

Rad52 function prevents chromosome loss and truncation in *Candida albicans*

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Summary

RAD52 is required for almost all recombination events in *Saccharomyces cerevisiae*. We took advantage of the heterozygosity of *HIS4* in the *Candida albicans* SC5314 lineage to study the role of Rad52 in the genomic stability of this important fungal pathogen. The rate of loss of heterozygosity (LOH) at *HIS4* in *rad52-ΔΔ* strains was $\sim 10^{-3}$, at least 100-fold higher than in Rad52⁺ strains. LOH of whole chromosome 4 or truncation of the homologue that carries the functional *HIS4* allele was detected in all 80 *rad52-ΔΔ* His auxotrophs (GLH – GL lab His⁻) obtained from six independent experiments. Isolates that had undergone whole chromosome LOH, presumably due to loss of chromosome, carried two copies of the remaining homologue. Isolates with truncations carried centric fragments of broken chromosomes healed by *de novo* telomere addition. GLH strains exhibited variable degrees of LOH across the genome, including two strains that became homozygous for all the heterozygous markers tested. In addition, GLH strains exhibited increased chromosomal instability (CIN), which was abolished by reintroduction of *RAD52*. CIN of GLH isolates is reminiscent of genomic alterations leading to cancer in human cells, and support the mutator hypothesis in which a mutator mutation or CIN phenotype facilitate more mutations/aneuploidies.

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Introduction

The opportunistic fungal pathogen *Candida albicans* is a diploid organism (Olaiya and Sogin, 1979) that, according to population studies, reproduces mainly by clonal propagation while exhibiting some recombination (Pujol *et al.*, 1993; Gräser *et al.*, 1996; Odds *et al.*, 2007). Nonetheless, phenotypic and genotypic variability can be extensive following growth *in vitro* or *in vivo* clonal propagation (Sudbery *et al.*, 2004; Magee, 2007). Phenotypic variability may be derived from (i) changes to the proteome due to flexibility of the genetic code (Gomes *et al.*, 2007), (ii) reversible changes in gene expression, such as the dimorphic transition (Brown, 2002), (iii) epigenetic heritable changes in gene expression, such as white/opaque switching (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006), or (iv) genetic alterations ranging from point mutations to extensive genome rearrangements that result in new combinations of alleles (Rustchenko and Sherman, 2003; Forche *et al.*, 2008).

Organisms with complete sexual cycles generate diversity during meiosis. In *C. albicans*, mating and karyogamy between cells of opposite mating type have been accomplished in the laboratory (Hull *et al.*, 2000; Magee and Magee, 2000), yet there is no evidence for a complete sexual cycle or for meiotic recombination. Rather, it appears that a parasexual process involving concerted chromosome loss (CL) results in the reduction of ploidy seen after diploids mate to form a tetraploid (Bennett and Johnson, 2003). Recent results indicate that this parasexual cycle is a source of genetic variability since it generates new combinations of the chromosomes present in the mating partners and yields a subset of progeny that underwent extensive genetic recombination between homologous chromosomes (Forche *et al.*, 2008). However, since recombination between homologues also occurs during the mitotic cycle (Lephart and Magee, 2006), the extent to which the parasexual cycle contributes to the variability detected in population studies is not known.

An important source of genetic variability in *C. albicans* derives from the high levels of heterozygous alleles (Whelan *et al.*, 1980; Whelan and Soll, 1982; Jones *et al.*, 2004) and the ability of the organism to undergo mitotic recombination events that result in loss of heterozygosity

(LOH). LOH is crucial for a number of processes that dramatically influence the biology, virulence and antifungal susceptibility of *C. albicans* (Larriba and Calderone, 2008). For instance, homozygosis of the *MTL* locus yields cells that can mate (Hull *et al.*, 2000; Magee and Magee, 2000) and LOH of specific *TAC1* and *ERG11* alleles underlies resistance to azoles in clinical isolates (White, 1997; Coste *et al.*, 2004; 2007). Following colonization of different individuals by the same strain, LOH events lead to microevolution (Lockhart *et al.*, 1995; Forche *et al.*, 2005). Similarly, isolates from a single patient are highly related, yet frequently show small changes (microvariations) due to LOH events (Bougnoux *et al.*, 2006; Odds *et al.*, 2006). Thus, local LOH events largely contribute to the expansion of diploid sequence types and, accordingly, to the variability/diversity of *C. albicans* (Odds *et al.*, 2007; Odds, 2010).

Processes leading to LOH are under the control of the DNA integrity network that includes the mitotic recombination systems (Paques and Haber, 1999). In wild-type diploid *Saccharomyces cerevisiae* cells that are hemizygous or heterozygous for a *URA3* marker, spontaneous LOH can occur by allelic recombination (crossing over, break-induced replication and local gene conversion), CL, or by chromosome aberrations caused by ectopic recombination between homologous sequences (Hiraoka *et al.*, 2000; Tourrette *et al.*, 2007). LOH is significantly increased in *S. cerevisiae* mutants defective in homologous recombination (HR), including *rad50*, *rad51* and *rad52* mutants, mainly because the frequency of CL and intragenic point mutation is increased, suggesting that, in the absence of HR, other mutagenic pathways generate genetic variability. An analysis of chromosomal alterations in HR mutants has provided clues about the molecular mechanisms leading to LOH in *S. cerevisiae* (Yoshida *et al.*, 2003).

In *C. albicans*, LOH at several loci may occur during propagation *in vitro* and during interaction with the host, but LOH accompanied by changes in karyotypes was only observed under the *in vivo* condition (Forche *et al.*, 2004; 2009a). Furthermore, adaptation of *C. albicans* to different stresses, including those caused by 5-fluorotic acid (5-FOA) or the antifungal compound fluconazole, is accompanied by specific gross chromosomal rearrangements (GCRs) as well as by additional uncharacterized aneuploidies that likely increase fitness in the presence of the drug (Selmecki *et al.*, 2006; Rustchenko, 2007; Bouchonville *et al.*, 2009; reviewed by Selmecki *et al.*, 2010). These observations suggest that adaptive mutations that allow survival under stress conditions often arise due to chromosome instability.

To know how LOH and GCR occur or are regulated in *C. albicans*, we analysed *C. albicans* Rad52, a member of the DNA integrity network that is crucial for DNA repair,

gene replacement and virulence in a murine model of haematogenously disseminated candidiasis (Ciudad *et al.*, 2004; Chauhan *et al.*, 2005; García-Prieto *et al.*, 2010). Here, we analysed the appearance of spontaneous His auxotrophs in *rad52* null homozygous (*Carad52-ΔΔ*) mutants. We found that *Carad52-ΔΔ* mutants exhibited an increased rate of LOH and a high frequency of genetic variability in the form of chromosome instability (CIN). CIN occurred by whole chromosome homozygosis, or by chromosome truncation (CT) and *de novo* telomere addition, but, unlike the case of *S. cerevisiae rad52* mutants, *Carad52-ΔΔ* strains did not undergo frequent translocations. These observations highlight the critical role of Rad52 recombination in protecting the genome from processes that generate extreme and anomalous genetic instability.

Results

Spontaneous generation of His auxotrophs by parental strains CAF2 and CAI-4 and rad52 null homozygous derivatives

Previously, we showed that *C. albicans* SC5314 and its derivative CAI-4 are heterozygous for the *HIS4* locus due to the presence of a SNP (G929T), such that one of the alleles (T) is inactive. Furthermore, all the UV-induced His auxotrophs derived from CAI-4 were complemented with a wild-type copy of *HIS4*, indicating that the heterozygous *HIS4* locus is responsible for histidine (His) auxotrophy in this *C. albicans* strain (Gómez-Raja *et al.*, 2008). Accordingly, the frequency of LOH at the *HIS4* locus can be measured by the appearance of His auxotrophs in the strain. When incubated in YPD broth, *rad52-ΔΔ* strains TCR2.1 (Uri⁺) and TCR2.1.1 (Uri⁻) (Table 1) produced spontaneous His auxotrophs (e.g. 6 out of 1100 colonies in one experiment), detected by replica-plating from complete medium to medium lacking histidine. Similar results were obtained with a second independent *rad52-ΔΔ* Uri⁻ strain (TCR2.2.1, Table 1) (not shown). In contrast, no His auxotrophs were detected in parallel cultures of the parental *RAD52/RAD52* strains (CAF2 or CAI-4). LOH rates were determined by fluctuation analysis (Table 2, Lea and Coulson, 1949; Reenan and Kolodner, 1992; Spell and Jinks-Robertson, 2004). For the parental Rad52⁺ strains, His auxotrophs were not observed among nearly 25 000 colonies of CAF2 (eight sets of ~3000 cells each, each set derived from a different colony), nor were they detected in ~100 000 colonies of CAI-4, indicating a frequency of spontaneous LOH at the *HIS4* locus of less than 1×10^{-5} events per viable cell. Similarly, no His auxotrophs were detected among ~25 000 colonies of a *RAD52* reintegrand (ABY0) (Table 1). In contrast, in the *rad52-ΔΔ* strain TCR2.1.1 we obtained an average of one

Table 1. Strains used in this study.

Strain	Genotype	Parental	Phenotype	Reference
SC5314	Wild type		Wild type	Gillum <i>et al.</i> (1984)
CAF2	<i>ura3::imm434/URA3</i>	SC5314	Wild type	Fonzi and Irwin (1993)
CAI-4	<i>ura3::imm434/ura3::imm434</i>	CAF2	Uri ⁻	Fonzi and Irwin (1993)
TCR2.1	<i>ura3::imm434/ura3::imm434 rad52::hisG/rad52::hisG-URA3-hisG</i>	CAI-4	Rad52 ⁻ Uri ⁺	Ciudad <i>et al.</i> (2004)
TCR2.1.1	<i>ura3::imm434/ura3::imm434 rad52::hisG/rad52::hisG</i>	TCR2.1	Rad52 ⁻ Uri ⁻	Ciudad <i>et al.</i> (2004)
TCR2.2.1	<i>ura3::imm434/ura3::imm434 rad52::hisG/rad52::hisG</i>	TCR2.2	Rad52 ⁻ Uri ⁻	Ciudad <i>et al.</i> (2004)
ABY0	<i>ura3::imm434/ura3::imm434 rad52::hisG/RAD52::URA3-hisG</i>	TCR2.1.1	Rad52 ⁺ Uri ⁺	This work
ABY1 to ABY7	<i>ura3::imm434/ura3::imm434 rad52::hisG/RAD52::URA3-hisG</i>	GLH1-1 to GLH1-7	Rad52 ⁺ Uri ⁺ His ⁻	This work

His auxotroph (GLH strains – GL lab Histidine auxotrophs) from each set of 500–1000 cells plated, although the frequency varied widely between the experiments and/or individual colonies (Table 2, Expts. 1 and 2; for details,

see Table S1). These results suggest that the frequency at which *C. albicans* cells undergo LOH, detected here as His auxotrophy, increased significantly in the absence of *RAD52*.

Table 2. Summary of fluctuation experiments measuring rates of loss of heterozygosity (LOH) at *HIS4*.

