The Candida albicans Ku70 Modulates Telomere Length and Structure by Regulating Both Telomerase and Recombination

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Abstract

The heterodimeric Ku complex has been shown to participate in DNA repair and telomere regulation in a variety of organisms. Here we report a detailed characterization of the function of Ku70 in the diploid fungal pathogen Candida albicans. Both Ku70 heterozygous and homozygous deletion mutants have a wild-type colony and cellular morphology, and are not sensitive to MMS or UV light. Interestingly, we observed complex effects of Ku70 gene dosage on telomere lengths, with the Ku70/ku70 heterozygotes exhibiting slightly shorter telomeres, and the ku70 null strain exhibiting long and heterogeneous telomeres. Analysis of combination mutants suggests that the telomere elongation in the ku70 null mutant is due mostly to unregulated telomerase action. In addition, elevated levels of extrachromosomal telomeric circles were detected in the null mutant, consistent with activation of aberrant telomeric recombination. Altogether, our observations point to multiple mechanisms of the Ku complex in telomerase regulation and telomere protection in C. albicans, and reveal interesting similarities and differences in the mechanisms of the Ku complex in disparate systems.

Introduction

Candida albicans, the most common fungal pathogen for humans, exhibits a number of unusual biological characteristics, including obligate diploidy, a high level of polymorphisms in its genome, and a predominantly clonal mode of reproduction [1]. In the absence of meiosis, the genetic variability of the species is primarily derived from shuffling of the highly polymorphic parental chromosomes during the parasexual cycle as well as from mitotic recombination events occurring during both the parasexual and the mitotic cycles [2,3]. There are two general pathways of recombination, homologous recombination (HR) and non-homologous end-joining (NHEJ). Homologous recombination requires the presence of a homologous partner, which is always present in an obligate diploid. In fact, in C. albicans, HR occurs during the mitotic cycle at a rate of 10^−6 events/cell/generation [4], causing loss of heterozygosity (LOH) in short or long stretches of DNA (through gene conversion or crossover/BIR, respectively), a process that may be clinically relevant [5]. C. albicans possesses homologues of the Rad52 epistasis group of HR-related proteins (Rad51, Rad52, Rad54, Rad55, Rad57 and Rad59) [6]. As in S. cerevisiae, Rad52 is involved in most homology-dependent recombination in C. albicans, and C. albicans Rad52 mutants exhibit the most severe phenotypes in recombination, DNA repair, and genetic instability [7–9].

The second recombination pathway, NHEJ, can rejoin the two ends of a DSB by simple ligation after little or no nucleolytic processing of the end. In budding yeast, the Yku70/Yku80 complex, Lig4 and its associated Lpl1/Nej1 complex, as well as the Mre11/Rad50/Xrs2 complex (MRX complex) are required for NHEJ [10]. Among the homologues of these genes in C. albicans, only Lig4 and Ku80 have been partially characterized [8,11]. In S. cerevisiae, NHEJ is repressed in diploids where the HR pathway is active. This is presumably advantageous because diploid cells contain one intact copy of each gene for the repair of damaged DNA through HR, a high fidelity repair mechanism. Therefore, the retention of NHEJ genes in an obligate diploid yeast such as C. albicans suggests that these genes may play important roles in non-NHEJ processes that are important for the biology of the fungus. In fact, besides its role in end-joining, the Ku proteins have been implicated in a variety of functions at telomeres [12–14].

Telomeres are nucleoprotein structures located at the ends of chromosomes that are crucial for maintaining chromosome stability. Telomeres in most organisms consist of G-rich repeats that terminate with a single-stranded 3’ overhang [15,16]. Conventional DNA polymerases cannot fully replicate the very ends of linear DNA molecules. Thus, without a specific compensatory mechanism, the ends of chromosomes shorten during each cell division. Most eukaryotes utilize telomerase, a specialized reverse transcriptase consisting of a catalytic protein subunit (Tert), an RNA template (Ter), and several accessory proteins for telomere addition [13]. Previous studies from yeast to mammals have implicated the Ku complex in performing a
multitude of functions at telomeres. In the budding yeast *S. cerevisiae*, the Yku70/Yku80 heterodimer has been shown to protect chromosomal ends from nucleolytic degradation; both yku70 and yku80 mutants accumulate high levels of single-stranded telomere DNA (G-tails) due to uncontrolled degradation of the C-strand [17]. In addition, the *S. cerevisiae* Ku complex promotes telomerase recruitment and telomere elongation through an interaction between Yku80 and telomerase RNA; both the yku70 and yku80 mutants possess shorter than normal telomeres [14]. Furthermore, binding of Ku to telomeres facilitates the recruitment of Sir3 and Sir4 to the subtelomeric regions to enhance telomere silencing [18–20].

