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Brh2 and Rad51 promote telomere maintenance in *Ustilago maydis*, a new model system of DNA repair proteins at telomeres

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Abstract

Recent studies implicate a number of DNA repair proteins in mammalian telomere maintenance. However, because several key repair proteins in mammals are missing from the well-studied budding and fission yeast, their roles at telomeres cannot be modeled in standard fungi. In this report, we explored the dimorphic fungus *Ustilago maydis* as an alternative model for telomere research. This fungus, which belongs to the phylum Basidiomycota, has a telomere repeat unit that is identical to the mammalian repeat, as well as a constellation of DNA repair proteins that more closely mimic the mammalian collection. We showed that the two core components of homology-directed repair (HDR) in *U. maydis*, namely Brh2 and Rad51, both promote telomere maintenance in telomerase positive cells, just like in mammals. In addition, we found that Brh2 is localized to telomeres *in vivo*, suggesting that it acts directly at chromosome ends. We surveyed a series of mutants with DNA repair defects, and found many of them to have short telomeres. Our results indicate that factors involved in DNA repair are probably also needed for optimal telomere maintenance in *U. maydis*, and that this fungus is a useful alternative model system for telomere research.

Keywords

Telomere; Ustilago maydis; Homology-directed repair; Brh2; Rad51

1. INTRODUCTION

The special structures located at the ends of linear eukaryotic chromosomes, known as telomeres, are critical for chromosome stability; they protect the terminal DNAs from degradation, end-to-end fusion, and other abnormal transactions [1,2]. In most organisms, telomere DNAs consist of copies of a short repetitive sequence, which are bound by a complex array of proteins through both DNA-protein and protein-protein interactions. The

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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integrity of the telomere nucleoprotein complex is crucial for chromosome stability and aberrant telomeres have been shown to induce chromosome rearrangement and cancer [3,4].

Broadly speaking, the maintenance of telomere DNA requires both efficient semi-conservative DNA replication by the replicative polymerases and “terminal repeat addition” by telomerase, a special reverse transcriptase [5,6]. The terminal repeat addition is necessary to compensate for the loss of DNA that is incurred as a consequence of incomplete end replication [7]. In the absence of telomerase, a recombination-based mechanism referred to as ALT can also lengthen telomeres, as has been demonstrated in certain cancer cells [8]. Each of these pathways has been shown to promote telomere maintenance in a wide range of organisms, including fungi. Indeed, extensive analyses of these pathways in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have provided major insights on the responsible factors and mechanisms [9,10], and accelerated studies of comparable pathways in mammalian cells.

Homology-directed repair (HDR) is a universal, recombination-based mechanism for DNA repair [11,12,13]. A variety of damaged DNAs can be processed to generate single stranded DNA (ssDNA), which can be manipulated by HDR proteins to pair with and invade a homologous DNA duplex. This is followed by a series of reactions including DNA synthesis and ligation of nicks, leading ultimately to restoration of the damaged DNA. Several HDR proteins are known to be major players in the ALT or ALT-like pathways of telomere maintenance, consistent with the notion that ALT is based on recombination [8,14]. Interestingly, recent studies indicate that even in the setting of active telomerase, some HDR proteins are needed for telomere maintenance in mammalian cells. Specifically, mutational inactivation or depletion of RAD54, RAD51D, RAD51, and BRCA2 each has been shown to engender telomere shortening and chromosome end-to-end fusion [15,16,17]. Because ALT is generally repressed in the presence of active telomerase, and because HDR proteins do not appear to regulate telomerase activity, these proteins may facilitate telomere maintenance by promoting semi-conservative replication rather than terminal repeat addition [15]. In contrast to these findings in mammals, the central factors of HDR in budding yeast (i.e., Rad51 and Rad52) do not appear to affect telomere length in telomerase positive cells [18,19], suggesting substantial differences in the telomeric functions of these proteins in yeast vis-à-vis mammals. In addition, a key HDR protein in mammalian cells, namely the BRCA2 tumor suppressor which mediates RAD51 delivery to single-stranded DNA, is conspicuously missing in both budding and fission yeast, making it difficult to assess the role of this protein at telomeres in standard model systems [13].