Expt.	Age of colony (h)	Size/morphology	NCS	Selected His auxotrophs from each experiment ^a	Total His ⁻ /total cells	Rate (events per cell per generation)
1	Nd	Not selected/filamentous	8	(GLH1-1, GLH1-2, GLH1-3, GLH1-4, GLH1-5, GLH1-6, GLH1-7) ^b	8/6971	1.59×10^{-3}
2	Nd	Not selected/filamentous	12	GLH2-1, (GLH2-2, GLH2-4) (GLH2-3, GLH2-5)	16/8155	2.04×10^{-3}
3	48	Large/filamentous	19	GLH3-1, GLH3-2, GLH3-3, GLH3-4, (GLH3-5, GLH3-6), GLH3-7, GLH3-8, GLH3-9	10/31 326	2.85×10^{-4}
4	72	Large	13	None	6/20 839	7.31×10^{-4}
	144	Small	13	None	49/10 942	5.95×10^{-3}
5	72	Large/smooth	6	None	3/6099	Nd
	144	Small/smooth	8	(GLH4-1, GLH4-2, GLH4-3, GLH4-4, GLH4-5), (GLH4-6, GLH4-7, GLH4-8, GLH4-9, GLH4-10), (GLH4-11, GLH4-12, GLH4-13, GLH4-14), GLH4-15	45/5871	5.44×10^{-3}
6	144	Small/filamentous	4	(GLH5-1, GLH5-2, GLH5-3, GLH5-4, GLH5-5, GLH5-6, GLH5-7, GLH5-8, GLH5-9, GLH5-10, GLH5-11), (GLH5-12, GLH5-13), GLH5-14	91/2415	Nd
	48	Large/filamentous	4	GLH6-1, GLH6-2	2/2360	Nd
	96	Not selected/filamentous	4	(GLH6-3, GLH6-4, GLH6-5, GLH6-6, GLH6-7, GLH6-8, GLH6-9, GLH6-10, GLH6-11), (GLH6-12, GLH6-13, GLH6-14, GLH6-15, GLH6-16), GLH6-17, (GLH6-18, GLH6-19, GLH6-20, GLH6-21, GLH6-22 and GLH6-23)	21/3319	Nd
7	144	Not selected/filamentous	4	(GLH6-24, GLH6-25, GLH6-26, GLH6-27, GLH6-28, GLH6-29 and GLH6-30)	18/1112	Nd
	144	Large/filamentous	4	None	3/1884	Nd

a. Only *rad52-ΔΔ HIS4* auxotrophs (GLH strains) selected for further studies are indicated. Strains indicated within parentheses derive from the same colony. Results corresponding to each colony are shown in Fig. S1. Nd, not determined.

b. Small colony.

Colonies of strain TCR2.1.1 were obtained by first streaking a –80°C stock culture onto YPD plates. Large and small colonies were initially selected when they were 48 h old. They were subsequently analysed for histidine auxotrophy when they reached approximately 1.5 mm diameter (about 72 h and 144 h of growth on YPD plates for large and small colonies respectively) regardless of whether they were initially large or small except in Expt. 6 and Expt. 7. In Expt. 6, colonies were not selected by size and, at the time of analysis, colonies 96 h and 144 h old had variable sizes and in Expt. 7, 144-h-old colonies were larger than 1.5 mm. NCS: number of *rad52-ΔΔ* colonies screened in each experiment for the presence of His auxotrophs. Rates were only calculated when the number of colonies screened was ≥ 8 .

To better understand the variability in the yield of His auxotrophs, we analysed the effect of the size, morphology and colony age on LOH frequencies. When samples from frozen cultures of a *rad52-ΔΔ* Uri⁻ strain (TCR2.1.1) were inoculated on YPD plates, they formed colonies of variable size (Fig. S1A). Large colonies yielded a lower rate of His auxotrophs (Table 2, Expt. 3), which was confirmed by a new fluctuation test: large colonies yielded 25-fold fewer His auxotrophs (0.03%) than small colonies (0.45%) (Expt. 4) ($P = 0.012$), which translated to a 10-fold lower LOH rate. Furthermore, within each group, the range of fluctuation in the rate of appearance of histidin auxotrophs was lower than for colonies that were not size-selected (Table 2, Expts. 3 and 4 respectively; for details, see Table S1).

Regardless of size, *rad52-ΔΔ* colonies also exhibited varying degrees of filamentation, from those that were smooth with no apparent filaments to the most abundant group: wrinkled colonies with filaments protruding into the medium (Fig. S1A) (Andaluz *et al.*, 2006). It is important to note that the distinction between smooth and filamentous colonies is simply operative, defining the appearance of the colony at the time of the selection. Not only do some apparently smooth colonies go on to produce filaments with increasing age, but, upon passage on YPD plates, most colonies become filamentous colonies regardless of the morphology assigned to the parent colony (Fig. S1A and B). Nonetheless, selected small smooth colonies as well as small filamentous colonies produced His auxotrophs at a rate similar to that calculated for the whole set of small colonies (Table 2, Expt. 5; Table S1). Six large smooth colonies as well as large filamentous yielded fewer His auxotrophs than smaller colonies. Thus, colony size appears to be more critical than colony morphology. Nonetheless, the frequency of appearance of His auxotrophs ranged widely in the small filamentous colonies; for one colony, 10% of the cells were His⁻, whereas 0–1% of cells from three other colonies were auxotrophs.

Overall, the number of His auxotrophs from unselected colonies of different ages did not show a consistent increase in LOH frequencies (Table 2, Expt. 6; Table S1), suggesting that colony age is not a major factor influencing the LOH frequency. In support of this, 144-h-old large colonies produced His auxotrophs at a similar frequency to younger large colonies sampled at 48 h and 72 h (Table 2, compare Expt. 7 with Expts. 3 and 4).

Methylene blue staining indicated that both large and small colonies had a similar, small proportion of dead cells (not shown), suggesting that the increased LOH rate observed for small colonies is associated with slow growth rather than with a higher cell mortality rate. Importantly, neither colony size nor colony morphology (filamentous growth) was a heritable phenotype, since upon transfer to new plates all colonies gave rise to both small

and large colonies, regardless of their size and degree of filamentation on the initial plate. Thus, small colonies produce His auxotrophs at a higher rate than large colonies, and there exists a substantial level of colony phenotypic variation which likely derives from the genetic instability associated with GLH strains.

His auxotrophs lose the functional allele of HIS4

To characterize the genetic alterations associated with the His⁻ phenotype, we analysed 80 *rad52-ΔΔ* His⁻ strains from six independent sets of isolates (GLH1 to GLH6; Table 2). Like the parental *rad52-ΔΔ* strain (TCR2.1.1) (Ciudad *et al.*, 2004), each GLH strain grew slower than wild-type strains. Colony morphology varied among the strains, but smooth, filamentous and sectorized colonies were observed in all six sets (not shown).

To ask if loss/inactivation of the functional *HIS4* allele accounted for the His auxotrophy in selected GLH isolates, we attempted to transform these strains with plasmid pRMH1 which carries the *HIS4* gene from strain 1001 of *C. albicans* and complements a *his4* null homozygote in a wild-type background (Gómez-Raja *et al.*, 2008). The results of these studies were inconclusive for two reasons. First, *rad52* null homozygous strains of *C. albicans* are refractory to transformation with standard *C. albicans* plasmids (our unpublished results). Second, the His auxotrophs frequently reverted to His⁺ (not shown), which complicated the analyses. Thus, instead, we focused on the structure of the *his4* alleles in the His auxotrophs to ask if the LOH events were due to alterations at this locus.

We next used an allele-specific PCR that discriminates between the two *HIS4* alleles in strain CAI-4 (Gómez-Raja *et al.*, 2008). In CAI-4 and in the parental *rad52-ΔΔ* strains (TCR2.1.1 and TCR2.2.1), both alleles were maintained. Importantly, in each of the 21 GLH isolates selected from Expts. 1 to 3, only the non-functional *his4* allele was retained (data not shown), implying that the His⁻ phenotype was due to loss of the functional *HIS4* allele rather than to point mutations within the functional allele. This could have occurred by (i) loss of the Chr4 homologue carrying the active *HIS4* allele, (ii) a large deletion removing *HIS4* and surrounding regions, or (iii) a HR event including cross-over on Chr4L, local gene conversion using the *his4* allele as the template, or break-induced replication (BIR) on Chr4L.

Most rad52-ΔΔ His auxotrophs exhibit altered karyotypes

Next, we analysed the 80 GLH isolates for chromosomal rearrangements by separating whole chromosomes on PFGE karyotype gels stained with ethidium bromide. Compared with the parental strain TCR2.1.1, GLH1 isolates showed dramatic reductions in chromosome (Chr) R mobil-

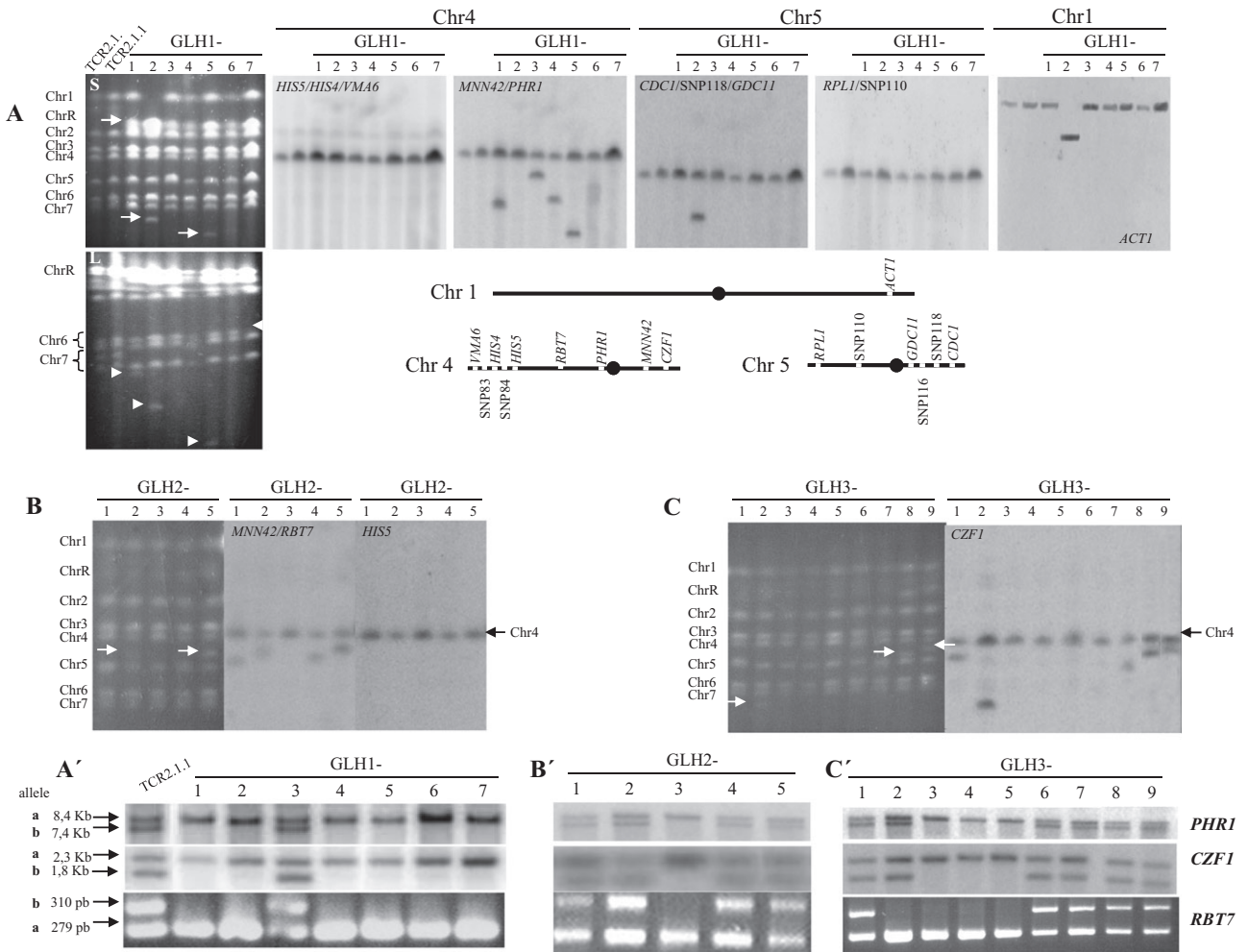


Fig. 1. A, B, C. Karyotype analysis by PFGE of GLH isolates from the first (A), second (B) and third (C) sets. Line drawing of Chrs1, 4 and 5 illustrated under (A) indicates the relative positions of probes used in Southern blots. Centromeres are indicated by dark circles. Within each set, membranes were stripped before being hybridized to the next probe. Southern blots labelled with more than one probe gave identical patterns when probed separately with each of the indicated probes. For the first set (A), Standard (separation of all the chromosomes; panel S) and Long run (separation of both homologues of the smaller chromosomes Chr6 and Chr7; panel L) electrophoresis are shown. Arrows indicate extra-bands not seen in the standard karyotype. Lane numbers refer to the GLH strains. For further explanation and details, see text. TCR2.1, *rad52-ΔΔ Uri*⁺; TCR2.1.1, *rad52-ΔΔ Uri*⁻.