Interestingly, Ku’s precise roles in telomere regulation appear to be somewhat divergent evolutionarily [13]. For example, in contrast to *S. cerevisiae*, the disruption of Ku in *Arabidopsis thaliana* results in long and heterogeneous telomeres [21]. Moreover, both the *A. thaliana* and human Ku complex have been shown to suppress the formation of extrachromosomal telomeric circles (t-circles) [22,23]. T-circles have been observed in a wide range of organisms, often in association with telomere dysfunction, and are believed to arise through telomere recombination [24]. High levels of t-circles are also characteristic of some cancer cells (ALT cells) that lack telomerase [25]. The elevation of t-circles in plant and human Ku mutants suggest that this complex suppresses the access or activity of recombination factors within the terminal repeats. In contrast to these organisms, a role for the budding yeast Ku complex in suppressing telomere recombination is less clear. In *S. cerevisiae*, the disruption of *IKU* genes alone have not been reported to cause higher telomere recombination, but the same disruption in a *cdl13-1* strain exacerbated aberrant telomere recombination [26]. In *K. lactis*, the loss of Ku80 induces a moderate increase in subtelomeric recombination [27]. In these two fungi, whether the Ku complex plays a role in suppressing recombination within the terminal repeats in wild type cells remains an open question.

In this work, we have characterized the phenotypes of Ku70 mutants of *C. albicans* with respect to growth, DNA damage-sensitivity, and telomere dysfunction. Our findings indicate a relatively minor role, if any, for the Ku complex in DNA damage repair, but revealed complex dosage-dependent effects of the Ku proteins in regulating telomere lengths and structure, including the accumulation G-tails and t-circles. We observed substantial functional distinctions between the *S. cerevisiae* and *C. albicans* Ku proteins with regard to telomere regulation. In addition, intriguing parallels were noted between the phenotypes of the *C. albicans* ku70 mutant and comparable mutants from plants and humans.

**Results**

**Identification of the *C. albicans* KU70 ortholog**

A BLAST search identified an open reading frame (ORF19.1135) in the genome of *C. albicans* strain SC5314 with significant similarity to the *S. cerevisiae* Ku70 protein. This ORF, with 2436 nucleotides, is located on the right arm of chromosome 1 and encodes a predicted protein of 811 amino acids. Clustal analysis revealed that CaKu70 was 24, 25, 16, 20, 19, and 19% identical to *Candida glabrata* (CgKu70), *S. cerevisiae* (ScKu70), *Aspergillus nidulans* (AnKu70), *Neurospora crassa* (NcKu70), *Arabidopsis thaliana* (AtKu70) and *Homo sapiens* (HsKu70) Ku proteins, respectively (Figure 1). Like these homologues, CaKu70 contains an N-terminal α/β domain, a central β-barrel domain, and a well-conserved C-terminal arm (Figure 1A and Figure S1). The central β-barrel domain of CaKu70 contains several long insertions relative to other homologues, thus accounting for its larger size. Based on the crystal structure of the human Ku70-Ku80 complex, these insertions are located near surface loops and are unlikely to disrupt the overall architecture of the proteins (data not shown) [20]. Also present in the C-terminal region of CaKu70 is the so-called SAP motif, a putative DNA/RNA binding structure found in several nuclear and cytoplasmic proteins. Interestingly, even though the SAP motif is conserved in many Ku70 proteins (e.g., *A. thaliana*, *H. sapiens*, *A. nidulans* and *N. crassa*), it has apparently been lost in a branch of budding yeast that includes *S. cerevisiae*, *C. glabrata*, and *K. lactis* (Figure 1A and Figure S1) and *Ashbya gossypii* (not shown) [29], which have significantly shorter Ku proteins (576-606 amino acids). A conserved sequence (ζ helix 5) that is crucial for NHEJ in *S. cerevisiae* is present in the N-terminal region of CaKu70 as well [30].

*C. albicans* is a diploid organism with a high degree of polymorphism; many genes exhibit allelic differences (http://www.candidagenome.org). As shown in Table 1 (see also Materials and Methods), in strain CA14, we identified two distinct alleles of *KU70*, referred to as A and B, which exhibit 13 SNPs in the coding region. Of these, eight were synonymous (positions +903, +969, +1041, +1044, +1779, +1827, +1839, and 1869) and five non-synonymous (positions +844, +866, +1697, +1722, and +2264). The latter SNPs result in differences in the protein sequence (Asn282 vs Asp282, Asn289 vs Ile289, Arg566 vs His566, His574 vs Gln574, and Gly755 vs Asp755, respectively). Only the first six SNPs (+844 to +1044) appear in the Assembly 21 of the *C. albicans* Database (strain SC5314) where the non-synonymous SNPs at positions +844 and +866 are designated R and W, respectively. Sequencing of alleles A and B present in the heterozygous strains LCD1A and LCD1B respectively revealed that the new SNPs were due to single base substitutions in allele A. In addition, three new SNPs, due also to changes in allele A, were detected in the terminator region of *KU70*. Therefore, the extra-SNPs detected here may be due to mutations occurring during the construction of strain CA14. Alternatively, they could have been eliminated by gene conversion in the SC5314 isolate used for sequencing.