The dimorphic fungus *Ustilago maydis* belongs to the phylum Basidiomycota, which is distinct from the phylum Ascomycota to which budding and fission yeasts belong [20]. *U. maydis* was developed by Robin Holliday as an experimental system for mechanistic analysis of recombination and repair. Like the standard budding and fission yeast, important pathways in *U. maydis* can be manipulated and dissected using a variety of molecular genetic and biochemical techniques, and its genome has been fully sequenced [21] and manually annotated (http://mips.helmholtz-muenchen.de/genre/proj/ustilago/). Notably, earlier studies have revealed greater resemblance of the HDR machineries in Basidiomycota to the mammalian machinery, especially as evidenced by the existence of a BRCA2 homolog named Brh2 in *U. maydis* and other basidiomycetes [22]. Moreover, in contrast to *S. cerevisiae* and *S. pombe*, which bear irregular and non-mammalian telomere repeats, *U. maydis* has a 6-base pair repeat that is identical to the mammalian repeat [23]. The total lengths of the terminal repeats have not been carefully determined, but sequencing of several clones indicates that some ends have at least 37–39 repeats (~230 bp) [23]. *U. maydis* also carries telomere-associated sequences (TASs), which are middle repetitive sequence elements located proximal to the terminal repeats. Two main classes of TAS have been

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characterized: UTASa, which is primarily restricted to chromosome ends, and UTASb, which is often found at interstitial sites [24]. These earlier findings provide a foundation for investigating the role of HDR proteins in telomere maintenance in *U. maydis*.

In this report, we show that *U. maydis* telomeres undergo dynamic growth and trimming during serial passage. We found that the two principal mediators of HDR in *U. maydis*, namely Brh2 and Rad51, both promote the maintenance of normal telomere lengths in telomerase positive cells, just like in mammals. In addition, we found that Brh2 is localized to telomeres *in vivo*, suggesting that it acts directly at chromosome ends. We surveyed a series of additional mutants with DNA repair defects, and found many of them to contain abnormally short telomeres. Finally, we showed by *in silico* analysis that *U. maydis* contains homologues of key components of the mammalian telomere nucleoprotein complex. Our findings demonstrate the potential of *U. maydis* as a new model system for telomere research.

2. MATERIALS AND METHODS

2.1. Construction of *U. maydis* strains

Standard protocols were employed for the growth and genetic manipulation of *U. maydis* ([25,26], and references therein). UCM350 was used as the parental strain for constructing the null mutant strains, most of which were freshly made as part of this study (Table 1). Null mutants were constructed by replacing the entire open reading frames with cassettes expressing resistance to hygromycin (Hyg^R), nourseothricin (Nat^R), or geneticin ($G418^R$) [27,28]. The strains used for ChIP analysis were generated by transforming the *brh2* null strain UCM565 with self-replicating plasmids that contain untagged or Myc_6-tagged Brh2. Expression of Brh2 and Myc_6-tagged Brh2 was under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and relied on hygromycin selection for plasmid retention [26,29].

2.2. Telomere analyses

U. maydis strains were passaged by repeatedly re-streaking for single colonies on YPD plates. For each re-streaking, only a small portion of the colony was captured and applied to a fresh YPD plate; the remainder was used for liquid culture inoculation and DNA isolation. Standard telomere Southern analysis was performed using established protocols with some modifications [30]. Briefly, chromosomal DNAs were digested with *Pst* I, and fractionated in 0.9–1.2 % agarose gels. Following transfer to nylon membranes, the telomere restriction fragments were detected using ^{32}P-labeled UmC8 probe that corresponds to eight copies of the *Ustilago* C-strand telomere repeat. Hybridization was performed in the Church Mix (0.5 M sodium phosphate buffer pH 7.2, 1 mM EDTA pH 8.0, 7% SDS, 1% BSA) at 50°C. Signals obtained by scanning the Phosphor plates were quantified using ImageQuant software (Molecular Dynamics Inc.). The hybridization intensities of TRFs were plotted against TRF lengths, and the length that divided the area under the curve into two equal halves was determined. This length was taken to be the approximate mean TRF length.

2.3. G- and C-strand overhangs analysis

The in-gel hybridization analysis was performed using a combination of established protocols with minor modifications [31]. The labeled UmG4 and UmC4 oligonucleotides, corresponding to four copies of the *U. maydis* telomeric G-strand and C-strand repeats, were used as the probes, and hybridization was performed in the Church Mix at 50°C.