A', B', C'. Analysis of Chr4 polymorphic markers in GLH1 (A'), GLH2 (B') and GLH3 (C') isolates respectively (see *Experimental procedures*).

ity (Fig. 1A, panel S, upper arrow). Furthermore, they carry only the smaller homologue of Chr7, detected by electrophoretic conditions that distinguish homologues of the smaller chromosomes (Fig. 1A, panel L). In contrast, in the GLH2 and GLH3 isolates, ChrR mobility was normal and both homologues of Chr6 and Chr7 were retained. Such differences in chromosome mobility are often due to changes in the size of repetitive DNA (e.g. rDNA on ChrR and MRS repeats on Chr7, as reviewed in Rustchenko and Sherman, 2003; Chibana and Magee, 2009).

GLH isolates exhibited additional types of chromosomal alterations. Isolate GLH1-2 had dramatically reduced sizes of both Chr1 homologues. Isolate GLH1-7 carried only the smaller homologue of Chr6 (Fig. 1A, panel L,

arrowhead, far right). Extrachromosomal bands smaller than Chr7 (supernumerary chromosomes, SNCs) (Chu *et al.*, 1993) were also evident in isolate GLH1-1 (Fig. 1A, panel L, arrowhead, far left) as well as in isolate GLH1-5 (Fig. 1A, panel S, arrow) and GLH2 and GLH3 strains (Fig. 1B and C, arrows). SNCs were also evident in GLH4, GLH5 and GLH6 strains (Figs S2, S3 and S4, respectively, arrowheads).

Since *HIS4* maps near the telomere of Chr4L, we first asked if any of the SNCs or chromosomes with altered mobility included DNA from Chr4. Southern blots of karyotype gels were probed with markers for each of the Chr4 arms (Fig. 1A). Probes for *HIS4* and *HIS5*, which are ~23 and ~113 kb proximal to the left telomere, respectively,

Table 3. Summary of Chr4 and Chr5 alterations in the GLH strains as deduced from Southern blot, CGH and polymorphism analyses.

Strains	Chr 4b	Chr 4a	<i>MTL</i>	SNP 116	Strains	Chr 4b	Chr 4a	<i>MTL</i>	SNP 116
CAI4	1	1	$\frac{a}{\alpha}$	Ht	GLH5-1 GLH5-2 GLH5-3 GLH5-4 GLH5-5 GLH5-6 GLH5-7 GLH5-8 GLH5-9 GLH5-10 GLH5-11 GLH5-12 GLH5-13 GLH5-14	CL	$\cong 1$	$\frac{a}{\alpha}$	Hma
TCR2.1.1	1	1	$\frac{a}{\alpha}$	Ht		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
{ GLH1-1 GLH1-2 GLH1-3 GLH1-4 GLH1-5 GLH1-6 GLH1-7	CL	2 + 4L ⁶⁹³	$\frac{a}{\alpha}$	Hmb		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	CL	2	$\frac{a}{\alpha}$	Hmb		4L ⁵⁰⁰	$\cong 1$	$\frac{a}{\alpha}$	Ht
	4L ³²²	2	$\frac{a}{\alpha}$	Hmb		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	CL	2 + 4L ⁵⁹⁰	$\frac{a}{\alpha}$	Hmb		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	CL	2 + 4L ¹⁰⁰⁰	$\frac{a}{\alpha}$	Hmb		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	CL	2	$\frac{a}{\alpha}$	Hmb		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	CL	2	$\frac{a}{\alpha}$	Hmb		4L ⁵⁰⁰	$\cong 1$	$\frac{a}{\alpha}$	Ht
{ GLH2-1 GLH2-2 GLH2-4 GLH2-3 GLH2-5	4L ³⁵⁰	2*	$\frac{a}{\alpha}$	Ht		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	4L ²⁵⁰	1*	$\frac{a}{\alpha}$	Hmb		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	4L ³²⁰	1*	$\frac{a}{\alpha}$	Hmb		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb		CL	$\cong 1$	$\frac{a}{\alpha}$	Ht
	4L ²²⁰	1*	$\frac{a}{\alpha}$	Hmb					
{ GLH3-1 GLH3-2 GLH3-3 GLH3-4 GLH3-5 GLH3-6 GLH3-7 GLH3-8 GLH3-9	4L ²³⁵	2	$\frac{a}{\alpha}$	Ht	{ GLH6-1 GLH6-2 GLH6-3 GLH6-4 GLH6-5 GLH6-6 GLH6-7 GLH6-8 GLH6-9 GLH6-10 GLH6-11 GLH6-12 GLH6-13 GLH6-14 GLH6-15 GLH6-16 GLH6-17 GLH6-18 GLH6-19 GLH6-20 GLH6-21 GLH6-22 GLH6-23 GLH6-24 GLH6-25 GLH6-26 GLH6-27 GLH6-28 GLH6-29 GLH6-30	4L ²⁵⁰	$\cong 1$	$\frac{a}{\alpha}$	Ht
	4L ⁷⁸⁶	2	$\frac{a}{\alpha}$	Ht		4L ⁴²⁰	$\cong 1$	$\frac{a}{\alpha}$	Hma
	CL	2	$\frac{a}{\alpha}$	Ht		CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb
	CL	2	$\frac{a}{\alpha}$	Ht		4L ¹⁰⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb
	CL	2	$\frac{a}{\alpha}$	Ht		4L ²⁵⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb
	CL	2	$\frac{a}{\alpha}$	Ht		CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb
	1	1	$\frac{a}{\alpha}$	Ht		CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb
	4L ⁴²⁰	2	$\frac{a}{\alpha}$	Ht		4L ⁶²⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb
	4L ³¹⁴	2	$\frac{a}{\alpha}$	Ht		CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb
4L ¹⁷⁵	1	$\frac{a}{\alpha}$	Ht	4L ⁶⁷⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb		
{ GLH4-1 GLH4-2 GLH4-3 GLH4-4 GLH4-5 GLH4-6 GLH4-7 GLH4-8 GLH4-9 GLH4-10 GLH4-11 GLH4-12 GLH4-13 GLH4-14 GLH4-15	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	4L ²⁵⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	4L ¹⁰⁰⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	4L ⁶⁵⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	4L ²⁰⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	CL+CT	$\cong 1$	$\frac{a}{\alpha}$	Ht	
	CL	$\cong 1$	$\frac{\alpha}{\alpha}$	Hma	4L ¹⁰⁰⁰	$\cong 1$	$\frac{a}{\alpha}$	Ht	
	CL	$\cong 1$	$\frac{\alpha}{\alpha}$	Hma	4L ⁶⁷⁰	$\cong 1$	$\frac{a}{\alpha}$	Ht	
	CL	$\cong 1$	$\frac{\alpha}{\alpha}$	Hma	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{\alpha}{\alpha}$	Hma	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	4L ²⁹⁰	$\cong 1$	$\frac{a}{\alpha}$	Hmb	
	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	CL	$\cong 1$	$\frac{a}{\alpha}$	Hmb	

Siblings are grouped by brackets. Chr5 was characterized for both *MTL* and the SNP116 marker. For details see text. Symbols: CL (chromosome loss) means homozygosity of all markers analysed. For Chr4a and Chr4b, superscripts indicate the size of the truncation in kb. For Chr4a, numbers indicate the copy number according to CGH (GLH1 and GLH3 isolates, except for GLH1-1, whose alterations were deduced from karyotype analysis) (Table 4) or to the intensity ratio yielded by the polymorphic markers (GLH2 isolates, *). Strain GLH3-6 likely carries a very small terminal deletion of Chr4a, restricted to little more than the *HIS4* locus or a loss-of-function mutation in *HIS4*. For SNP116, Hm, homozygote (the allele found is indicated); Ht, heterozygote.

labelled only Chr4 in all the GLH1 isolates. Interestingly, probes for *MNN42on* Chr4R, or *PHR1* which is near the centromere (CEN) on Chr4L hybridized to the intact Chr4 band and also hybridized to smaller SNCs in four strains (GLH1-1, GLH1-3, GLH1-4 and GLH1-5) (Fig. 1A). Together, these data suggest that one homologue of Chr4, including *CEN4* and Chr4R, is missing the distal

portion of Chr4L that encodes *HIS4*. Based on the assumption that these SNCs arose only from Chr4 DNA, we suggest that 693, 322, 590 and 1000 kb of Chr4L were lost from strains GLH1-1, GLH1-3, GLH1-4 and GLH1-5 respectively (summarized in Table 3).

A similar analysis of the karyotypes of the other GLH isolates using the same or additional probes (*CZF1*)

indicated that 80% (4 of 5) of the GLH2 isolates (GLH2-1, GLH2-2, GLH2-4 and GLH2-5) (Fig. 1B) and 55% (5 of 9) of the GLH3 isolates (GLH3-1, GLH3-2, GLH3-7, GLH3-8 and GLH3-9) (Fig. 1C), as well as 14% (2 of 14) of the GLH5 isolates (Fig. S3) and 53% (16 of 30) of the GLH6 strains carried SNCs that hybridize to genes from Chr4 (Fig. S4). In contrast, no SNCs were detected with Chr4 probes in the GLH4 isolates (Fig. S2). A summary of truncations affecting Chr4, with the deletion size indicated, is provided in Table 3.

To ask if alterations also occurred on other chromosomes, we probed Southern blots of karyotype gels with probes of Chr5. Three probes from Chr5R (*GCD11*, *CDC1* and SNP118) detected a SNC smaller than Chr7 in strain GLH1-2, in addition to the expected detection of Chr5-sized bands. However, Chr5L probes (SNP110 and *RPL1*, the ORF closest to the telomere) detected only intact Chr5 bands. Therefore, the SNC was most likely generated by deletion of ~250 kb from Chr5L.

Other chromosome rearrangements were also detected in GLH1 isolates, including a ~1 Mb deletion of Chr1 in strain GLH1-2, that did not delete *ACT1* which maps near the Chr1R telomere (Fig. 1A). In contrast, probes from Chr6L (*orf19.5525* and *COX12*) and Chr7R (*ARG4*) detected only Chr6 and Chr7 bands, respectively, suggesting that these chromosomes had not undergone rearrangements (data not shown).

To better characterize deletions of Chr4, we used the *HIS4* gene itself (24 kb from the telomere) and *VMA6* (the ORF closest to the telomere) (Fig. 1A) to probe karyotypes of all GLH1 isolates. For all strains, these two Chr4L probes labelled only intact Chr4, and none of its shorter derivatives (Fig. 1A and data not shown), implying that the shorter Chr4 SNCs arose via a chromosome break telomere-proximal to *PHR1*. We assume that the chromosome break was followed by addition of a telomere sequence to the centric fragment to form a stable truncated chromosome (e.g. Selmecki *et al.*, 2005). Similarly, we assume that the Chr5b SNC in GLH1-2 arose via CT and telomere addition. Taken together, these results indicate that, in addition to causing an increase in the LOH rate, the absence of Rad52 results in a high occurrence of CT that forms SNCs.

Comparative genomic hybridization (CGH) identifies segmental aneuploidies and reveals chromosome break points

While PFGE analysis of SNC size provides a good estimate of the presumed truncation and Southern analysis points to the likely chromosome fragments involved, comparative genomic hybridization (CGH) analysis (Fig. 2) provides a more comprehensive view of changes in genome copy number (reviewed by Selmecki *et al.*,

2010). Importantly, aneuploidy is widespread in the GLH isolates as revealed by CGH performed with microarrays carrying two copies of each *C. albicans* ORF (Selmecki *et al.*, 2005). For example, among the GLH1 and GLH3 isolates, 71% (5 of 7) and 55% (5 of 9), respectively, had segmental aneuploidy for one or more chromosomes (Table 4), implying that chromosome breaks had occurred. Strains with truncated Chr4 SNCs were usually trisomic for the DNA on the SNC and disomic for the region missing from the SNC (Fig. 2B), with the exception of strain GLH3-9, which was disomic for the retained region and monosomic for the region that had been truncated.

