism in dilute media. Cells containing GFP under the control of the *ACT1* promoter were grown in different concentrations of SCD and analyzed for mean fluorescence intensity by flow cytometry. Note that dilution of the media did not compromise cellular growth rates until media was 25% or lower in concentration (data not shown), so that changes in growth rates were not observed until culture conditions limited general cell metabolism. Data are combined from three experiments. Error bars represent SE.

slower than wild-type yet exhibits hyperswitching to opaque (a strain expressing fluorescently labeled H2B/Tub2; Tables 3 and 4) showed only a 10% decrease in total RNA levels ($p = 0.48$; Figure 3C). These studies confirm a close relationship exists among cellular generation times, RNA and protein synthesis, and rates of white-opaque switching.

In a second series of experiments, we further analyzed the connection between cell metabolism and white-opaque switching. In this case, strains were grown on nutrient-depleted medium to decrease their rate of growth. SCD medium was defined as being 100% and dilutions of this medium were prepared from 5 to 100% (agar concentration held constant at 2%). As the concentration of nutrients decreased, rates of colony growth decreased in parallel as expected. However, although cell growth was slowed, no increase in white-opaque switching was observed at any of the lower concentrations of SCD tested (Figure 4A). These experiments demonstrate that slowing cell growth by limiting nutrients does *not* lead to increased rates of white-to-opaque switching in *C. albicans*. In addition, we examined the effect of varying nutrient concentrations on strains that normally undergo hyperswitching to the opaque state. For example, partial complementation of $\Delta/\Delta rad52$ mutants (see Supplemental Figure S3 and Table 3 for mutant characterization) produced strains that undergo switching at elevated rates and display clear white-opaque sectoring (strains CAY10 and CAY11; Figure 4A). When these hyperswitching strains were grown on decreasing medium concentrations, a significant decrease in white-to-opaque switching frequencies was observed (Figure 4A). Decreased rates of switching were not due to instability of opaques under these conditions, as plating of opaque cells onto diluted medium led to the formation of opaque colonies at the same frequency as on standard SCD medium (Figure 4B). These experiments reveal that high frequency switching to the opaque state requires an adequate supply of nutrients, presumably to maintain high levels of RNA and protein synthesis. We note that Wor1p levels in white cells were below the level of detection by western blotting or fluorescent microscopy, so that direct determination of Wor1p concentrations in these cells was not possible (data not shown).

To confirm that depleting nutrients from the media results in compromised protein expression, a GFP reporter was placed under control of the *ACT1* promoter as a sensitive indicator of protein expression levels in the cell. As media concentrations decreased, cells showed a concomitant decrease in fluorescence intensity, particularly in media concentrations between 100 and 25% (Figure 4C). This result confirms that protein expression is reduced in diluted media conditions, consistent with our hypothesis of nutrient limitation affecting general cellular metabolism. Thus, strains that grow slowly due to low nutrients do not switch to opaque at high frequency due to reduced RNA and protein expression in the cell.

Oxidative Stress also Induces Efficient White-Opaque Switching

Our experiments suggest a general model whereby conditions that slow cell division (without compromising protein expression) lead to increased rates of white-opaque switching. We decided to test whether other environmental conditions could affect switching frequency, particularly stresses that could be encountered in the mammalian host. Neutrophils often target invading microbes by production of an oxidative burst (Nathan, 2006); therefore, we analyzed

Figure 5. Oxidative stress induces white-to-opaque switching. white cells from wild-type SC5314 *a/a* or *α/α* strains (RBY1153/DSY247) were plated on SCD medium containing increasing amounts of hydrogen peroxide and grown at room temperature for 7 d. Colonies were then counted and scored for opaque sectors. Data are combined from a minimum of two independent experiments for each strain. Error bars represent SE. * $p < 0.01$ compared with no treatment control. ** $p < 0.0001$ compared with the control.

whether oxidative stress in the form of hydrogen peroxide modulates white-opaque switching in SC5314 strains.