**Phenotypic analysis of ku70 mutants**

To initiate a functional analysis of CaKu70, we generated a series of heterozygous and homozygous mutants by using a URA-blaster cassette. Several *C. albicans* DNA repair mutants have been shown to exhibit reduced growth rates or abnormal morphology. We therefore first analyzed our *KU70* mutants with respect to these phenotypes [7,8]. We found that CaKu70 is not essential for viability; the generation time of the null homozygous strain (LCD2A, 67 min) was only slightly longer than those of the heterozygote (LCD1A, 65 min) and wild type (CAF2, 63 min) (Figure 1B). When grown on YPD plates, the null strain exhibited a colony and cellular morphology that is indistinguishable from the wild type. In liquid YPD medium at 30°C, only yeast cells were observed in both parental and null strains (Figure 1C). Thus, the growth behaviors of the *ku70* mutants resemble those of the *ku80* mutants [30] and *lig4* mutants, which, similar to *ku70*, are presumably defective in NHEJ [11,12]. In contrast, mutants defective in HR (*rad52* and *rad51*) or in genes common to both HR and NHEJ (*rad59, mre11*) exhibit filamentous morphology and grow as wrinkled colonies under the same conditions [7,9]. When grown on solid Spider and M-199 plates, *ku70* mutants formed filaments similar to those produced by wild type strains. Finally, in comparison to the wild type strain (CAF2), no difference in filamentation was observed in both parental and null strains (Figure 1C). Thus, the *ku70* mutants resemble those from the *S. cerevisiae* *rad50, mre11* mutants. At both 37°C and 30°C, only yeast cells were observed in both parental and null strains (Figure 1C). Thus, the growth behaviors of the *ku70* mutants resemble those of the *ku80* and *lig4* mutants, which, similar to *ku70*, are presumably defective in NHEJ [11,12]. In contrast, mutants defective in HR (*rad52* and *rad51*) or in genes common to both HR and NHEJ (*rad59, mre11*) exhibit filamentous morphology and grow as wrinkled colonies under the same conditions [7,9]. When grown on solid Spider and M-199 plates, *ku70* mutants formed filaments similar to those produced by wild type strains. Finally, in comparison to the wild type strain (CAF2), no difference in filamentation was observed when this morphologic form was induced by addition of serum to *C. albicans* wild type strain (CAF2), no difference in filamentation was observed when this morphologic form was induced by addition of serum to CaKu70 wild type. In liquid YPD medium at 30°C, only yeast cells were observed in both parental and null strains (Figure 1C). Thus, the growth behaviors of the *ku70* mutants resemble those of the *ku80* mutants [30] and *lig4* mutants, which, similar to *ku70*, are presumably defective in NHEJ [11,12]. In contrast, mutants defective in HR (*rad52* and *rad51*) or in genes common to both HR and NHEJ (*rad59, mre11*) exhibit filamentous morphology and grow as wrinkled colonies under the same conditions [7,9]. When grown on solid Spider and M-199 plates, *ku70* mutants formed filaments similar to those produced by wild type strains. Finally, in comparison to the wild type strain (CAF2), no difference in filamentation was observed when this morphologic form was induced by addition of serum to CaKu70 wild type.
42°C, both a heterozygous KU70/ku70::hisG (LCD1A.1) and a null ku70::hisG/ku70::hisG (LCD2A.1) behaved like the wild type (CAI4) strain (Figures 2A and 2B). The same was true for the heterozygous and null strains constructed in the BWP17 background (not shown). Thus, the C. albicans ku70 null mutant is not thermo-sensitive.

We have reported that rad52 null strains of C. albicans, which are defective in HR, are very sensitive to the alkylating agent MMS [7]. As shown in Figure 2C, both heterozygous (LCD1A1) and null ku70 (LCD2A1) strains were indistinguishable from its parental CAI4 when grown in MMS, even at concentrations as high as 0.03%. A previous analysis indicates that the Saccharomyces yku70 mutant is sensitive to MMS only in the absence of recombination [32]. We therefore analyzed a C. albicans ku70 rad52 double mutant (JLT2.1), but found that this mutant did not exhibit greater MMS sensitivity than the rad52 mutant (TGR2.1.1); both strains showed some growth at 0.0025% MMS (Figure 2D), but were killed by 0.01% MMS (not shown). The ku70 mutant strains were also treated with 25 J/m² of UV radiation. At this dose of irradiation, no difference in viability was detected between the ku70 mutant and the wild type CAI4 strain. In contrast, all rad52 mutants (single and double mutants) exhibited increased sensitivity (not shown). Thus, NHEJ is not a major pathway for the repair of MMS or UV-induced lesions.

Role of KU70 in telomere length regulation

Because the SNP differences between the KU70 alleles, we generated a collection of heterozygous (LCD1A and LCD1B, each with a different allele) and reconstituted strains (see below) and analyzed the transcript levels of KU70 as well as the telomere phenotypes of these strains. The level of the KU70 mRNA was decreased by one half in the heterozygous strains LCD1A (retains allele A) and LCD1B (retains allele B), whereas no message was detected in the null strain (Figure 3). A reconstituted strain LCD3A, constructed by reintroducing allele B into LCD1A (i.e., it carries both alleles of KU70), regained the wild type expression level, whereas its counterpart LCD3A.1, constructed by reintroducing allele B into the null strain LCD2A1 (i.e., it carries a single copy of allele B), exhibited a level similar to the heterozygous strain LCD1B (allele B) (not shown). Reintroduction of a second copy of allele B into LCD3A.1 (strain SAT1.6) increased the amount of the KU70 message to wild type levels. Therefore, the amount of the KU70 transcript is roughly proportional to the gene dosage.