For some GLH1 isolates (e.g. GLH1-3 and GLH1-5), the trisomic regions corresponded well with the sizes of the SNCs. In other isolates, the correspondence with the SNC size predicted by Southern analysis (Fig. S5) was less clear (Table 4 and Fig. S5). For instance, for GLH1-2, PFGE analysis suggested altered sizes for Chrs1, R and a SNC including Chr5R DNA (Fig. 1A), while CGH revealed trisomies in selected regions of Chr1, R and 6 (Table 4, Fig. S5). This highlights the different aspects of genome rearrangements detected with the two techniques: PFGE detects changes in chromosome size and geometry, but requires Southern analysis with a large number of probes to determine exactly which portions of each chromosome are included in specific bands; CGH identifies those genome regions that are present in altered copy number, but does not reveal their geometry (Selmecki *et al.*, 2010). In this case, several chromosome fragments may have broken and now run with a mobility similar to that of other chromosomes, which makes their detection more difficult (e.g. a fragment of Chr1, detected with the *ACT1* probe, has a mobility like that of ChrR, and Chr6 appears to contain extra DNA, Fig. 1A), or are too small and may have been lost from the gel (e.g. the ~300 kb trisomic region of ChrR). Notably, all the trisomic fragments detected by CGH carry a CEN region which allows them to be stably maintained. However, because the PFGE and CGH analyses required transfer of the strains between two laboratories, we cannot rule out (and indeed it is entirely possible as described below) that some GLH1 isolates underwent additional chromosome alterations between the two analyses. Analysis of chromosomal instability helped to explain the conflicting results between PFGE and CGH in some isolates (see below). Simple rearrangements in GLH3 strains resulted in clear correlations between the karyotype gel analysis and the CGH results; alternatively, the GLH3 strains were simply more stable than those from the first set.

Notably, CGH analysis pointed to specific break points of the truncated chromosomes, which were confirmed for strains GLH1-3 and GLH1-5 by Southern blot hybridization using probes predicted to be within and outside the

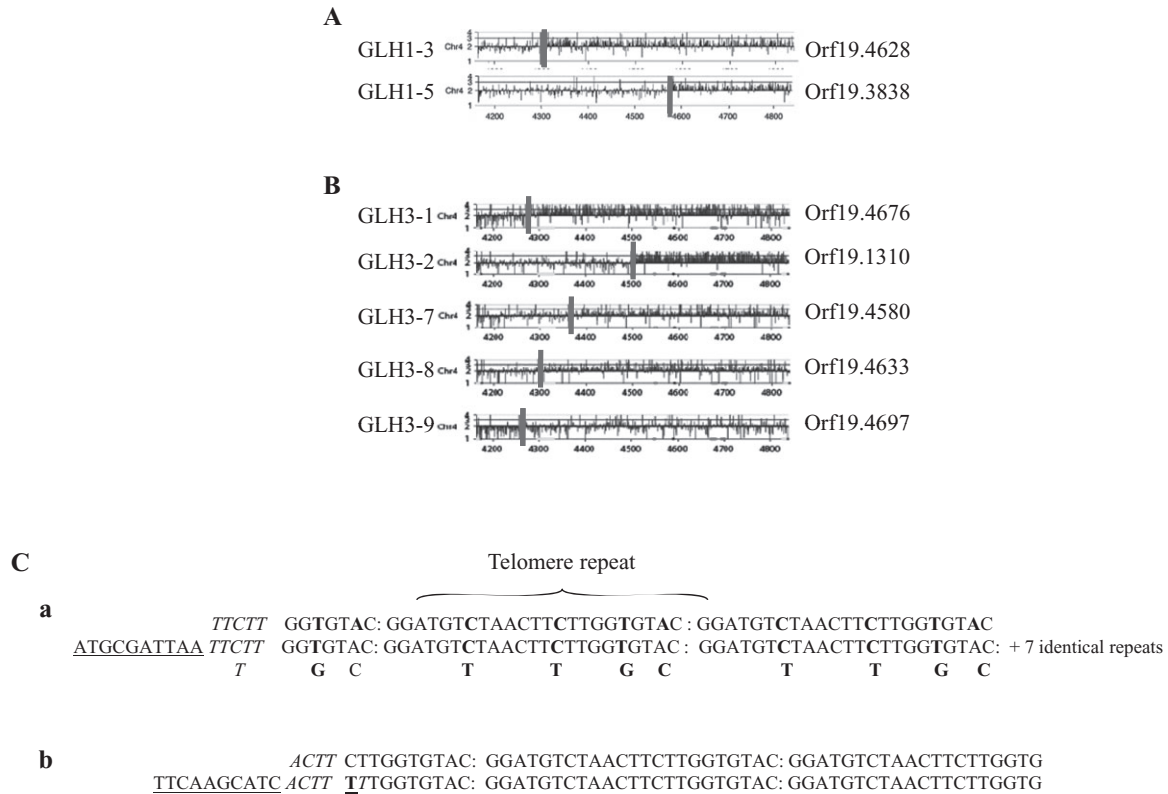


Fig. 2. A and B. CGH for Chr4 of the indicated His⁻ strains. GLH from set 1 (A) and set 3 (B). The last genes present in the aneuploid (extra copies) region at the position of the vertical bar are indicated.

C. Alignment of the telomere repeat (top line) and the *de novo* added telomere (bottom line) found in strains GLH1-3 (a) and GLH1-5 (b). Unambiguous chromosomal sequences are underlined. The junction sequence (italics) carries bases that could be derived from the chromosome or the telomere (Putnam *et al.*, 2004). In isolate GLH1-3 the sequence yields two bases (C/T) at the middle of the junction sequence **TTC/TTT**. Note also the presence of two bases, one identical to its counterpart in the canonical telomere sequence and the other different (in bold) at four specific positions of the sequenced *de novo* added telomere of strain GLH1-3 (see text). Telomere repeats are separated by colons. In strain GLH1-5, either there was a mutation in the first base after the putative junction sequence (underlined) or the junction sequence is reduced to the next T (italicized).

truncation (summarized in Table 4). To further evaluate if the SNCs had been generated by CT and telomere addition, we performed PCR using a primer complementary to sequences telomere-proximal to the last probe that hybridized to the truncated chromosome, and a primer from the telomere. In both isolates, GLH1-3 and GLH1-5, we isolated PCR fragments and sequenced them, confirming that telomere addition to truncated chromosome ends was a common mechanism to rescue broken chromosome ends. For strain GLH1-3, the junction sequence was (**TTC/TTT**), where *TTCTT* is common to both the promoter of the *orf19.4629* and the telomere. In contrast, in GLH1-5 we found no more than a single bp of homology to the template sequence single T (italicized in Fig. 2C, b) or a sequence with only five nucleotides of homology (Fig. 2C, a and b) within six nucleotides of the telomere template. Resequencing of the template region of the gene that encodes the telomerase RNA component (McEachern and Hicks, 1993; Hsu *et al.*, 2007) did not detect any ambiguity in the sequence in these strains.

Given that the *rad52-ΔΔ* strains are defective in HR (an alternative mechanism of telomere addition in wild-type cells; Le *et al.*, 1999), we propose that telomerase elongated the broken chromosome despite minimal homology to the template sequence.

His auxotrophy in rad52-ΔΔ cells occurred through loss or truncation of Chr4

Although PFGE karyotype and CGH analyses indicate that several His⁻ strains carry a truncated Chr4, we could not determine whether the deletion was preceded by CL. To address this issue, we took advantage of the polymorphism of three genes on Chr4, *CZF1*(Chr4R) and *PHR1*and *RBT7*(Chr4L) (Yesland and Fonzi, 2000; Chen *et al.*, 2004) (Fig. 1). For a given chromosome, homozygosity of markers located on both sides of the centromere supports the idea of whole chromosome homozygosity, and is usually taken as an evidence of CL. Even though the definitive proof of CL requires the analysis of addi-

Table 4. Summary of DNA copy number from CGH analysis.

Strain	Copy change	Chromosome	Region of aneuploidy (orf19)
TCR2.1.1	None		No aneuploidies detected
GLH1-1	None		No aneuploidies detected
GLH1-2	3×	R	19.6119–19.7279
	3×	1	19.6248–19.4883
	3×	6	19.3474–right end
GLH1-3	3×	4	19.4628–right end
GLH1-4	3×	R	19.4349.6–19.7281
GLH1-5	3×	4	19.3838–right end
GLH1-6 ^a	3×	R	19.3848–right end
	3×	1	19.4451–right end
GLH1-7	None		No aneuploidies detected
GLH3-1	3×	4	19.4676–right end
GLH3-2	3×	4	19.1310–right end
GLH3-3	None		No aneuploidies detected
GLH3-4	3×	R	19.5286–19.449; 19.413.1–right end
GLH3-5	None		No aneuploidies detected
GLH3-6	None		No aneuploidies detected
GLH3-7	3×	4	19.4580–right end
GLH3-8	3×	4	19.4633–right end
GLH3-9	1×	4	Left end–19.4697

a. In ChrR of this strain, there is also partial trisomy between orfs 19.6306 and 19.6640, then it increases slightly to trisomy between orfs 19.6640 and 19.3848.

tional markers, this reasoning is especially appropriate for strains lacking Rad52, in which allelic conversion events are unlikely. A RFLP (restriction fragment length polymorphism) analysis (Fig. 1A') indicated that the parental strain TCR2.1.1 was heterozygous at all three loci, whereas all three markers had become homozygous in six of the seven GLH1 strains. In all cases, the same allele was identified. Further analysis of two SNPs of Chr4 (see below) indicated that this allele corresponds to 'homologue a' (Legrand *et al.*, 2008), which therefore is the one that carries the non-functional copy of *HIS4*. In 3 strains (GLH1-1, GLH1-4 and GLH1-5) the Chr4b homologue was lost and three copies of Chr4a were present: two were full length and the other was truncated. In strain GLH1-3 two intact copies of Chr4a (based on CGH results) and one truncated copy of Chr4b was retained, as determined from the heterozygosity of all three RFLP markers (Fig. 1A') and homozygosity of two SNP markers (SNP83, SNP84) located within 300 kb of the left telomere (Fig. 1A, summarized in Table 3 and Fig. 3B). Therefore, for six out of seven (86%) isolates, His auxotrophy was due to loss of both arms of Chr4b; and in three of these six Chr4a was truncated as well. The isolate that remained heterozygous, GLH1-3, became His⁻ by truncation of the Chr4b homologue, resulting in loss of the functional *HIS4* allele.

These same types of genetic events, loss of the Chr4b homologue or truncation of Chr4L, were responsible for His auxotrophy in GLH2 through GLH6 isolates as well. Interestingly, GLH4 and GLH5 isolates, which, like GLH1, were derived from small colonies (Table 2), gave rise to

isolates that exhibited high levels of Chr4b loss (100%, 15 of 15, and 86%, 13 of 15 respectively) (Table 3 and Figs S2 and S3). This suggests that slow growth of colonies was associated with CL. Consistent with this idea, GLH strains derived from large colonies were less likely to have undergone CL (20%, 1 of 5, and 33%, 3 of 9, for GLH2 and GLH3 isolates respectively). Finally, 53% (16 of 30) of the GLH6 isolates (Table 2, Expt. 7), which were not size-selected, carried Chr4 SNCs, and most also included SNCs composed of other chromosome fragments; the remaining 14 GLH6 isolates apparently lost the Chr4b homologue (Fig. S4 and Table 3). These results indicate that in GLH isolates LOH occurred by either CL and/or CT, and the frequency of the two types of events fluctuated in different sets of strains, with CL being a more frequent event in small colony isolates. Importantly, additional CTs that were not responsible for generating His auxotrophy, often arose in the isolates.