Switching assays were performed on *a/a* or *α/α* derivatives of SC5314 plated on SCD medium supplemented with increasing concentrations of hydrogen peroxide (see *Materials and Methods*). Although ~1% switching occurred on control SCD plates, switching was significantly increased in plates containing gradually higher concentrations of hydrogen peroxide. For example, 0.8 mM hydrogen peroxide induced switching in 42% of colonies, whereas 1 mM hydrogen peroxide induced switching in >70% of colonies (Figure 5). In addition, at the higher concentrations of hydrogen peroxide (0.8 and 1 mM) colonies often contained multiple opaque sectors. Increasing hydrogen peroxide concentrations even higher (>1 mM) led to a reduction in colony counts due to increased levels of cell death in the population (data not shown). Notably, concentrations of hydrogen peroxide that led to increased switching also resulted in reduced growth rates. For example, strains grown on SCD medium containing 1 mM hydrogen peroxide grew on average 46% slower than those on control SCD medium.

These results establish that oxidative stress can also induce high rates of white-to-opaque switching, and that this increased switching again correlates with slower growth of cells under these culture conditions.

Clinical Isolates P37005 and L26 Exhibit High-Frequency White-Opaque Switching

Greater than 90% of all naturally occurring isolates of *C. albicans* are heterozygous at the *MTL* locus, containing both *a* and *α* copies of the locus (Lockhart *et al.*, 2002; Odds *et al.*, 2007). Several natural isolates have been identified, however, that are homozygous *a/a* or *α/α* at the *MTL* locus and thus are competent to undergo white-opaque switching (Lockhart *et al.*, 2002; Pujol *et al.*, 2002; Daniels *et al.*, 2006; Srikantha *et al.*, 2006; Yi *et al.*, 2008). We analyzed several of these clinical isolates to determine whether there is a corre-

Figure 6. Naturally occurring *a/a* isolates show high rates of white-to-opaque switching due to slow growth. (A) Switching frequency of clinical isolates. White cells from the *a/a* clinical isolates P37005 and L26 were plated on SCD medium and grown at room temperature for 7 d. Colonies were counted and analyzed for white-to-opaque switching events. (B) Growth rate analysis of faster growing isolates. The *a/a* clinical isolate P37005 was subjected to serial passaging in YPD media for 7 d. In total, nine independent faster growing isolates (KAY326–334) were subjected to further analysis. The growth rates of these faster growing strains in liquid YPD media at room temperature compared with the original P37005 isolate. (C) Switching analysis of faster growing isolates. White cells from each of the isolates were plated on SCD medium and grown at room temperature for 7 d. Colonies were counted and analyzed for white-to-opaque switching events. Data are combined from a minimum of three independent experiments. Error bars represent SE. ** $p < 0.0001$ compared with SC5314-derived strains.

lation between their generation times and their rates of white-opaque switching.

In isolates P75063 and P78048, these strains grew at rates near that of SC5314 and also switched to opaque at similar frequencies of 1–2% (data not shown). In contrast, however, isolates P37005 and L26 were found to exhibit both elevated rates of white-opaque switching and significantly longer generation times compared with SC5314. The growth rates of P37005 and L26 were 16 and 8% slower, respectively, than wild-type SC5314 (Table 3). In P37005, ~45% of colonies accumulated opaque sectors, a 30-fold increase over SC5314-derived *a* or α strains. In L26, 28% of colonies showed opaque sectors, a nearly 20-fold increase over SC5314 (Figure 6A). Both increases in switching frequencies were statistically significant ($p < 0.0001$). These results indicate that P37005 and L26 switched at frequencies similar to that of SC5314-derived strains grown either under conditions of genotoxic stress or lacking *RAD51/RAD52*. Similar generation times are also seen in comparing these hyperswitching strains. For example, P37005 grew at rates close to that of $\Delta/rad51$ mutants of SC5314 (16 and 19% slower than wild-type SC5314, respectively) and also showed similar switching frequencies (>45% of colonies contained opaque sectors).

To establish a link between growth rate and switching frequencies in clinical isolates, P37005 was serially passaged for 7 d and subsequently plated for single colonies. Faster

growing colonies were obtained by this method, presumably due to accumulation of suppressor mutations. Nine colonies from three independent experiments were selected for additional analysis, and these isolates were found to grow at rates nearly 10% faster than the parental P37005 isolate (Figure 6B). Strikingly, when these faster growing derivatives were analyzed in switching assays, all nine switched at reduced levels compared with the parental strain, and in fact switched at rates very similar to wild-type SC5314 (Figure 6C). This result confirms a close association between growth rates and switching frequencies even in clinical isolates of *C. albicans*.