To analyze the effect of C. albicans KU70 deletion on the length and distribution of telomeres, we compared the wild type, the heterozygous and the null homozygous strains after repeated passages (Figure 4). All of the strains analyzed in this experiment
Table 1. Identification of the SNPs present in the two alleles of the KU70 gene of strain CAI4.

| Relative position within ORF | SNPs | Amino acids |
|-----------------------------|------|-------------|
| +844                        | A    | G           |
| +866                        | A    | T           |
| +903                        | A    | T           |
| +969                        | C    | T           |
| +1041                       | G    | T           |
| +1044                       | A    | G           |
| +1697                       | T    | A           |
| +1779                       | T    | C           |
| +1827                       | G    | A           |
| +1839                       | G    | A           |
| +1869                       | T    | C           |
| +2264                       | G    | A           |
| +2488                       | G    | T           |
| +2512                       | G    | A           |
| +2726                       | G    | A           |

The ORF of CaKU70 is from positions +1 to +2436. SNPs at positions +2488, +2222, and +2436 are in the terminator region.

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carry the URA3 gene (U61+). The heterozygous strains containing either allele of KU70 (LCD1A and LCD1B) maintained on average slightly shorter telomeres than the parental strain (CAF2). In contrast, the ku70 null strain contained telomeres that are much longer than the parental strain (Figure 4). The distribution of telomere fragments also differs between the various strains. Specifically, distinct clusters of telomere bands can be discerned in the wild type and heterozygous, but not in the null strain, indicating that the latter strain has increased telomere length heterogeneity (Figure 4). To avoid the potential effects of the position in the genome of the marker gene (URA3), we also compared the U61− derivatives of the Ku mutants to the parental strain CAI4, as well as strains generated from BWP17 (another reference strain), and found similar telomere phenotypes for the heterozygous and null mutants (data not shown). Several features of the telomere phenotypes are worthy of further comments. First, the shortening of telomeres in the heterozygous mutants were apparent in the earliest passage (~75–100 generations after the derivation of the mutant) and did not change appreciably afterwards, suggesting rapid kinetics that is distinct from other telomere mutants such as telomerase-null strains. However, because telomere shortening in the KU70+/− heterozygous strain is very modest, it may take a relatively small number of generations to reach to the new equilibrium distribution even if the rate of shortening is as low as in other mutants. In addition, because of the modest length difference between the telomeres of the wild type and heterozygous strains, more studies will be necessary to confirm this defect definitively. Finally, the long and heterogeneous telomeres of the homozygous knockout strain suggest the possible existence of aberrant telomere DNAs that do not migrate into the gel during electrophoresis (found e.g., in the rap1−/− mutant, which also has long and heterogeneous telomeres [33]). However, Southern analysis using blots that included the wells of the gel did not reveal such aberrant telomeric DNA in the ku70−/− mutant (Figure 2S).

Next, we used in-gel hybridization to analyze the levels of G-strand overhangs in the various strains. Interestingly, we observed an increase in the amount of G-strand overhangs in the ku70 heterozygotes, but not in null strain relative to the parental CAI4 (Figure 3S). The reason for this somewhat paradoxical finding is not understood, but similar results were reported for human KU86 heterozygous and homozygous null mutants [34,35]. The accumulation of G-strand overhangs in the heterozygous strain is reminiscent of C. albicans telomerase mutants as well as S. cerevisiae yku mutants, and consistent with a role for the C. albicans Ku complex in protecting telomeres against G-strand degradation [36,17]. Altogether, our findings suggest that C. albicans KU70 regulates telomere length and structure in a dosage-dependent manner.

We then analyzed the consequences of re-integrating the KU70 gene back into the heterozygotes and null strains. Surprisingly, the telomere defect of the heterozygote was not suppressed by reintroducing a missing allele, as shown for strain LCD3A (Figure 4). Similarly, reintroducing one copy of KU70 (allele B) into the null strain LCD2A did not completely restore the telomere phenotype of the heterozygote. Although the telomeres of the resulting strain LCD3A.1 showed reduced heterogeneity, they remained elongated through many passages (Figure 4 and Figure S4). Introducing a second copy of KU70 into LCD3A.1 caused a progressive decline of telomere length followed by stabilization (SAT1.5 and SAT1.6 in Figure 4, see also Figure S4). These complex effects of KU70 reintroduction reinforce the notion that the regulation of telomere length by KU70 is dosage dependent. They also suggest that the initial telomere lengths of the strain influence the final equilibrium length reached after alterations in KU70 dosage.

The mechanisms of telomere elongation in the ku70 null strain

To determine the mechanisms of telomere elongation in the ku70 mutant, we created various combination mutants and compared their telomeres to the ku70 single mutant. As shown in Figure 5A, telomeres of the ku70 tert combination mutant are similar in size to the tert mutant and substantially smaller than the ku70 single mutant, indicating that the shortening is telomerase-dependent. However, in terms of size heterogeneity, the ku70 tert combination mutant resembles more closely the ku70 mutant, suggesting that telomerase is not responsible for generating telomere size heterogeneity in the combination mutant. Thus, at least two mechanisms are acting to create the characteristic telomeres of the ku70 mutant. Interestingly, whereas neither the ku70 nor the tert single mutant displayed growth defect at 40°C, the growth of a ku70 tert double mutant was significantly affected at this temperature. This, unlike the S. cerevisiae mutant, the thermosensitivity of the C. albicans ku70 mutants was only observed in the absence of telomerase (Figure S5). It has been shown earlier that the ts phenotype of the S. cerevisiae yku mutants is caused by short telomeres [37]. Hence the difference in temperature sensitivity between the S. cerevisiae and C. albicans ku mutants can be explained by the fact that the C. albicans ku70−/− strains have long rather than short telomeres.