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2.4. Chromatin immunoprecipitation

Chromatin immunoprecipitation of Myc_6-tagged Brh2 was carried out using a previously published procedure with slight modifications [32]. Briefly, formaldehyde-fixed cells were resuspended in lysis buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 150 mM NaCl, and protease inhibitors), and broken by glass beads. The lysates were sonicated to shear DNAs to a mean length of ~600 base pairs. Extracts were adjusted to 1–1.5 mg/ml protein in 700 μl lysis buffer and then incubated with monoclonal anti-Myc antibody (9E11 from Covance, 1:300 dilution) overnight at 4°C. Five percent of each cell extract was set aside and used as the input sample. Each extract-antibody mixture was subjected to immunoprecipitation at 4°C for 2 hrs. After stringent washes, IP samples were eluted in 500 μl of 1% SDS, 0.1 M $NaHCO_3$ and crosslinks were reversed at 65°C for 5 hrs. The DNA samples were recovered by treatment with RNase A and proteinase K followed by extraction with phenol/chloroform and precipitation with ethanol. The DNA samples were then subjected to either PCR or dot blot analysis. For PCR analysis, the level of an UTASa fragment (generated by primer UT-F [5′-GATCGGCTGTTCGTATGCAC-3′] and UT-R [5′-AGCCGTCCAGCGGTCAGCGC-3′]) was used to assess telomere enrichment and an *act1* fragment (generated by primer ACTIN-F [5′-TTGGCTCAACAGCTCGTTAT-3′] and ACTIN-R [5′-GGGTACTTGAGCGTCAAAAT-3′]) was used as the control. In the case of dot blot analysis, the membrane was probed with the same ^{32}P-labeled UTASa and *act1* fragments.

2.5. Bioinformatic analysis

To identify putative *U. maydis* shelterin or CST components, we initially used the mammalian or *S. pombe* proteins as the queries and performed iterative PSI-BLAST searches of the non-redundant NCBI database. If a *U. maydis* hit was not identified in this manner, the search was repeated using just the most conserved domain of the query protein (e.g., the OB fold domain of TPP1). Alternatively, if a hit was not found in *U. maydis*, but was found in a closely related fungal branch (e.g., *Cryptococcus* or *Coprinopsis*) by the initial hunt, searches were repeated using such hits as the query. Each *U. maydis* candidate protein was also used as the query in a PSI-BLAST search. The recovery of *bona fide* shelterin or CST components was taken as providing strong support for the validity of the identification. It should be noted that the protein hits obtained from the NCBI searches were largely derived the initial Broad Institute analysis. The sequences of these proteins have been curated manually by the MIPS *Ustilago maydis* database [21], and the updated sequences were used for the analysis presented in Table 2 and Supp. Fig. 3.

3. RESULTS

3.1. *U. maydis* telomeres undergo dynamic length changes during serial passage

Our immediate goal was to investigate the role of HDR and other DNA repair proteins in *U. maydis* telomere maintenance. Before initiating such studies, we first examined telomere length and contents in the wild type strain UCM350 by telomere Southern. Chromosomal DNA was cleaved with *Pst* I to liberate the terminal restriction fragments (TRFs), and an oligonucleotide (UmC8) corresponding to eight copies of the *U. maydis* telomere C-strand repeat was used as the probe (Fig. 1a). The *Pst*I enzyme was chosen because it cleaves a site in UTASa that is close to the terminal repeats. The TRF fragments detected by our assay ranged in length from 0.4 to 2.5 kb, with the major clusters observed between 0.6 and 1.2 kb (Fig.1b, lane 1, right panel). To confirm that these signals were derived from terminal telomere repeats rather than internal regions of the chromosomes, we tested their sensitivity to *Bal*31, a nuclease that nibbles DNA from the ends. As predicted, the majority of fragments shortened and exhibited reduced hybridization signals with increasing duration of *Bal*31 treatment (Fig. 1b, right panel). Notably, the putative TRFs were completely degraded

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after 20 min of *Bal*31 treatment, whereas the bulk of chromosomal DNA as well as a few interstitial fragments were largely unaffected (Fig. 1b, lane 4 in both left and right panels; interstitial fragments in the right panel are marked by asterisks).

We then used two different methods to estimate the sizes of terminal repeat tracts within the TRFs. First, we cloned and sequenced a number of UTASa-type telomeres by employing a previously described linker-ligation/PCR strategy [33]. The sizes of the terminal repeat tracts vary considerably between the different clones: some are similar to the previously reported 200 bp [23], whereas others are as long as 500 bp (data not shown). Because a *Pst*I site is located ~140 bp away from the terminal repeats in UTASa-type telomeres, these telomeres are presumed to yield TRFs of 340 to 640 bp, which are consistent with the sizes of the smaller TRFs in our analysis. Second, we estimated the terminal repeat tract lengths of the relatively long TRFs by comparing the TRF length reduction with the loss of hybridization intensity in the *Bal*31 assays. In one comparison, the shortening of the 1.7 kb TRF cluster by 200 bp (to 1.5 kb) correlated with an ~50% reduction in the hybridization intensity, suggesting that the terminal repeats are ~400 bp (Fig. 1b, lane 1 and 2, TRF clusters marked by a bent arrow; Supp. Fig. 1). Other comparisons yielded similar results (data not shown). Thus, the longer TRFs are most likely due to non-UTASa telomeres where the *Pst*I sites are further away from the terminal repeats. Taken together, our data suggest that *U. maydis* telomeres are probably heterogeneous with an average length of 300–400 bp.