We note that a recent study also used the P37005 isolate to analyze white-to-opaque switching but observed only low switching frequencies (<1%) by using this isolate (Ramirez-Zavala *et al.*, 2008). Analysis of this isolate in our laboratory, however, has found that it also grows much faster (>10%) than our parental P37005 strain. We therefore suggest that passaging of this isolate has led to growth suppressors that also dramatically reduce the efficiency of switching in this background.

Modulation of White-Opaque Switching in Response to Varying Levels of *Wor1*

We speculate that changes in cell growth rates influence *Wor1* protein levels and hence white-opaque switching frequencies (see *Discussion*). Previous studies have shown that

Figure 7. Copy number of *WOR1* influences white-to-opaque switching frequencies. White cells from strains containing either one, two, or three copies of *WOR1* under the control of the native promoter were plated on SCD medium and grown at room temperature for 7 d. Colonies were counted and analyzed for white-to-opaque switching events. Data are combined from a minimum of three independent experiments. Error bars represent SE. ** $p < 0.0001$ compared with two copies of *WOR1*.

Wor1p is a master regulator of switching; ectopic expression of *WOR1* causes cells to switch en masse to opaque, whereas $\Delta\Delta\text{wor1}$ mutants are unable to form opaques (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006). To determine the sensitivity of the switch to Wor1p levels, *WOR1* copy number was manipulated in the SC5314 background. White-opaque switching has already been shown to be sensitive to *WOR1* copy number in clinical isolates of *C. albicans* (Ramirez-Zavala *et al.*, 2008).

Our experiments were performed in a ura derivative of a SC5314 strain that has been shown to exhibit a higher background level of white-opaque switching (~7%) (Alby and Bennett, unpublished observations; Miller and Johnson, 2002; Zordan *et al.*, 2007). In comparison, *wor1*/*WOR1* heterozygotes from this strain were found to switch to opaque at a frequency of 0.5% (Figure 7). Significantly, integration of a third copy of the *WOR1* gene under its native promoter (integrated at the *ACT1* locus) resulted in switching increasing to 42% (a sixfold increase over the parental strain). These results confirm that relatively small changes in the levels of Wor1p expression can have a dramatic effect on rates of switching from white to opaque.

Together, our experiments demonstrate the following. 1) *C. albicans* strains exhibiting different growth rates have associated differences in white-opaque switching frequencies. 2) A window exists for optimal white-opaque switching; the cell cycle of SC5314-derived strains must be extended to enter this window, whereas clinical isolates P37005 and L26 naturally grow at slower rates that promote efficient switching to opaque. 3) Cell cycle delays caused by cell stress (or by genetic manipulation of the strain) have the potential to significantly increase switching frequencies. 4) Efficient white-opaque switching requires efficient cell metabolism, including high levels of RNA and protein synthesis. 5) The switch to opaque is dependent on Wor1p, and relatively small changes in Wor1p levels can have a significant effect on the frequency of white-opaque switching.

DISCUSSION

The white-opaque switch represents a paradigm for studying a bistable genetic switch in a pathogenic eukaryote. This switch regulates multiple features of *C. albicans* biology (see *Introduction*), yet the molecular mechanism regulating the white-opaque transition has only recently begun to be elucidated. Central to the switch is the role of Wor1 protein, which has been shown to be the master regulator of white-opaque switching (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006). In white cells, expression of Wor1p is either low or is completely repressed by the heterodimer $\alpha 1/\alpha 2$. To switch to the opaque state, Wor1p expression must increase until it reaches a critical threshold level at which point stable Wor1p expression is maintained by positive feedback on its own promoter. In this manner, stable opaque formation is achieved and is inherited by future generations (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006).