We have shown earlier that the absence of Rad52 also resulted in an increase in the telomere lengths [7]. As shown in Figure 5B, this effect, although reproducible, was rather modest as compared with that caused by the absence of Ku70. A double mutant ku70 rad52 has telomeres that resemble the ku70 null strain, suggesting...
that the ku70 is epistatic to rad52 with regard to telomere lengthening and the induction of telomere length heterogeneity.

We next asked whether the effect caused by the absence of Ku70 was paralleled by the absence of Lig4, another component of the NHEJ pathway. Telomere fragments of the lig4 mutant were similar in length to those of the parental strain, although they appeared slightly sharper. Deleting the LIG4 gene in the ku70 background did not modify the length or the heterogeneity of the telomeres (Figure 5C). Therefore, the lengthening and the increased heterogeneity of ku70 telomeres appear unrelated to NHEJ.

Detection of circular telomeric DNA in the ku70 null mutant

The finding that the absence of Ku70 caused an increase in both the heterogeneity and length of telomeres in C. albicans, although contrary to the results obtained in S. cerevisiae, mimics the situation reported for A. thaliana, where loss of this protein also caused telomeres to be 2 to 3-fold longer and much more heterogeneous than the wild type cells [22]. In A. thaliana, Ku70 suppresses ALT (Alternative Lengthening of Telomeres), an HR-based mechanism that results in the formation of telomeric circles (t-circles). To determine if this mechanism operates also in C. albicans, we analyzed the structural properties of telomeric DNA in the ku70 null strain. For this purpose, AluI and NlaIII-digested genomic DNA was resolved using two-dimensional (2D) gel electrophoresis and then the telomeric DNA detected using a specific probe. As shown in Figure 6A, the parental strain BWP17 showed arcs corresponding only to linear DNA whereas its isogenic ku70 null strain showed arcs corresponding to both circular DNA and linear DNA. By contrast, the level of ribosomal circles was unaffected by KU70 deletion [Figure 6A]. Thus, similar to A. thaliana, the C. albicans Ku70 plays an important role in specifically suppressing the formation of t-circles.

As noted earlier, the absence of Lig4 in the ku70 background did not alter the length or the heterogeneity of telomeres. 2D analysis revealed similar or slightly elevated levels of t-circles in the lig4 ku70 double mutant in comparison to the ku70 single mutant (Figure 6B). This phenotype is stable for at least ~150 generations (six passages). Thus, Lig4 does not influence significantly t-circle formation in the ku70 mutant.

The formation of t-circles in the ku70 mutants is presumably mediated through a recombination-based mechanism. Because, like other budding yeast, Rad52 is apparently the most important gene for HR in C. albicans [7], we analyzed the level of t-circles in the ku70 rad52 double mutant. Interestingly, the abundance of t-circles in this double mutant is again similar or slightly higher than that of the ku70 single mutant, indicating that Rad52 is dispensable for t-circle maintenance (Figure 6B).
Other non-linear DNAs such as branched molecules are also known to migrate slower in the second dimension in the 2D gel system. To confirm our designation of t-circles, we analyzed the ku70-/− DNA sample together with a nicked circular DNA plasmid (Figure 6C). The signal for the nicked circular plasmid indeed localized to the putative circular arc, supporting our assignment.

**Discussion**

Although CaKu70 exhibited a low level of sequence identity/similarity to other homologues, it retains the shared motifs and most likely the same structural organization as other Ku70 proteins. In the present study, we have characterized the potential roles of CaKu70 based on previous findings in other organisms. In contrast to S. cerevisiae ku70 mutants, C. albicans ku70 was not thermosensitive. Furthermore, Ku70 does not play any significant role in the repair of lesions caused by MMS or UV light in *C. albicans*. However, our analyses did reveal complex regulation of telomere length and structure by CaKu70.

The *C. albicans* Ku complex acts in both telomerase regulation and telomere protection

Studies in various organisms have revealed disparate impacts of *KU* gene mutations on telomere lengths. In baker’s yeast, as well as in organisms ranging from fission yeast, humans to trypanosomes, deletion of *KU* genes results in telomere shortening [14,35,38,39]. In contrast, Ku deficient *Arabidopsis* cells exhibit very long and heterogeneous telomeres [21]. These findings suggest that depending on the experimental system, Ku can play either a positive or negative regulatory role in telomere length regulation. Remarkably, we observed complex effects of *KU70* gene dosage on *C. albicans* telomere length, indicating that the *C. albicans* protein may simultaneously play both a positive and negative regulatory role. Disruption of one copy of *KU70* resulted in telomeres that were slightly shorter than those of wild type cells and this effect was both reproducible and independent of the allele present in the heterozygotes. This observation is reminiscent of the short but stable telomeres of haploid *S. cerevisiae* yku70 and yku80 mutants upon prolonged passage [40,41]. The positive regulatory function of Yku70 and Yku80 involves a direct interaction between Yku80 and telomerase RNA [14]. However, we have not been able to detect a comparable interaction between CaKu70 or CaKu80 and *C. albicans* telomerase RNA (data not shown). Further studies will be necessary to determine the molecular basis of the telomere elongation function of CaKu70 suggested by the phenotype of the heterozygous mutant. In stark contrast to the heterozygous strains, homozygous *C. albicans* ku70 deletion strains exhibit long and heterogeneous telomeres resembling those of the corresponding *Arabidopsis* ku70 mutants. Such a phenotype has been attributed to loss of telomere capping, leading to aberrant access of telomerase