Several rad52-ΔΔ His⁻ strains simultaneously lost one copy of most, if not all, chromosomes

To further evaluate the extent of the homozygosity of GLH1 strains, we analysed LOH of the remaining chromosomes using the *C. albicans* SNP map constructed by Forche *et al.* (2004). We selected 24 SNPs distant from each other and on both arms of each chromosome when possible. As shown in Fig. 3A (which is based on Table S2), the parental *rad52-ΔΔ* strain TCR2.1.1 conserved all but two of the 24 heterozygosities previously reported for strain SC5314 (Legrand *et al.*, 2008). Chr6

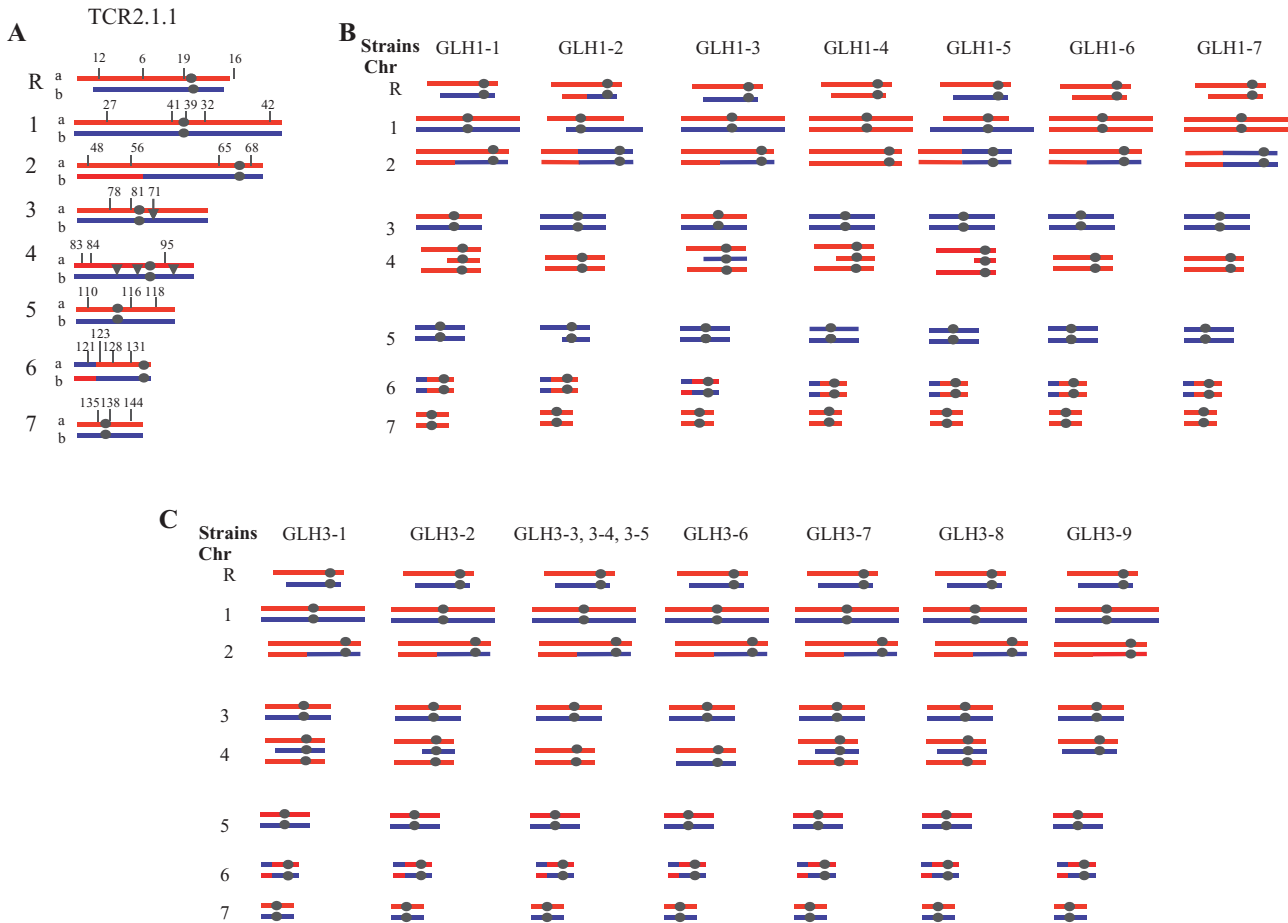


Fig. 3. A. A summary of the SNPs found in the parental *rad52*- $\Delta\Delta$ (TCR2.1.1). SNPs (numbers) and heterozygous markers (present in both red and blue) analysed are shown. Arrowheads indicate *RBT7*, *PHR1* and *CZF1* (see Fig. 1). The arrow indicates the homozygosity of SNP71. B and C. A summary of the SNPs detected His⁻ strains of the GLH1 (B) and GLH3 (C) sets. For a detailed description of the SNP markers analysed in (B) and (C), see Table S1. Colours denote homologues 'a' (red) and 'b' (blue) (Legrand *et al.*, 2008). Centromeres are indicated by full black circles. Rearrangements in Chr1 from strains GLH1-2 and GLH1-5 are only based on the SNP marker results and thus, are provisional. Note that Chr2 has undergone a rearrangement in parental strain TCR2.1.1. Therefore, strain GLH1-4 has lost the rearranged homologue 'b', whereas strains GLH1-2, GLH1-5 and GLH1-7 have lost homologue 'a'. For GLH1-1 we have used the PFGE/Southern blot instead of the CGH results. When a pair of chromosomes are homozygous but carry segments of both homologues, the segments ascribed to each homologue are also provisional. *RBT7*, *PHR1* and *CZF1* alleles present in the homozygous strains have been ascribed to homologue 'b'.

exhibited a rearrangement that suggests that a cross-over in the left arm likely caused a reciprocal exchange between homologues or more complex events. Because either alternative includes events that are Rad52-dependent, it is likely that they occurred before the deletion of both *RAD52* alleles (TCR2.1.1), as indicated in Fig. 3A. A large region of Chr2L was homozygous (SNPs 48 and 56), due to a cross-over or a BIR event that occurred in the CAI-4 strain used in our laboratory. Curiously, the same event was also observed in a diploid segregant derived via concerted CL from a tetraploid product of a parasexual cross as well as in a strain recovered from a mouse following experimental infection (Forche *et al.*, 2008; 2009a), suggesting there may be a recombination hot spot in the region between SNPs 65 and 56.

All of the GLH1 isolates underwent some degree of genome-wide homozygosity. Most dramatic are two strains (GLH1-4 and GLH1-7) that became completely homozygous for all the markers tested (Fig. 3B). In contrast, strain GLH1-3 became homozygous for markers on Chr5, Chr7 and the Chr4L distal region but remained heterozygous for most markers on the remaining chromosomes. By combining the SNP marker profiles with the Southern blot hybridization results and using the haplotype map of strain SC5314 as a reference (Legrand *et al.*, 2008), we inferred the chromosome complement of the GLH1 isolates (Fig. 3B and Table S2). Interestingly, homozygosity of the smaller chromosomes was very common: Chr5 and Chr7 were homozygous in all seven GLH1 isolates, and Chr4 and Chr6 were homozygous in six of them. Importantly, each chromosome became

homozygous in at least one strain, indicating that homozygosity of a portion of any chromosome is possible. Interestingly, the homologue that was retained was the same for each chromosome (homologue *a* for Chrs R, 1, 2, 4, 7 and most of Chr6; homologue *b* for Chrs 3 and 5), suggesting that recessive deleterious or lethal alleles may be present on the homologue that was lost.

SNP markers were used to detect LOH and/or aneuploidies in GLH2, GLH3 and GLH4 isolates. GLH2 and GLH3 isolates (Table 3, Fig. 3C) remained generally heterozygous, like the parent strain TCR2.1.1, although two alleles on Chr5 (*MTL* and SNP116) were homozygous in five GLH2 isolates, suggesting that the Chr5 homologue carrying *MTL* α had been lost. GLH4 isolates exhibited a level of homozygosity intermediate between GLH1 and GLH3 isolates (Table 3, Fig. S9). The same homologues were homozygous for Chrs 4, 6 and 7, whereas Chr5 homozygosity was mixed (5 with homologue *a* and 10 with homologue *b*). This is consistent with previous work showing that both alleles of Chr5 can be lost (Magee and Magee, 2000; Lephart *et al.*, 2005; Wu *et al.*, 2005). We conclude that all chromosomes can become at least partially homozygous, that the degree of homozygosity varies in different isolates, and that for most chromosomes, there appears to be a preference to retain a particular homologue.

Chromosomes frequently become unstable in rad52- Δ His⁻ strains

GLH1 strains displayed a significant number of chromosomal rearrangements (i.e. loss of the larger homologue of Chr7; a dramatic reduction in the length of ChrR), suggesting that, as also indicated by the CGH results, they had acquired an exceptional level of CIN. This was further confirmed by the karyotype analysis of different culture samples of these strains. The GLH1 isolates were stored in two series of frozen cultures (A and B), each stored from parallel liquid cultures inoculated with the same original colony of each His auxotroph. Three samples derived from frozen culture A (A1, A2 and A3), and one from frozen culture B (B1) (see *Experimental procedures*) were analysed by PFGE and Southern analyses using the *CZF1* probe from Chr4R. Chr4 SNCs seen in the initial samples were generally retained in these cultures (Fig. S6, right panels). In addition, new SNC bands appeared in several strains (arrowheads in Fig. S6; summarized in Table 5), including a new Chr4 SNC that appeared in isolate GLH1-6 (Fig. S6, sample B1, right arrow). Because GLH1-6 samples A1 and A2 also exhibited a diffuse band of the same size, we suggest that the starting isolate likely contained subpopulations of cells with different karyotype changes, and that the new Chr4 SNC became more prevalent in the B1 culture than in the

Table 5. Summary of the chromosomal alterations identified in GLH1 isolates.

Trains	A1	A2	A3	B1
TCR2.1.1				
GLH1-1	Chr4L ⁶⁹³	Chr4L ⁶⁹³	Chr4L ⁶⁹³	Chr4L ⁶⁹³
GLH1-2	Chr1L [?]	Chr1L [?]	Chr1L [?]	Chr1L [?]
	Chr5 ³⁵⁰	—	—	—
	—	Chr? ^{Chr2–Chr3}	—	—
		Chr? ^{Chr4–Chr5}	—	—
		Chr? ^{–Chr7*}	—	Chr? ^{<<Chr7*}
GLH1-3	Chr4L ³²²	Chr4L ³²²	Chr4L ³²²	Chr4L ³²²
GLH1-4	Chr4L ⁵⁹⁰	Chr4L ⁵⁹⁰	Chr4L ⁵⁹⁰	Chr4L ⁵⁹⁰
		ChrR ^{Chr2}	ChrR ^{Chr2}	ChrR ^{Chr2}
		Chr? ^{–Chr7}	—	—
GLH1-5	Chr4L ¹⁰⁰⁰	Chr4L ¹⁰⁰⁰	Chr4L ¹⁰⁰⁰	Chr4L ¹⁰⁰⁰
GLH1-6	—	—	—	Chr4L ⁵²⁰
GLH1-7	—	—	—	—

*The chromosomal band likely corresponds to a fragment of Chr6 (see Fig. 4B).

Column A1 summarizes karyotype analysis shown in Fig. 1A. Columns A2, A3 and B1 are different samples of the GLH strains from the first set and correspond to the analysis shown in Fig. S6. For additional detail, see the text. Superscripts indicate the approximate size of the deletions (kb).

A1 and A2 cultures. Despite some instability in the cultures, isolates often retained distinguishing karyotype features (e.g. GLH1-2 retained a truncated Chr1).

To follow the stability of GLH1 strains over time we serially streaked each strain 10 times using two protocols. Protocol I was designed to maintain the original population diversity whereas protocol II followed cumulative changes in individual isolates (see *Experimental procedures*). We then analysed the strains on karyotype gels. Regardless of the protocol used, the Chr4 SNCs were usually retained, and new alterations in karyotypes continued to be detected (Fig. 4A and B; summarized in Table 6, where prominent changes are highlighted). It should be noted that: (i) the three colonies from GLH1-6 (protocol I) had the Chr4L⁵²⁰ fragment (Fig. 4A, lower left and lower right, arrows) first seen in sample B (see above, Fig. S6), suggesting that the subpopulation represented by these three clones was initially present in both series, A and B, (ii) GLH1-7 did not exhibit obvious karyotype changes, and (iii) fewer changes were detected during passages following protocol II as compared with protocol I.