In the current work, we demonstrate that multiple conditions that delay cell cycle progression (without also compromising protein synthesis) lead to high rates of white-to-opaque switching in *C. albicans*. During growth on standard laboratory medium, homozygous *MTLa/a* or *MTLa/ α* strains derived from SC5314 normally switch to opaque at low frequency (~1%). However, cells experiencing genotoxic or oxidative stress that slow growth undergo high rates of switching that are 20- to 50-fold higher than in untreated controls. A similar increase in switching frequencies was seen in a number of different slow growing mutants of *C. albicans*, as genetic deletions in SC5314 (including $\Delta\Delta\text{rad51}$, $\Delta\Delta\text{rad52}$, and $\Delta\Delta\text{kar3}$) caused slower growth and high rates of switching to the opaque state.

To explain these observations, we propose a model for how growth rates influence white-opaque switching frequencies due to their effect on Wor1p levels in the cell. We hypothesize that although Wor1p synthesis positively promotes switching to opaque, frequent cell division acts to limit the accumulation of Wor1p in these cells. Thus, in wild-type SC5314 strains in which cell generation times are short, frequent cytokinesis acts to keep Wor1 protein concentrations low, reducing the chance that sufficient Wor1p will be present to drive the transition to opaque. However, conditions that slow cell cycle progression (either intrinsic factors such as genetic mutations or extrinsic factors such as environmental stress) promote the accumulation of Wor1p by delaying cell division. This will increase the chance that Wor1p levels will reach the threshold necessary for stable switching to opaque. Consistent with this model, we found that reverse switching (i.e., opaque to white) is decreased in slow-growing strains. The clinical isolate P37005 and a slow growing derivative of SC5314 (RSY240/247) both switched from opaque to white at a lower frequency (<2.5%) than wild-type SC5314-derived strains (6.5% switching) (Alby and Bennett, unpublished data). Again, slower growing strains are expected to accumulate higher concentrations of Wor1p and thereby shift the white-opaque equilibrium toward the opaque state.

Evidence that the white-opaque switch is exquisitely sensitive to the levels of Wor1 protein came from analysis of SC5314 strains containing different copy numbers of the *WOR1* gene under its native promoter. These experiments showed that losing one copy of *WOR1* in a diploid strain decreased white-opaque switching 14-fold, whereas the addition of a third copy of *WOR1* could increase switching sixfold over the parental strain. Thus, even small fluctuations in the levels of Wor1 protein are predicted to have a

significant effect on the overall frequency of white-opaque switching.

Direct support for the proposed model comes from experiments in which growth of *C. albicans* was artificially manipulated via regulated expression of the B-type cyclin gene *CLB4*. When *CLB4* expression was decreased, cells grew more slowly due to an extended delay in G2/M (Bensen *et al.*, 2005), and they exhibited a concomitant increase in switching from white to opaque. A further reduction of *CLB4* expression, however, reduced white-opaque switching frequencies back to basal levels. This decrease in switching correlated with lower RNA expression, because total RNA levels in $\Delta\textit{Clb4}$ mutants were significantly reduced compared with those in wild-type strains. Thus, intermediate growth rates led to high rates of white-opaque switching in the SC5314 background, whereas slow rates of growth produced only limited switching, presumably due to reduced Wor1 protein expression under those conditions.

We further demonstrate that efficient cellular metabolism is required for white-opaque switching by analysis of strains grown on nutritionally depleted media. Although a hyperswitching *rad52* mutant strain switched from white to opaque at frequencies 40-fold higher than wild-type strains on standard SCD medium, this strain did not switch at high frequency when cultured on diluted SCD medium. Cell metabolism was monitored by quantification of GFP expression from the *ACT1* promoter and confirmed a general decrease in protein expression on nutrient-depleted media. Thus, strains grown under these conditions have longer cell generation times, but they exhibit no increase in white-to-opaque switching levels. These results demonstrate that cell metabolism has a direct impact on the frequency of switching; slowing cell growth increases switching to opaque unless protein expression is also compromised, in which case switching occurs only at basal levels.

Additional support for a connection between white-opaque switching and cell doubling times comes from analysis of clinical isolates. Although several clinical isolates grew at similar rates and switched at similar frequencies to SC5314-derived strains, this was not the case for L26 and P37005. These isolates grew significantly slower than SC5314 and showed high rates of white-opaque switching (28 and 45%, respectively), further illustrating that the frequency of white-opaque switching is related to the growth rate of the strain. This hypothesis was confirmed by selection of faster growing suppressors in the P37005 isolate; all of the faster growing derivatives from P37005 grew at rates similar to SC5314 and also switched to opaque at similar frequencies. This result establishes the close relationship between growth rates and switching frequencies even in isolates of *C. albicans* from different genetic backgrounds.