Next, we analyzed telomere dynamics of the strain during serial passages at two different temperatures (Fig. 1c). The majority of TRF clusters appear to undergo progressive lengthening at both 25°C and 30°C, and this visual impression is consistent with quantitative estimates of mean TRF lengths of the samples (Fig. 1d). Interestingly, this trend was interrupted in late passages of the cultures. Loss or shortening of long TRF fragments was also evident during these passages (Fig. 1c, several TRFs that appear to shorten in subsequent passages are marked by open arrowheads), implying that telomeres can experience sudden truncation when they reach certain lengths. Taken together, these results indicate that *U. maydis* telomeres undergo dynamic growth and trimming during passage.

3.2. *U. maydis rad51* and *brh2* mutants have shorter than normal telomeres

Human BRCA2, which functions together with RAD51 to promote HDR, has recently been shown to play a crucial role in telomere capping and preventing telomere loss [15]. The *U. maydis* BRCA2 homologue Brh2 interacts with Rad51 in HDR, allowing the latter to overcome the barrier imposed by RPA and access single stranded DNA [34]. It was therefore of substantial interest to determine if the Brh2 and Rad51 proteins are required for telomere maintenance in *U. maydis*. Accordingly, we analyzed the TRF lengths of the *U. maydis brh2* and *rad51* null mutants. Because the wild type *U. maydis* strain exhibits telomere growth and trimming during passage, we generated pristine knockouts of the *brh2* and *rad51* genes and carefully followed telomere dynamics in these naïve strains starting with early generations of mutant strains. As shown in Fig. 2a and 2b, the mean TRF lengths of the *U. maydis brh2Δ* and *rad51Δ* mutants were consistently shorter than normal telomeres, by approximately 100 bp. This difference was observed in the earliest passages of the mutants analyzed (~75 generations after construction) and was maintained over time. Growth and trimming of individual TRF clusters can still be observed in both mutants, suggesting that the responsible factors and reactions are unaffected by Brh2 and Rad51. Because telomere maintenance mutants sometimes exhibit elevated levels of single stranded DNAs, we also analyzed the levels of unpaired G- and C-strand telomere DNAs in the mutants by in-gel hybridization. However, we detected normal levels of single stranded DNA in the mutants, indicating that neither protein is needed to prevent the accumulation of single-stranded DNA (data not shown). Our data clearly demonstrate that both Brh2 and Rad51 promote normal telomere maintenance in telomerase-positive cells. In addition, the

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similarity between the phenotypes of the mutants suggests that Brh2 and Rad51 may function in the same pathway of telomere maintenance.

3.3. Brh2 is localized to telomeres *in vivo*

To investigate if Brh2 and Rad51 act directly at telomeres, we checked the telomere association of Brh2 *in vivo* by chromatin immunoprecipitation (ChIP). *U. maydis* strains containing untagged Brh2 or Myc_6-tagged Brh2 were subjected to formaldehyde crosslinking, chromatin isolation and immunoprecipitation using anti-Myc antibodies. The levels of a telomere-associated sequence (UTASa) and an actin fragment (*act1*) in the precipitates and inputs were then analyzed by PCR. As shown in Fig. 3a, the strain bearing Myc_6-tagged Brh2 is as resistant to UV irradiation as the strain bearing untagged Brh2, suggesting that tagging does not affect Brh2 function. PCR analysis of the IP samples indicates that Myc_6-tagged Brh2 specifically associated with higher levels of UTASa but not the *act1* control (Fig. 3b). No UTASa PCR signal was detected in anti-Myc antibody immunoprecipitates from an untagged strain. In addition, two independently derived, Myc_6-tagged strains yielded the same result in ChIP analyses with regard to the telomere localization of Brh2, indicating that the enrichment of telomeres in the IP samples is Myc_6-tag-dependent. We note that PCR amplification for UTASa resulted in two bands of around 350 bp. This may be due to the presence of an alternative annealing site for one of the primers within the UTASa region. To complement the PCR analysis, we also assayed the levels of UTASa and *act1* DNA using dot blot hybridization. Again, the subtelomeric fragment was preferentially enriched in the IP samples derived from strains with Myc_6-tagged Brh2 (Fig. 3c). Taken all together, our data indicate that the Brh2 is preferentially associated with subtelomeric or telomeric DNA *in vivo*, supporting the idea that Brh2 acts directly at telomeres.