CIN was most extreme in GLH1-2, the most unstable strain (Fig. 4C). The original version of Chr5 (Chr5L³⁵⁰, Fig. 1A), which had the faster electrophoretic mobility, was not detected in the new clones from protocol I (Fig. 4C, b); instead, a new SNC migrating far below (Fig. 4C, a, arrows), identified as Chr6 truncated by ~400 kb (Chr6L^{~400}; Fig. 4C, c) that was similar to the SNC in sample B1 (Fig. S5), was detected. This result is consistent with CGH results that detected aneuploidies of

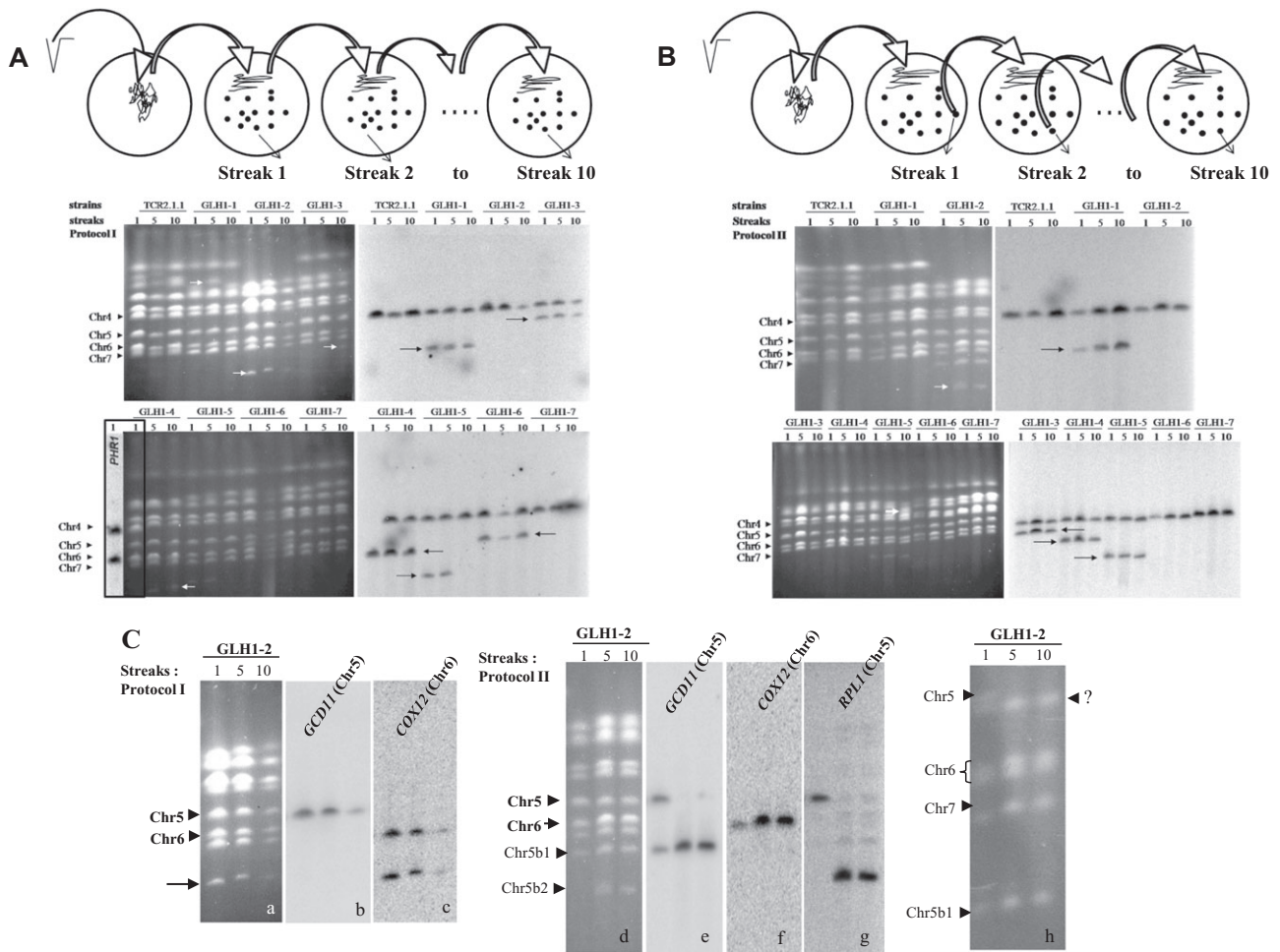


Fig. 4. A and B. Electrochromosomes of the GLH1 strains (series A) following passages according to protocols I (A) (1, 5, 10) and II (B) (1, 5, 10) and Southern blots (right panels) using the *CZF1* probe, which is located on the right arm of Chr4 (see Fig. 1). The membranes were also hybridized to *PHR1*, *VMA6*, *VID21* and *SNP19* probes (not shown). A drawing illustrating the difference between protocols I and II is shown above the corresponding karyotype gels (for details see *Experimental procedures*). Note that in protocol II the same colony was used for inoculation as well as for karyotype analysis. White arrows on the karyotype gels indicated SCNs that were not detected in Southern blots. C. Karyotypes of isolate GLH1-2 in the successive passages as in (A) using protocols I (left) and II (right). Membranes were hybridized to *COX12* (Chr6L), *GCD11* (Chr5R) and *RPL1* (adjacent to Chr5L telomere) probes.

Chr6 in this strain. The original Chr5L³⁵⁰, but not Chr6L^{~400}, was present in the three clones from protocol II (Fig. 4C, d–f). In addition, a smaller SNC, identified as a truncated Chr5R (e.g. labelled with *RPL1* – Fig. 4C, g – but not with *GCD11* – Fig. 4C, e), appeared in clones from passages 5 and 10 (Fig. 4C, d; arrowheads). Since neither of these Chr5 probes labelled an intact Chr5 in these two clones, it is likely that before passage 5, the remaining full Chr5 homologue underwent a truncation that covered most of Chr5R. Surprisingly, a Chr5-sized band was detected in the EtBr stained gels (Fig. 4C, d). PFGE using conditions that separate the smaller chromosomes detected two Chr5-sized bands in sample 1: one corresponded to Chr5 and the other was slightly smaller (Fig. 4C, h). The latter is likely derived from one of the larger chromosomes.

Karyotypes continued to change throughout the passages for the GLH2 through GLH5 isolates as well; SNC bands, likely derived from breakage of the larger chromosomes, were detected in the EtBr stained gels (Figs S2, S3, S7 and S8 – white arrowheads; summarized in Table 6). Levels of karyotype change varied among the starting strains and individual isolates, consistent with the idea that CIN arises stochastically. For example, Chr4 SNCs were relatively stable among GLH3, GLH4 and GLH5 isolates and were highly unstable in GLH2 isolates (Table 6). The size of ChrR frequently varied during the passages, presumably due to ribosomal DNA rearrangements (Iwaguchi *et al.*, 1992; Rustchenko *et al.*, 1993) that occurred despite the absence of Rad52. Consistent with this, rDNA size alterations were reported for a S.

Table 6. Summary of truncations identified in the karyotypes of GLH strains throughout the passages.

Strains	Changes in passages					
	Protocol I (streaks)			Protocol II (streaks)		
	1	5	10	1	5	10
TCR2.1.1	None	None	None	None	None	None
GLH1-1	Chr4L ⁶⁹³	ChrR*	Chr4L ⁶⁹³	Chr4L ⁶⁹³	Chr4L ⁶⁹³	Chr4L ⁶⁹³
GLH1-2	Chr1L [?]	Chr1L [?]	Chr1L [?]	Chr1L [?]	Chr1L [?]	Chr1L [?]
	–	–	–	Chr5L ³⁵⁰	Chr5L ³⁵⁰	Chr5L ³⁵⁰
	–	–	–	–	–	–
GLH1-3	Chr6 ^{–400**}	Chr6 ^{–400**}	Chr6 ^{–400**}	–	Chr5 ^{–600}	Chr5 ^{–600}
	Chr4L ³²²	Chr4L ³²²	Chr4L ³²²	Chr4L ³²²	Chr4L ³²²	Chr4L ³²²
	–	–	Chr(?) ^{<Chr7}	–	–	–
GLH1-4	Chr4L ^{590†}	Chr4L ⁵⁹⁰	Chr4L ⁵⁹⁰	Chr4L ⁵⁹⁰	Chr4L ⁵⁹⁰	Chr4L ⁵⁹⁰
	Chr4R ¹⁰⁰	–	–	–	–	–
GLH1-5	Chr4L ¹⁰⁰⁰	Chr4L ¹⁰⁰⁰	–	Chr4L ¹⁰⁰⁰	Chr4L ¹⁰⁰⁰	Chr4L ¹⁰⁰⁰
	–	–	–	–	–	Chr(?) ^{Chr2–Chr3}
GLH1-6	Chr4L ⁵²⁰	Chr4L ⁵²⁰	Chr4L ⁵²⁰	–	–	–
GLH1-7	–	–	–	–	–	–
GLH2-1	Chr4L ³⁵⁰	–	–	Chr4L ³⁵⁰	–	–
	–	Chr(?) ^{Chr4–Chr5}	Chr(?) ^{Chr4–Chr5}	–	–	–
GLH2-2	Chr4L ²⁰⁰	–	–	Chr4L ³⁵⁰	Chr4L ³⁵⁰	Chr4L ³⁵⁰
	–	–	Chr(?) ^{<<Chr7}	–	–	–
	–	Chr(?) ^{<<Chr7}	Chr(?) ^{<<Chr7}	–	–	–
GLH2-3	–	Chr(?) ^{Chr4–Chr5}	–	–	–	–
GLH2-4	Chr4L ³⁰⁰	–	–	Chr4L ³⁰⁰	Chr4L ³⁰⁰	Chr4L ³⁰⁰
GLH2-5	Chr4L ²¹⁰	Chr4L ²¹⁰	–	Chr4L ²¹⁰	Chr4L ²¹⁰	Chr4L ²¹⁰
GLH3-1	Chr4L ²³⁵	Chr4L ²³⁵	Chr4L ²³⁵	–	–	–
GLH3-2	Chr4L ⁷⁸⁶	Chr4L ⁷⁸⁶	Chr4L ⁷⁸⁶	–	–	–
GLH3-3	–	–	–	–	–	–
GLH3-4	–	–	–	–	–	–
GLH3-5	–	–	–	–	–	–
GLH3-6	–	–	–	–	–	–
GLH3-7	Chr4L ⁴²⁰	Chr4L ⁴²⁰	Chr4L ⁴²⁰	–	–	–
GLH3-8	Chr4L ³¹⁴	Chr4L ³¹⁴	Chr4L ³¹⁴	–	–	–
GLH3-9	Chr4L ¹⁷⁵	Chr4L ¹⁷⁵	Chr4L ¹⁷⁵	–	–	–
GLH4-1	Chr(?) ^{<Chr7}	Chr(?) ^{<Chr7}	Chr(?) ^{<Chr7}	–	–	–
GLH4-2	Chr(?) ^{Chr4–Chr5}	Chr(?) ^{Chr4–Chr5}	Chr(?) ^{Chr4–Chr5}	–	–	–
GLH4-3	–	–	–	–	–	–
GLH4-4	Chr(?) ^{Chr4–Chr5}	–	Chr(?) ^{Chr4–Chr5}	–	–	–
GLH4-5	Chr1L [?]	Chr1L [?]	Chr1L [?]	–	–	–
	–	–	Chr4La ¹⁷⁵	–	–	–
	–	–	Chr4Lb ⁷⁸⁶	–	–	–
	Chr(?) ^{<<Chr7}	–	Chr(?) ^{<<Chr7}	–	–	–
GLH4-6	Chr(?) ^{Chr2–Chr3}	Chr(?) ^{Chr2–Chr3}	Chr(?) ^{Chr2–Chr3}	–	–	–
GLH4-7	–	–	–	–	–	–
GLH4-8	–	–	–	–	–	–
GLH4-9	–	–	–	–	–	–
GLH4-10	–	Chr(?) ^{Chr4–Chr5}	Chr(?) ^{Chr4–Chr5}	–	–	–
GLH4-11	–	–	–	–	–	–
GLH4-12	Chr1L [?]	Chr1L [?]	Chr1L [?]	–	–	–
GLH4-13	–	–	–	–	–	–
GLH4-14	–	–	–	Chr(?) ^{Chr5–Chr6}	–	–
GLH4-15	–	–	–	–	–	–
GLH5-1	Chr(?) ^{<<Chr7}	–	–	–	–	–
GLH5-2	–	–	–	–	–	–
GLH5-3	–	–	Chr(?) ^{Chr2–Chr3}	–	–	–
GLH5-4	–	Chr(?) ^{Chr5–Chr6}	–	–	–	–
GLH5-5	Chr4L ⁵⁰⁰	Chr4L ⁵⁰⁰	Chr4L ⁵⁰⁰	–	–	–
GLH5-6	–	Chr(?) ^{Chr4–Chr5}	–	–	–	–
GLH5-7	Chr(?) ^{<<Chr7}	–	–	–	–	–
GLH5-8	–	Chr(?) ^{Chr4–Chr5}	Chr(?) ^{Chr4–Chr5}	–	–	–
GLH5-9	–	–	–	–	–	–
GLH5-10	Chr4L ⁵⁰⁰	Chr4L ⁵⁰⁰	Chr4L ⁶³⁰	–	–	–
GLH5-11	–	–	–	–	–	–
GLH5-12	–	–	–	–	–	–
GLH5-13	–	–	–	–	–	–
GLH5-14	–	–	–	–	–	–

*Increase in size.