The model for how cell growth modulates white-opaque switching is outlined in Figure 8. The critical factor determining switching efficiency is the effect of growth conditions on Wor1 protein levels. We have confirmed that white-opaque switching is absolutely dependent on Wor1p in our experiments, because genotoxic and oxidative stress, as well as mutants in DNA repair genes, failed to induce switching to opaque when tested in a $\Delta\textit{Wor1}$ background. We also ruled out a role for the cell cycle checkpoint pathway mediated by Rad53p, because activation of Rad53p was not observed in all hyperswitching mutants. The simplest model, therefore, is that lengthening the cell cycle is directly responsible for increased rates of switching due to a greater accumulation of Wor1p before cell division. This model suggests that most, if not all, stressful environments could potentially induce white-opaque switching by inhibiting rates of cell

Figure 8. Model for stress-induced phenotypic switching. A model for how variations in cell growth affect rates of white-to-opaque switching in *C. albicans*. In particular, we propose that increases in cell generation times (e.g., in response to genotoxic or oxidative stress) promote white-to-opaque switching, whereas decreases in cell metabolism (including RNA and protein synthesis) inhibit white-to-opaque switching.

growth. It also has important implications for other studies on phenotypic switching, as changes in switching frequencies previously observed (e.g., in response to UV irradiation; Morrow *et al.*, 1989) may simply be due to indirect effects of the test conditions on cell generation times.

Phenotypic switching provides a rapid means for adaptation in many microorganisms (Dubnau and Losick, 2006), and the same holds true for white-opaque switching in *C. albicans*. Studies have shown that white and opaque cells show marked differences in their relationship with the host, as exemplified by differences in both tissue specificity and in interactions with immune cells. For example, white cells are phagocytosed more efficiently than opaque cells by macrophages, and white cells, but not opaque cells, secrete a chemoattractant for leukocytes (Geiger *et al.*, 2004; Lohse and Johnson, 2008). The current work shows that white-to-opaque switching rates are modulated in response to multiple forms of cell stress, perhaps conferring a selective advantage during hostile conditions. In particular, strains exhibit an increased tendency to switch to opaque in response to oxidative stress, and this could potentiate escape from the immune system. Host neutrophils often target microbial invaders by release of oxidants, and thus the ability to sense these agents and switch to an alternative form could promote survival, particularly given that host cells differentially sense white and opaque cells (Kolotila and Diamond, 1990; Geiger *et al.*, 2004; Lohse and Johnson, 2008). In addition, increased switching to opaque may provide a benefit during colonization and infection of specific host niches. Other microbial flora can release oxidants such as hydrogen peroxide into the environment (Jakubovics *et al.*, 2008); thus, *C. albicans* could respond to these stimuli by switching to opaque. Opaque cells could be intrinsically advantageous under these conditions or could promote entry into the program of mating and sexual reproduction, as opaque cells are the mating-competent form of *C. albicans* (Miller and Johnson, 2002). Thus, recombinant forms may be generated that are more suited to survival under hostile environmental conditions.

Finally, recent studies have also shown that increasing rates of phenotypic switching in *S. cerevisiae* can offer a distinct fitness advantage for adaptation to a constantly fluctuating environment (Acar *et al.*, 2008). This is of interest because we show that at least two *C. albicans* strains, L26 and

P37005, have undergone selection for increased rates of spontaneous white-to-opaque switching in spite of a concomitant reduction in cell growth rates. L26 and P37005 were originally recovered from different host niches (1 oral and 1 vaginal; Lockhart *et al.*, 2002), indicating that hyper-switching strains are not limited to one environmental niche. We are therefore interested in determining what advantages increased rates of phenotypic switching may provide to *C. albicans* (whether induced by extracellular signals or intrinsic to the strain), with particular emphasis on the role of switching in directing the outcome of host-pathogen interactions.

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