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**Figure 3. Analysis of the KU70 transcript levels in the mutant strains.** (A) A Northern blot analysis of KU70 transcripts in wild type CAF2 (KU70/KU70), heterozygotes LCD1A and LCD1B (KU70/ku70Δ), null mutant LCD2A (ku70Δ/ku70Δ) and reconstituted strains LCD3A (KU70/ku70::KU70) and SAT1.6 (ku70::KU70/ku70::KU70). The levels of actin (ACT1) mRNA were used as a loading control (lower panel). (B) The relative KU70 transcript levels were calculated (Material and Methods) and plotted.

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at telomere ends [42]. Consistent with this idea, the telomere elongation in the C. albicans ku70 mutant appears to be mostly telomerase dependent.

In addition to allowing uncontrolled telomerase action at telomeres, the C. albicans ku70 mutant resembles the corresponding Arabidopsis Ku70 and human ku86 mutants in possessing high levels of t-circles [22,23]. These t-circles are thought to derive from aberrant recombination, and are hallmarks of the ALT pathway described in human cancers. The induction of both telomerase and recombination activities at ku70 telomeres suggests that the Ku complex may directly regulate both pathways by facilitating the formation of a protective structure. However, a recent study of human cells indicates that t-circle levels can be elevated by telomerase overexpression, suggesting that long telomeres by themselves are sufficient to induce telomere recombination [43]. Hence the elevated telomere recombination of the ku70-/- mutant could be a secondary consequence of uncontrolled telomerase activity. In any case, it is clear that loss of Ku70 leads to phenotypes that are quite reminiscent of those caused by the loss of other telomere binding proteins such as Rap1, Stn1 and Ten1 [33,44].

More findings in support of a protective function for Ku at telomeres came from analysis of single-stranded G-tails. Previous studies indicate that deprotected telomeres are often subjected to preferential degradation of the C-strand, leading to accumulation of G-tails. Remarkably, we found that G-tail accumulation in C. albicans KU70 mutants was also dosage-dependent, with the heterozygous mutant manifesting high levels of G-tails and the homozygous null mutant exhibiting no evident accumulation. These observations, while paradoxical, are actually identical to those described for the human Ku86 mutants [23,34]. One speculative explanation is that longer G-tails are formed in the homozygous null mutant, but are more quickly processed by recombination or C-strand fill-in synthesis due to greater accessibility of the tails in the null mutant. More studies will be necessary to address this possibility.

Notably, the distinctive telomere phenotypes of the ku70 mutant in S. cerevisiae and C. albicans indicate that even between closely related budding yeast, the telomere maintenance factors have undergone significant evolutionary divergence and mediate slightly different functions. The S. cerevisiae telomere complex has been studied extensively and utilized as a model system for understanding human telomeres. However, the C. albicans Ku complex appears to exhibit more functional similarities to the human complex from the standpoint of suppressing aberrant recombination and suppressing G-tail formation. Further analysis of this genetically tractable diploid fungus may thus provide insights on Ku mechanisms that could not be obtained from S. cerevisiae.

T-circles and telomere recombination in the ku70 mutant

The phenotypes of ku70 lig4 and ku70 rad52 combination mutants provide further insights on the function of KU70 and raise interesting questions concerning the mechanisms of t-circle formation and maintenance. First, neither LIG4 nor RAD52 deletion significantly altered the lengths of telomeres in the ku70 mutant, consistent with telomerase (rather than NHEJ or HR) being largely responsible for the observed elongation. Additionally,
the level of the t-circles observed in the ku70 mutant was not modified by the subsequent deletion of LIG4 or RAD52. Our failure to eliminate t-circles in the rad52 ku70 combination mutant is surprising at first glance, since t-circles formation is thought to be mediated by an aberrant, HR-related mechanism [25] and RAD52 is important for most HR pathways in both S. cerevisiae and C. albicans. However, it is possible (though unlikely) that t-circles may replicate autonomously in C. albicans. Because our combination mutant was derived from sequential disruption of KU70 and RAD52, our results can be explained by the proposition that RAD52 is required for the generation but not the maintenance of t-circles. In addition, many putative Arabidopsis recombination genes, including MRE11 and RAD51 paralogs, have been shown to be dispensable for t-circle accumulation [22]. Finally, a RAD52-independent recombination pathway for telomere maintenance named ILT was recently uncovered in S. cerevisiae, again underscoring the multiplicity of recombination mechanisms and their disparate requirements [45].

In summary, we have shown that CaKu70 regulates C. albicans telomeres through multiple mechanisms, some of which are quite distinct from those of the Yku proteins in S. cerevisiae. Continued exploration of this alternative model system should lead to broader insights on the mechanisms and evolution of the Ku complex in budding yeast and other organisms.