3.4. Many *U. maydis* DNA repair mutants exhibit shorter than normal telomeres

To extend the observations on Brh2 and Rad51, we surveyed several additional DNA repair genes for their roles in telomere maintenance in telomerase positive cells. Four additional DNA repair-deficient strains were generated. These included mutants deleted of DNA ligase IV (*dnl4*), which is involved in non-homologous end joining (NHEJ) [35], Exonuclease I (*exo1*), which functions in HDR to resect DNA ends in preparation for strand invasion [12], and *rec1*, which functions in DNA damage response as part of the heterotrimeric 9-1-1 complex [36], In addition the double mutant *dnl4Δ/brh2Δ* defective in both HDR and NHEJ was analyzed. These mutants and the *rad51* and *brh2* null strains were passaged and their telomeres analyzed in parallel. Consistent with previous findings, telomeres in the *brh2Δ* and *rad51Δ* mutants were about 100 bp shorter than normal (Fig. 4a and 4b). All four of the new DNA repair mutants displayed abnormal telomere phenotypes as well (Fig. 4a and 4b). Mean TRF lengths of the *exo1Δ* and *rec1Δ* mutants were also about 100 bp shorter than normal, whereas the *dnl4Δ* mutant and *dnl4Δ/brh2Δ* double mutant exhibited telomere shortening of about 50 and 180 bp, respectively. Our results indicate that many DNA repair proteins have significant roles in maintaining normal telomeres in *U. maydis*. It was interesting to note that deleting the *brh2* and *dnl4* genes appeared to cause an additive effect on telomere length, suggesting that they affect different pathways at telomeres. Similar to the *rad51* and *brh2* null strain, the ability of individual telomere clusters to undergo slow elongation during passage appears to be retained in the other DNA repair mutants. Further mechanistic and functional analysis of the impact of DNA repair genes on telomere dynamics will be necessary to understand the specific reactions and pathways governed by these genes at telomeres.

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3.5. *U. maydis* has a telomere nucleoprotein complex that closely resembles the mammalian complex

The telomere nucleoprotein complexes in different taxa exhibit impressive evolutionary divergence, driven by as yet incompletely understood factors [37]. In mammals, a six-protein complex named shelterin comprised of TRF1, TRF2, POT1, TPP1, TIN2 and RAP1 is critical for both telomere protection and maintenance [38] (Supp. Fig. 2). Collectively the subunits of this complex bind telomere DNAs as well as protein targets to suppress DNA rearrangements and enhance DNA synthesis. In addition, a trimeric complex named CST (comprised of CTC1, STN1 and TEN1) was recently shown to interact with shelterin and to regulate telomere replication and terminal repeat addition [39] (Supp. Fig. 2). Previous bioinformatic analysis indicated the existence of *U. maydis* POT1 and TRF1/2 homologues, but the full extent of telomere protein conservation between Basidiomycota fungi and mammals has not been determined [40]. To gain evolutionary insights and provide a foundation for experimental studies, we queried the NCBI and Broad Institute databases for *U. maydis* homologues of each of the shelterin and CST subunits (see Materials and Methods for a more detailed description of our strategies). Notably, with the sole exception of TIN2, we were able to identify the putative orthologues of all of the mammalian shelterin components (Table 2)(Supp. Fig. 2). TIN2 is thought to be a mammalian-specific subunit of shelterin, just as Poz1 and Ccq1 are believed to be confined to fission yeast. Consistent with the lineage-specific nature of these telomeric proteins, homologues of Poz1 and Ccq1 could not be identified in *U. maydis*. By contrast, we discovered plausible STN1 and TEN1 orthologues in the *U. maydis* genome. Each identified candidate not only exhibits substantial sequence similarity to the query, but also possesses conserved domains found in other family members. For example, an N-terminal BRCT and a central SANT domain are located in the putative *Um*Rap1, precisely as predicted for this family of proteins (Supp. Fig. 3)[32]. Likewise, the putative *Um*Tpp1 contains an N-terminal OB fold, which is predicted by the HHpred program to resemble structurally the OB fold of mammalian TPP1 (Supp. Fig. 3) [41]. Overall, our analysis revealed substantial similarities between the telomere complexes of basidiomycetes and mammals, and suggests that further analysis of the *U. maydis* telomeres could provide insights on telomere maintenance in mammals.