**The size of this band varied in the passages.

†Based on hybridization of the new band to *PHR1* and *VAM6* probes, but not to *CZF1* and *VID21* (Fig. 4A, bottom left panel, left lane).

Superscripts indicate the approximate size of the deletions observed (kb), according to the CGH results (Table 4) when possible. Because the size of ChrR is not fixed in wild type, no references to this chromosome are made except for strains GLH1-1, passage 6 of the first protocol, and GLH1-5, passage 10 of the second protocol where a sudden increase in size and an additional truncation, respectively, of ChrR were observed.

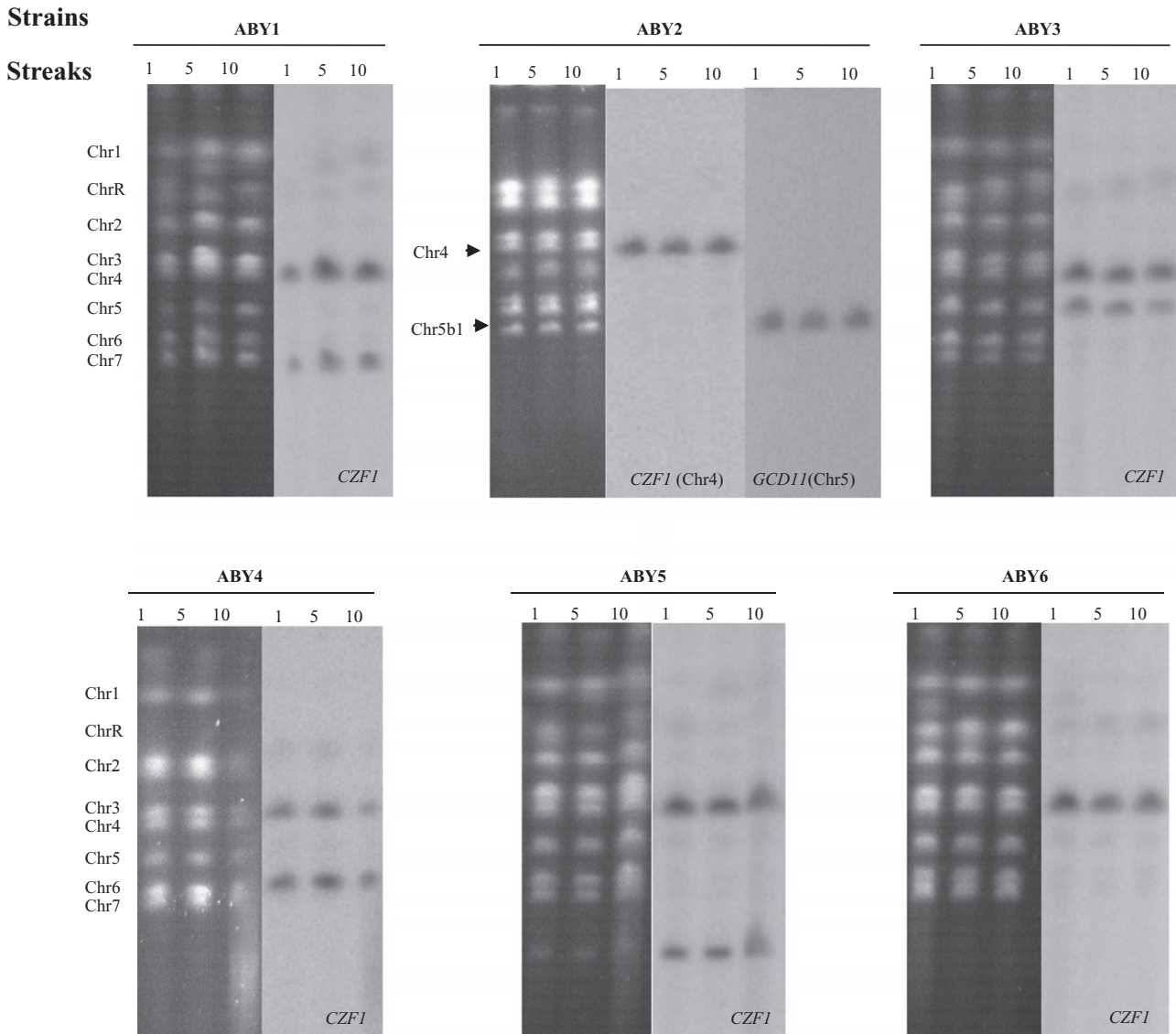


Fig. 5. Karyotypes of the *RAD52* reintegrants (ABY1–ABY7) from GLH1 strains after successive streaks and Southern blot using the *CZF1* (Chr4) and/or *GCD1* (Chr5) probes.

cerevisiae rad52 wine strain (Carro *et al.*, 2003). We conclude that GLH isolates of *C. albicans* exhibit continuing karyotype instability.

Reintegration of RAD52 restores the chromosomal stability of unstable strains

To investigate whether the chromosomal instability was a consequence of the absence of Rad52, we reintegrated one copy of *RAD52* into the GLH strains (GLH1-1 through GLH1-6) and randomly selected one reintegrant from each. As shown previously, *RAD52* can be reintegrated into *rad52* strains, presumably because, once inside

the cell, exogenous *RAD52* is expressed and the protein synthesized is sufficient to allow integration by HR (Ciudad *et al.*, 2004). All of the reintegrants (ABY1 to ABY7) recovered colony and cell morphology of the wild type (CAF2) (not shown). We then passaged these reintegrants using protocol I and analysed the karyotypes of the resulting isolates (Fig. 5). Importantly, the reintegrant strains ABY1 through ABY6 retained the extra-bands present in the parental strains. Importantly, the karyotypes did not change with the passages, except for the general tendency for both homologues of ChrR to increase in size. This suggests that a Rad52-dependent pathway is responsible for karyotype stability.

Discussion

We studied the effect of *RAD52* on genome stability in *C. albicans* by exploiting the heterozygosity of *HIS4* in laboratory strains derived from SC5314 (Gómez-Raja *et al.*, 2008) by measuring the rate of appearance of spontaneous His auxotrophs in *rad52-ΔΔ* isolates. Overall, the *rad52-ΔΔ* mutants produced more than 100-fold more His auxotrophs than the wild-type *RAD52/RAD52* strains. Furthermore, the LOH rate at the *HIS4* locus was ~10-fold higher for small colonies than for large colonies, and small colony size is a reflection of a slow growth rate. We propose that progenitors of small colonies acquired aneuploidies that caused enhanced genome instability and therefore were more prone to generate His auxotrophs or other types of mutants, based on previous observations that small colonies isolated from laboratory strains often carry monosomies or other aneuploidies (Janbon *et al.*, 1998; Rustchenko, 2007). Similarly, the instability of the colony size phenotype upon restreaking suggests that cells from small colonies regain a 'wild-type' growth rate, presumably by regaining a more balanced chromosome complement. In contrast, small smooth colonies that remain stably small likely acquired irreversible damage events such as point mutation or loss of critical alleles.

In a selective assay that permitted the analysis of a very large number of colonies, the *in vitro* LOH rate at the heterozygous *GAL1/gal1* locus was 6×10^{-6} recombination events per cell per generation (Forche *et al.*, 2009a). Because His auxotrophy is determined by screening rather than by counter-selection, it was not possible to analyse the large number of colonies necessary to detect the low frequency of events that occurs in the wild-type strain. Nonetheless, it is clear that the frequency of spontaneous events became high enough to be detectable in *rad52-ΔΔ* strains. Furthermore, our phenotypic screen did not utilize selective reagents such as 5-FOA (Chen and Kolodner, 1999; Yoshida *et al.*, 2003), which can lead to additional genetic changes (Wellington and Rustchenko, 2005) that may be difficult to distinguish from those caused exclusively by the absence of *RAD52*. Interestingly, 61 *S. cerevisiae* homozygous diploid deletion strains, including strains lacking *RAD50*, *RAD51* and *RAD54*, but no mutants lacking *RAD52*, were identified in a genetic screen for increased LOH rate (twofold increase compared with wild type) (Andersen *et al.*, 2008).

Chr4b loss or truncation caused most His auxotrophy in *rad52-ΔΔ* cells

With one possible exception, LOH leading to His auxotrophy in the *rad52-ΔΔ* strains always arose either by CL of the *Chr4b* homologue or by truncation of the left arm of the *Chr4b* homologue, including the functional *HIS4* allele. In diploid *S. cerevisiae rad52* null homozygous

strains, CL has been described (Mortimer *et al.*, 1981; Haber and Hearn, 1985; Yoshida *et al.*, 2003), and the CL frequency is chromosome-specific (Santos-Rosa and Aguilera, 1994; Hiraoka *et al.*, 2000) and is rarely followed by chromosome reduplication (CRD) (Yoshida *et al.*, 2003). However, in this study of *C. albicans rad52-ΔΔ* cells, CL was usually followed by CRD and is reminiscent of the CL followed by CRD detected for Chr5 in wild-type *C. albicans* (Janbon *et al.*, 1998; Wu *et al.*, 2005). Here, we extend the analysis using more comprehensive CGH analysis. One possibility is that the signal that triggers CRD is a reduction in the fitness of the strain, which in turn would depend on both the nature of the missing genes (i.e. essential, non-essential, haploinsufficient, etc.) and concomitant alterations in other chromosomes.

Loss of one homologue was not limited to Chr4; it was detected on other chromosomes. For example, loss of one homologue of Chr5 generates *MTL* homozygous strains, which are found in 3–7% of clinical isolates (Lockhart *et al.*, 2002; Legrand *et al.*, 2004; Wu *et al.*, 2005). In GLH strains the frequency of CL for Chr5 was much higher than this (64%, 51 out of 80) suggesting that Chr5 stability may be particularly affected by the stress conditions imposed by loss or truncation of Chr4b, at least in the absence of Rad52. Consistent with this, 20 randomly selected TCR2.2.1 (*rad52-ΔΔ*) colonies (with apparently intact Chr4 according to PGFE) were heterozygous for *MTL*. In contrast, copy number variation or truncation of Chr5 does not seem to influence CL or CT of Chr4, since both homologues of Chr4 were maintained in Rad52⁺Sou⁺ and Rad52⁻Sou⁺ strains that lost one copy of Chr5 (Andaluz *et al.*, 2007). Chr5 truncation was relatively rare among the His auxotrophs, since this event was only seen in GLH1-2 and GLH6-17 isolates.

Although speculative, an attractive explanation for the high levels of homozygosity observed in some GLH isolates is that during mitosis chromosomes rather than chromatids separate as in the first meiotic division. It has been recently reported that Rad51 associates with the centromeres of fission yeast (Nakamura *et al.*, 2008). Since Rad52 and Rad51 form supramolecular complexes, it is possible that, in the absence of Rad52, centromeric chromatin assembly is defective, resulting in frequent chromosome non-disjunction events.