**Materials and Methods**

**Strains and media**

The C. albicans strains used in this study are listed in Table S1. C. albicans cells were grown routinely at 30°C in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) or SC (0.67% yeast nitrogen base, 2% glucose) missing or containing uridine (25 μg/ml), histidine (90 μg/ml), arginine (89 μg/ml). Uri<sup>+</sup> cells were selected in SC medium lacking uridine and Uri<sup>-</sup> auxotrophs on SC plates containing 0.1% 5-FOA and 25 μg/ml uridine. Nourseothricin-resistant (Nou<sup>+</sup>) transformants were selected on YPD agar plates supplemented with 200 μg/ml of nourseothricin (Werner Bioagents, Jena, Germany) [11,46,47]. For testing hyphal formation in C. albicans, the cells were grown in liquid YPD containing 10% pre-heated bovine serum at 37°C or on solid M-
Cloning of KU70

ORF19.1135, the candidate for CaKU70 (see Results) was amplified by PCR of genomic DNA isolated from CAI4. Primer pairs for PCR (with restriction sites for SacI and HindIII, respectively) were complementary to positions +2416 to +2395 and +7963 to +7882 in relation to the first nucleotide of the ORF (Table S2). The products were purified, inserted in between the SacI and HindIII site of pGEM7Zf(+), and then transformed into E. coli.

Several independent transformants were cultured for DNA isolation. Southern blot hybridization of electrophoretic karyotype blots [9] of strain CAI4 with an internal fragment of KU70 (HDF-A2F and HDF-B3R primers, Table S2) confirmed its location on chromosome 1 (not shown).

Disruption of KU70

We used the C. albicans strains CAI4, BWP17, CEA2.5 and a tert null mutant for the sequential disruption of this ORF by the ‘URA-blaster’ method [48]. Briefly, a PCR fragment containing 416 bp of the noncoding upstream sequence and 233 bp 5’ ORF (amplified using the AF and AR primer, Table S2) was inserted 5’ to the URA-blaster cassette, and a PCR fragment containing 244 bp of 3’ ORF and 445 bp of downstream sequence (amplified using the BF and BR primer, Table S2) was inserted 3’ to the URA-blaster cassette. As a consequence, the disruption cassette was expected to replace 1959 bp of ORF19.1135.

C. albicans transformations were carried out as described [49]. Uri+ transformants were first isolated on SC-uridine and then tested for the correct integration of the URA-blaster cassette by Southern analysis. Uri- clones were selected by their ability to grow on 5-FOA plates and used for the disruption of the second allele with the same cassette. The loss of both alleles was confirmed by Southern analysis using HindIII-digested genomic DNA and the...
transformants were selected on YPD plates with 200 μg/ml of nourseothricin, and correct re-integrants were identified as +/− mutants using the Random Primed DNA Labeling Kit (Roche). Other experiments were performed using Ur1 mutants and the Ur2 control (CAF2).

Reintegration of KU70 at its native locus in the ku70 mutants

In order to reintegrate a cloned allele of KU70 into the heterozygote and null mutant, these mutants were transformed with a cassette containing a copy of cloned KU70 gene and the URA3-hisG. Thus, this ORF was directed to its own locus by the homology between the cassette and the disrupted ku70Δ::hisG allele. The transformation was carried out as described previously [49] and Ur1 transformants were selected on SC-uridine plates. To screen for the desired re-integrants, genomic DNA was digested by BamHI and analyzed by Southern blots using a HindIII-SacII fragment of the cloned C. albicans KU70 as the probe. Reconstituted strains derived from a heterozygote mutant contain two copies of ORF 19.1135 and reconstituted strains derived from a null mutant contain only one copy of the ORF. All the reconstituted strains are prototrophic for uridine.

To construct a KU70/KU70 strain from the null mutant, the strains with one re-integrated KU70 gene were transformed with a cassette containing a copy of KU70, a SAT1 marker and a 3′ fragment from KU70 [47]. Nourseothricin-resistant (Nou R) transformants were selected on YPD plates. To screen for the desired re-integrants, genomic DNA was digested by BamHI and analyzed by Southern blots using a HindIII-SacII fragment of the cloned C. albicans KU70 as the probe. Reconstituted strains derived from a heterozygote mutant contain two copies of ORF 19.1135 and reconstituted strains derived from a null mutant contain only one copy of the ORF. All the reconstituted strains are prototrophic for uridine. The KU70 genes of the heterozygous strain LCD1A and all the reintegrants were sequenced to verify that no mutations in the KU70 ORF had been introduced during the genetic manipulations.

Characterization of polymorphism in KU70

The KU70-containing plasmid was sequenced using eight pairs of oligonucleotides (eight forward and eight reverse) (Table S2). These primers are evenly spaced along the ORF to yield reliable and overlapping sequences that allow the assembly of the entire ORF. The data from the sequencing reactions were analyzed using the SeqMan software (DNASTar, Lasergene), and the consensus sequence from our clone was compared with the database sequence using the MegAlign software (DNASTar, Lasergene). Several potential SNPs were identified. In order to confirm the SNPs, genomic DNA of CAF2 was again amplified by PCR to obtain fragments encompassing residue +782 to +1260 of the ORF. The fragments were sequenced directly. In addition, sequencing of the heterozygous strain LCD1A and KU70-reintegrant LCD3A (see Results) indicated the presence of additional SNPs, whose presence was traced to strain CAF2.