4. DISCUSSION

The chief significance of the current study resides in the development of *U. maydis* as a new model system for telomere research. Specifically, we characterized the basic telomere dynamics of this fungus and confirmed the role of several HDR and DNA repair proteins in telomere maintenance. We also identified in the *U. maydis* genome a set of shelterin-like proteins, which provide a foundation for future analysis. The implications of our findings are discussed below.

4.1. Telomere growth and trimming during passage

The individual telomeres of *U. maydis* exhibit progressive elongation with occasional trimming such that the average telomere lengths are relatively stable in late passages. The slow and steady growth of telomeres has been observed in other systems and is thought to be mediated by telomerase [42,43]. For example, *Candida albicans* telomeres lengthen substantially when the strains are propagated at high temperatures [42]. The *Tetrahymena* macronuclear telomere length increases by 3–10 bp per generation in cultures maintained in log-phase growth [44]. This length increase is probably due to telomerase because it was eliminated when telomerase components were knocked down [45]. Similar growth of telomeres is also known to occur in cancer cells overexpressing telomerase [46]. Presumably in these systems and in *U. maydis*, the frequency and extent of telomere elongation by telomerase more than compensate for the loss incurred by incomplete end replication,

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leading to a net lengthening. However, the longer telomeres in *U. maydis* also have a greater probability of undergoing sudden shortening, perhaps through a mechanism akin to telomere rapid deletion (TRD) that has been described in budding yeast [47]. Telomere homeostasis, then, may be achieved through a balance of telomerase and TRD in *U. maydis*. Notably, TRD does not appear to play a significant role in normal budding and fission yeast [48]. Instead, the longer telomeres in these fungi become resistant to telomerase by adopting a more inaccessible conformation, thereby achieving length equilibrium [49]. The existence of a trimming mechanism in normal *U. maydis* suggest that this fungus will be a useful model system for identifying factors and pathways responsible for this mechanism, which may also operate in tumor cells with high levels of telomerase [46].

4.2. The role of HDR proteins in telomere maintenance

There is accumulating evidence that HDR proteins are required for telomere maintenance in mammalian cells even in the setting of positive telomerase. On the other hand, deletion of the two principal HDR proteins in budding yeast, i.e., Rad51 and Rad52, had little effect on telomere length [18]. Because HDR proteins have not been shown to influence telomerase activity, the findings in mammals suggest a role for these proteins in semi-conservative telomere replication. However, the precise reactions executed by these proteins at telomeres remain to be determined. We have shown that, unlike budding yeasts, mutation in HDR proteins has significantly detrimental effects on telomere maintenance in *U. maydis,* and that the key HDR protein Brh2 is localized to telomeres *in vivo*. The difference between *S. cerevisiae* and *U. maydis* is especially striking in the case of Rad51, which plays major roles in HDR in both organisms, yet affect telomere maintenance only in *U. maydis*. What could account for the difference? Unlike *S. cerevisiae*, *U. maydis* has a highly G-rich canonical telomere repeat unit that is identical to the mammalian repeat unit. This telomere sequence has been shown to dynamically adopt a series of folded structures that may impede replication fork progression [50]. Hence it is conceivable that the forks at mammalian and *U. maydis* telomeres may frequently stall and collapse, requiring HDR proteins for stabilization and repair. By contrast, the irregular repeat of budding yeast telomeres may present less obstacles for the replication machinery, thus obviating the need for HDR proteins. In this regard, we note that budding yeast engineered to possess “humanized telomeres” exhibits significant growth defects when subjected to replication stress [51]. The strain is also especially sensitive to the loss of Rad51 or Rad52 [51]. Though many factors could account for these phenotypes, one contributing factor may be increased difficulties in replication through the mammalian repeat. Regardless of the precise underlying cause of differences between *S. cerevisiae* and *U. maydis*, our findings indicate that the latter will be an especially informative model system for understanding the mechanisms of HDR proteins at telomeres. Besides HDR, other DNA repair pathways may also participate in telomere maintenance in *U. maydis*. For example, we observed significant telomere loss in strains bearing deletions in *dnl4*, which functions in NHEJ rather than HDR. Further studies will be necessary to gain a more complete understanding of the roles of various repair proteins at telomeres.