The frequent aneuploidies and chromosome breaks observed in GLH isolates are likely due to additional defects due to absence of Rad52. In *S. cerevisiae*, chromosome fragmentation induces the DNA-damage checkpoint, which arrests cells prior to anaphase, providing time for DNA repair while chromatids remain cohered so that checkpoint functions can act synergistically with the HR (Klein, 2001; Myung *et al.*, 2001; Myung and Kolodner, 2002; 2003). Interestingly, in wild-type cells at metaphase, the two halves of the broken chromosome are held

together by components of the recombination machinery, including the Rad50–Mre11–Xrs2 complex and Rad52, which bind and tether DNA ends. Elimination of these components promotes the loss of the intrachromosomal association between the centric and acentric fragments upon entry into metaphase, but maintains the interchromosomal association that tethers broken sister chromatids (cohesins). This situation results in missegregation of both the acentric (CT) and, to a lesser extent, the centric fragments (non-disjunction, CL) (Kaye *et al.*, 2004; Lobachev *et al.*, 2004). We propose that similar mechanisms operate in *C. albicans rad52-ΔΔ* mutants.

CT occurs at a high rate in C. albicans rad52-ΔΔ cells

Interestingly, spontaneous CT occurred at a very high frequency in *C. albicans rad52-ΔΔ* cells (40% of the His auxotrophs analysed), while CT was not reported in *S. cerevisiae rad52-ΔΔ* diploid strains (Yoshida *et al.*, 2003). Furthermore, while CT contributed to 45% of GCRs in a haploid wild-type *S. cerevisiae* strain (Chen and Kolodner, 1999; Motegi and Myung, 2007), in a *rad52* derivative of the strain, the frequency of GCR increased, but translocations using microhomology were the most frequent events and telomere additions were detected only in the absence of Pif1, an inhibitor of telomerase, or in the absence of Lig4, the ligase involved in non-homologous end-joining (NHEJ) (Schultz and Zakian, 1994; Myung *et al.*, 2001; Pennaneach *et al.*, 2006). Why is telomere addition to truncated chromosomes much more frequent in *C. albicans rad52-ΔΔ* than in *S. cerevisiae rad52* strains, while translocations involving a non-homologous chromosome are frequent in *S. cerevisiae* but not detected in *C. albicans*? One possibility is that the DNA damage checkpoint in *C. albicans rad52-ΔΔ* cells (Andaluz *et al.*, 2006) facilitates telomere addition; alternatively, Pif1 inhibition of telomerase or the Lig4-dependent NHEJ pathway could be less effective or absent in *C. albicans*. In *S. cerevisiae*, the NHEJ pathway is active in haploids but not in diploids (Daley *et al.*, 2005). In *C. albicans*, an obligate diploid, the importance of the NHEJ pathway is less well understood (Andaluz *et al.*, 2001). It is also possible that translocations in *C. albicans* require Rad52 since they usually involve regions of extensive homology such as the major repeat sequence (e.g. MRS) (Chu *et al.*, 1993) or other repeats (Selmecki *et al.*, 2006).

It should be emphasized that Chr4b loss or truncation was frequently accompanied by simultaneous loss and/or truncations of a homologue of other chromosomes, which were unrelated to the scored mutation. Chr4 seems to be particularly prone to undergo truncation events. It is clear that some of these truncations arose because of selection in our screen; however, others truncations arose in strains

that were already His⁻ (GLH1-1, GLH1-4, GLH1-5 and GLH4-5), suggesting an abundance of fragile sites in Chr4 (Arlt *et al.*, 2006), which might arise if genes on Chr4 were more actively transcribed genes (Aguilera, 2002). This situation has not been described in *S. cerevisiae* and may reflect the higher tolerance of *C. albicans* to aneuploidy (Bouchonville *et al.*, 2009). In support of this idea, *C. albicans* exhibits a higher frequency of multiple aneuploid chromosomes and chromosomal rearrangements in the presence of fluconazole as compared with an *S. cerevisiae* diploid strain (Anderson *et al.*, 2004; Selmecki *et al.*, 2006, 2009).

The frequency of CIN varies among the rad52-ΔΔ His⁻ strains

Variation in both the level of CIN and the nature of the associated GCR among different GLH strains supports the mutator hypothesis initially advanced to explain the appearance of cancer cells in higher eukaryotes. These cells must acquire a mutator mutation that may facilitate more mutations in order to account for a high rate of accumulation of genetic changes (Kolodner, 1996; Kolodner *et al.*, 2002; Motegi and Myung, 2007). While the CIN phenotype of *rad52-ΔΔ* cells contributes to additional mutations (a process that would be facilitated by the high level of polymorphisms present in the genome of *C. albicans*) and/or aneuploidies (which are prone to more non-disjunction; Bouchonville *et al.*, 2009) that could exacerbate genetic instability, the restoration of chromosome stability in the *RAD52* reintegrants indicates that instability is likely a direct effect of the absence of Rad52. Interestingly, when analysed using protocol II karyotypes of GLH1 and GLH2 isolates appeared to be more stable than when using protocol I. This suggests that (i) variants arise at a measurable frequency within the population, and (ii) if the mutation confers a higher growth rate, it may out-compete the original strain upon passaging. Selection can occur along the passages of the cell mass (protocol I) but is prevented when individual colonies are passaged (protocol II). In summary, our results suggest that the mutator phenotype of *rad52-ΔΔ* cells facilitated the acquisition of a CIN phenotype which always manifested as CL and/or CT. Given its obligate diploidy and tolerance to aneuploidy, *C. albicans* appears to be a better model than *S. cerevisiae* for studying CIN processes that occur in mammalian mutator strains.

Interestingly, the break points detected here have not been observed previously in either clinical or laboratory strains of *C. albicans* (although similar GCRs have been reported in clinical isolates of *Candida glabrata* – Poláková *et al.*, 2009), even when they were subjected to evolution in the presence of fluconazole (Selmecki *et al.*, 2010). Conversely, *C. albicans* strains adapted to grow in

the presence of fluconazole (Flu^R) exhibited a recurrent break point on Chr5 that was not observed here. Since *rad52-ΔΔ* strains are more sensitive to fluconazole than wild-type cells (Legrand *et al.*, 2007; our unpublished results), CaRad52 may be a useful target for the design of antifungal compounds.

Finally, this study identified strains that appear to be homozygous for markers across all eight chromosomes. This suggests that there is no particular region of the genome that must be heterozygous for the survival of the organism. However, it is interesting to note that, for most of chromosomes, homozygosity always involved loss of the same homologue and retention of the other. Indeed this could explain the discrepancy between our work and that of Tagaki *et al.* (2008), who reported that *C. albicans rad52-ΔΔ* strains were defective in both spontaneous and UV-induced LOH based upon homozygosity of *ade2::URA3/ADE2*. Perhaps the *ADE2* allele disrupted by *URA3* could not be lost because it is in the Chr3 homologue that cannot be lost. Importantly, the completely homozygous isolates obtained here will be useful for studies of the general importance of heterozygosity in *C. albicans*.

Experimental procedures

Strains and media

The *C. albicans* strains used in this study are indicated in Table 1. *C. albicans* cells were grown routinely at 28°C in YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose). For the isolation of His auxotrophs, cells from each strain were plated on YPD medium at a density \leq 200 cells per plate. At the indicated times or colony size, colonies were replica-plated on SC without histidine and incubated for 24 h (CAI-4) or 48 h (*rad52-ΔΔ*). Each His auxotroph isolate was re-isolated in YPD and checked for His auxotrophy. One of the new colonies derived from each auxotroph was used to inoculate one flask containing liquid YPD. The culture was harvested, divided in small lots and kept at -80°C. For His auxotrophs from set 1, each colony was used to inoculate two parallel flasks. Each culture was harvested independently (series A and B), and processed as above.

Reintegration of *RAD52* into the GLH isolates was performed essentially as described before (Ciudad *et al.*, 2004). A large fraction of *rad52-ΔΔ* colonies have a spiny appearance and contain filamentous cells whereas those of the reintegrants are smooth and contain regular yeast cells (Andaluz *et al.*, 2007). Randomly selected smooth colonies were analysed for the correct reintegration of *RAD52* into its own locus using PCR and Southern blot (ABY strains) (Ciudad *et al.*, 2004).

Fluctuation test

Several fluctuation tests were performed to calculate the rate of the production of His auxotrophs in the *rad52-ΔΔ* strain (Table 2). For this purpose, colonies isolated on YPD from a

-80°C culture, selected by size and/or filamentous/smooth morphology, were excised from the plate and resuspended in sterile water. Appropriate dilutions were then plated on YPD medium at a density \leq 200 cells per plate. At the indicated times or colony size (Table 2), colonies were replica-plated on SC without histidine and incubated for 48 h (*rad52-ΔΔ*) to identify and score His auxotrophs, which were further verified. When possible (i.e. when all or near all the cultures had mutants), we used the method of Lea and Coulson (1949), as described by Reenan and Kolodner (1992), to determine the rate of appearance of His auxotrophs. When a large fraction of cultures had no mutants, the mutation rate was estimated by an alternative method (Lea and Coulson, 1949; Spell and Jinks-Robertson, 2004).

DNA extraction and analysis

Extraction of genomic DNA, Southern blot hybridization, chromosomal preparations and electrophoretical separations of chromosomes (PFGE), and PCR analysis of the *MTL* loci were performed as described (Andaluz *et al.*, 2007; Gómez-Raja *et al.*, 2008). For Southern analysis, probes consisted of markers of Chr5 (*CDC1*, *GCD11*), Chr4 (*HIS5* and *YJR61/MNN42*), Chr1 (*ACT1*), Chr6 (*COX12* and *orf19.5525*) and Chr7 (*ARG4*) kindly provided by BB Magee (Chu *et al.*, 1993), and PCR products of markers of Chr4 (*HIS4*, *PHR1*, *VMA6*, and SNP markers SNP83 and SNP84), Chr5 (*RPL1*, *VID21*, SNP110 and SNP118) and ChrR (SNP19), synthesized using appropriate oligonucleotides. These primers can be provided upon request. The Quantity ONE-1D Software (Bio-Rad) was used to determine the intensity bands in Southern blots. The presence of one or both homologues of each chromosome was investigated by analysing previously characterized single-nucleotide polymorphisms (SNP). For these experiments, the regions containing the polymorphisms were amplified using the appropriate oligonucleotides and the PCR product sequenced. Primer sequences can be obtained upon request from Anja Forche (<http://albicansmap.ahc.umn.edu/html/snp.html>). Analysis of the indicated SNP-RFLP markers was carried out as described (Forche *et al.*, 2009b). Other polymorphic markers were analysed as described (Gómez-Raja *et al.*, 2008).

Comparative genomic hybridization (CGH)

Comparative genomic hybridization analysis was performed as described previously (Selmecki *et al.*, 2005). All strains were compared with the same reference control strain, SC5314. Genomic DNA from the experimental strain was labelled with Cy3-dUTP and DNA from the reference control strain was labelled with Cy5-dUTP (Roche). Arrays were printed in-house, and one array per strain was analysed. Chromosome_map, written in Matlab (Selmecki *et al.*, 2005), was used to plot all microarray data points as a function of gene position along the eight *C. albicans* chromosomes. All CGH data (mean fluorescence ratios) were plotted on a log₂ scale and clipped to the range corresponding to one to four gene copies. Break points were predicted from an increase or decrease in gene copy number and were determined using a running average over 2.3 ORFs per chromosome.

Analysis of karyotype instability in *His⁻* strains

To address this issue, we first looked for possible variations in two series of cultures derived from the GLH1 isolates kept at -80°C (series A and B; see *Strains and media*). Three samples were taken from series A (A1, A2 and A3), and one from series B (B1). In each case, electrokaryotypes were obtained from a randomly selected colony from the first plate and then subjected to Southern blot hybridization with a probe of Chr4 (*CZF1*). In a second set of experiments, we analysed the karyotypes shown by the descendants of TCR2.1.1 and the indicated GLH isolates according to two theoretically complementary protocols (illustrated in Fig. 4). Protocol I was designed to determine random non-cumulative changes in the population. For this purpose, cellular mass from a culture kept at -80°C was streaked onto a YPD plate. Cell mass taken from the region of confluent growth was successively passaged by streaking it sequentially, at 3-day intervals, over 10 passages. Protocol II was designed to detect cumulative changes in individuals within the population. A new YPD plate from a frozen culture stock from series A was prepared as above, but single colonies, rather than cell mass were used to inoculate each successive passage. Single colonies from the successive passages of each protocol were then grown in liquid YPD for the preparation of frozen culture stocks. Liquid cultures from passages 1, 5 and 10 of each protocol were also processed for DNA and karyotype analysis.

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