DNA extraction and analysis

Chromosomal DNA was isolated from protoplasts obtained by incubation of cells with Zymolyase [11] or by the ‘Smash and Grab’ method [50]. For Southern analysis, genomic DNA was digested with restriction enzymes, subjected to electrophoresis into an agarose gel, transferred to nitrocellulose or nylon membranes and probed with the indicated DNA fragments (labeled with 32P using the Random Primed DNA Labeling Kit (Roche). The membrane was mostly analyzed using a Molecular Imager FX (Bio-Rad). Alternatively, an X-Omat X-AR film (Kodak) was used to visualize the bands.

RNA extraction and Northern analysis

Total RNA was isolated by extraction with hot acidic phenol from 30 ml of C. albicans cultures in the exponential phase of growth (OD600 = 0.5 approximately). ORF19.1135 expression levels in the ku70 mutants were measured by Northern using a 32P labeled internal fragment of KU70 gene (HDF-A2F and HDF-B3R primers, Table S2) as the probe. The membrane was analyzed as described for Southern blots and the bands were quantified using the Corel Draw Graphics Suite X3 software.

MMS and UV light treatment

Cultures of the various strains were grown at 30°C until OD600 = 1. For MMS-sensitivity analysis, 5 μl aliquots of the original suspension and of 5-fold serial dilutions of each mutant were applied onto YPD plates containing varying concentrations of MMS. For UV-sensitivity analysis, the cell suspensions and serial dilutions were applied to YPD plates, and the plates were irradiated with 25 J m−2 of UV light. Both the MMS-containing and UV-irradiated plates were incubated between 48 and 72 hours at 30°C and then photographed.

Analysis of telomere length and structure

For analysis of telomere lengths over multiple generations, cells were streaked on YPD plates, grown for 2 days at 30°C, and one colony was picked for further re-streaking. Following the desired number of passages, a colony was inoculated into 20 ml YPD, and the culture grown at 30°C for 24 hours. Genomic DNA was isolated as described previously and then digested with a combination of AluI and MbolI [51]. Restriction fragments were subjected to electrophoresis into a 2% agarose gel and transferred to a nylon filter. Telomeric DNA was detected by hybridization at 50°C to a 5′-labeled oligonucleotide containing two copies of the C. albicans telomere repeat (Table S2) [52]. Single-stranded telomere overhangs were analyzed by in-gel hybridization using a previously described protocol [36]. T-circles were detected using 2D gels as described by Brewer and Fangman with modifications by Cohen and Lavi (1996) [53]. AluI- and MbolI-digested or undigested genomic DNA was first separated according to size in a 0.5% agarose gel at 1 V cm−1 for 22 hours. Strips of the gel containing DNAs that range in size from about 250 bp to slightly larger than 10 kb were excised, and then subjected to electrophoresis in the orthogonal direction in a 1.2% agarose gel containing 0.3 μg/ml ethidium bromide at 6 V cm−1 for 4 hours. Circular DNA molecules are expected to migrate slower during the second round of electrophoresis, forming a separate arc from linear molecules. The DNA was transferred onto a nylon filter and hybridized with Candida telomere or rDNA probes (Table S2). The hybridization condition was the same as previously described for telomere Southern blots.

Supporting Information

Figure S1 Multiple sequence alignment of Ku70 proteins from C. albicans, C. tropicalis, S. cerevisiae, C. glabrata, K. lactis, A. nidulans, N. crassa, A. thaliana and H. sapiens; the α/β domain, β-barrel, C-terminal arm and SAP domains are highlighted in different colors. (TIF)
Figure S2 Analysis of telomere lengths in various Ku70 mutants. Duplicate Genomic DNA samples were prepared from the indicated strains following 2 streaks on YPD plates, and subjected to telomere Southern analysis. The entire gel (including the wells) was subjected to transfer and hybridization. The location of the well in the PhosphorImager scan is indicated by an arrow.

(TIF)

Figure S3 Analysis of G-strand overhangs in the ku70 heterozygous and homozygous mutants. (A) Genomic DNA samples from cultures of the indicated strains were prepared from different passages, and subjected to in-gel hybridization analysis of telomeric G-strand overhangs as described (36). The Ku70+/− samples were prepared following 2, 4, 6, 8 and 10 streaks, whereas the ku70+/− samples were prepared following 2, 4, 6, 8 and 10 streaks on plates. Both the Ku70+/− strain (LCD1A.1) and the ku70+/− strain (LCG2A.1) are derived from the CA4 parental strain. (B) After the detection of G-strand overhangs, the DNA fragments in the gel were denatured and hybridized again with the same probe to identify all terminal restriction fragments.

(TIF)

Figure S4 Analysis of the stability of telomere lengths in reconstituted strains. Genomic DNA samples were prepared from the indicated strains following 2, 4, 6, 8, 10 and 12 streaks on YPD plates, and subjected to telomere Southern analysis.

(TIF)

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8. Table S4 Analysis of telomere Southern analysis of Ku70 mutants. (TIF)

9. Table S5 Analysis of telomere Southern analysis of Ku70 mutants. (TIF)

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