4.3. The evolution of the telomere nucleoprotein assembly

Studies of telomeres in mammals and fungi in recent years have revealed two distinct paradigms for the telomere nucleoprotein assembly, as epitomized by the shelterin complex in mammals and the Rap1 and CST complexes in budding yeast [37] (Supp. Fig. 2). One major difference between the two paradigms resides in the mechanism of G-tail protection, which is mediated by POT1/TPP1 in mammals and the CST complex in budding yeast. (Even though the CST complex is present in mammals, its main function appears to be in telomere replication rather than G-tail protection [52]). Another major difference resides in the factors that bind duplex telomere repeats, which are TRF1 and TRF2 in mammals and

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Rap1 in budding yeast. The fission yeast telomeres possess at least four of the shelterin subunits (Taz1(=TRF1/2), TPP1, Rap1, Pot1), suggesting that they are structurally more similar to the mammalian paradigm. This in turn suggests that the common ancestor of fungi and metazoans has a shelterin-like telomere complex, and that the Rap1/CST paradigm in budding yeast represents a more recent invention. Our identification of a suite of shelterin-like proteins in the basidiomycete *U. maydis* is entirely consistent this evolutionary model, and provides further support for the prevalence of shelterin-like telomere protective complexes in diverse organisms.

A more interesting and unresolved set of issues concerns the evolution of the CST complex, specifically the lineages of the largest subunit (Cdc13 in budding yeast and CTC1 in mammals). Cdc13 and CTC1 are both rapidly evolving proteins [52,53]. Hence our inability to identify a plausible homologue of either protein in *U. maydis* is probably due to the low level of sequence similarity between the homologues rather than the absence of such a factor. Further studies of the CST complex in basidiomycetes may provide significant insights on the evolution of this complex.

4.4. U. maydis as a model system for telomere research

Our findings and other recent developments provide strong support for the utility of investigating telomere maintenance mechanisms in *U. maydis*. First, the *U. maydis* genome has been fully sequenced and annotated, and bioinformatic analysis has uncovered a collection of telomere-related proteins that bear strong resemblance to the mammalian factors [21,40] (also the current study). Second, *U. maydis* has a telomere repeat unit that is regular and identical to the canonical mammalian repeat (TTAGGG/CCCTAA). Because G-rich DNAs are capable of forming non-Watson/Crick structures that require resolution [50,54], the precise sequence and composition of the telomere G-strand could impact on the formation of such structures and the cellular response. Hence, the identical telomere repeat unit in *Ustilago* and mammals ensures that the telomere machineries deal with DNAs with the same physical-chemical properties. Finally, *U. maydis* has an HDR machinery that exhibits greater similarity to the mammalian machinery, and as we have shown, the HDR proteins in both systems appear to play similar roles in telomere maintenance in telomerase positive cells. Especially in light of the second and third considerations, *U. maydis* appears to offer advantages that are not available in standard budding and fission yeast, and thus untapped potentials for dissecting the mechanisms of telomere maintenance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- *U. maydis* telomeres undergo progressive elongation and abrupt shortening.
- Brh2 and Rad51 are required for telomere maintenance in telomerase positive cells.
- Brh2 is localized to telomeres *in vivo*.
- The *U. maydis* telomere protein complex is similar to the mammalian complex.

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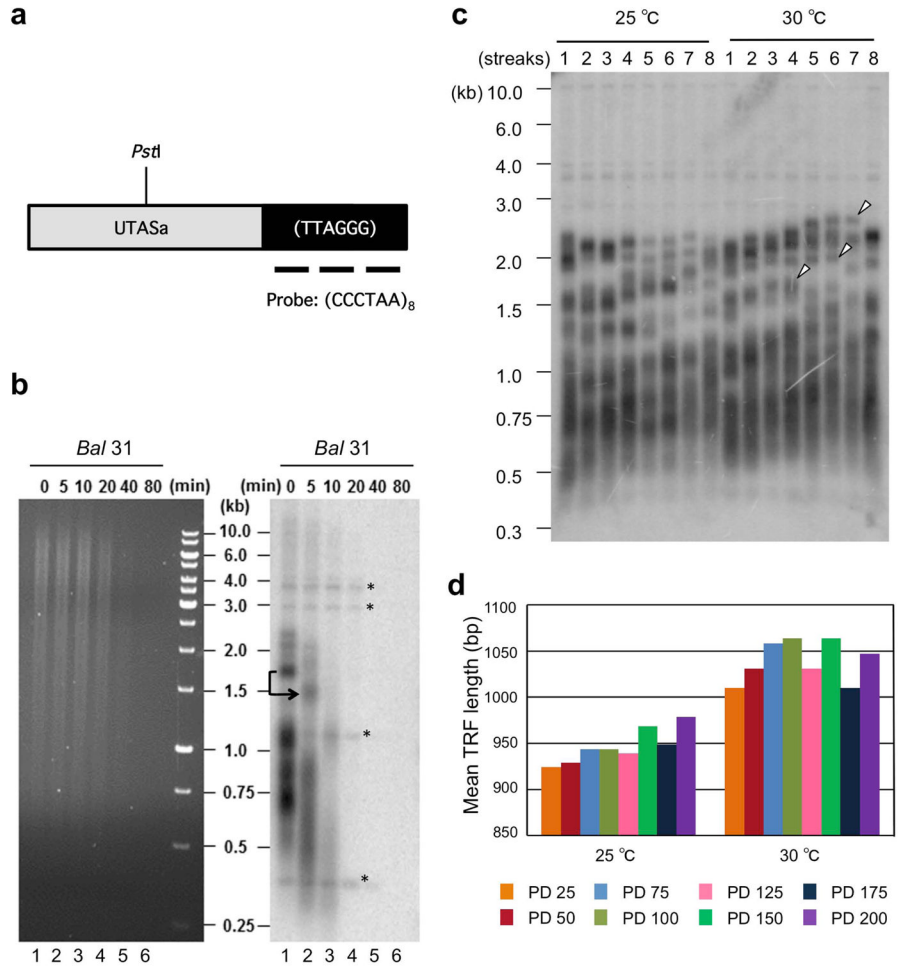

Fig. 1. Characterization of telomere restriction fragment and telomere dynamics in U. maydis

a, A schematic representation of *U. maydis* telomeres with the UTASa subtelomeric element is shown. **b,** *Bal*31 sensitivity assay. Chromosomal DNA from the wild type strain UCM350 was treated with 1 unit of *Bal*31 endonuclease for increasing durations followed by telomere Southern analysis. The ethidium bromide-stained gel and the phosphorImager scan are shown on the left and right, respectively. Putative interstitial fragments are indicated by asterisks and the TRF clusters used for estimating terminal repeat tract lengths marked by a bent arrow. **c** and **d**, dynamic changes of telomere during passage and mean TRF length. Cells were grown at two different temperatures, 25°C and 30°C. Each streak represents approximately 25 generations of growth. Mean TRF length was estimated using the method described in Materials and Methods. Several TRFs that evidently shortened in subsequent generations are marked by open arrowheads. For example, the longest TRF in streak 7 disappeared in streak 8, most likely because it experienced sudden truncation.

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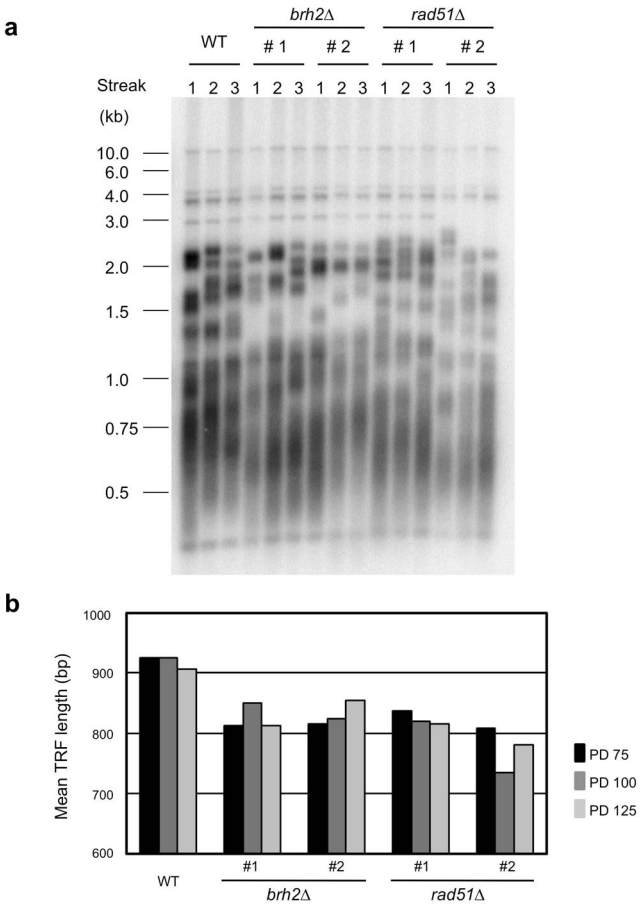

Fig. 2. Telomeres lengths in the rad51 and brh2 mutants

a, TRF analysis of *brh2* and *rad51* mutants. Two independent *brh2* or *rad51* null strains were generated and passaged at room temperature. Chromosomal DNAs from PD 75 to PD 125 were analyzed for telomere lengths. **b**, Mean TRF lengths of the samples shown in **a** were calculated and plotted.

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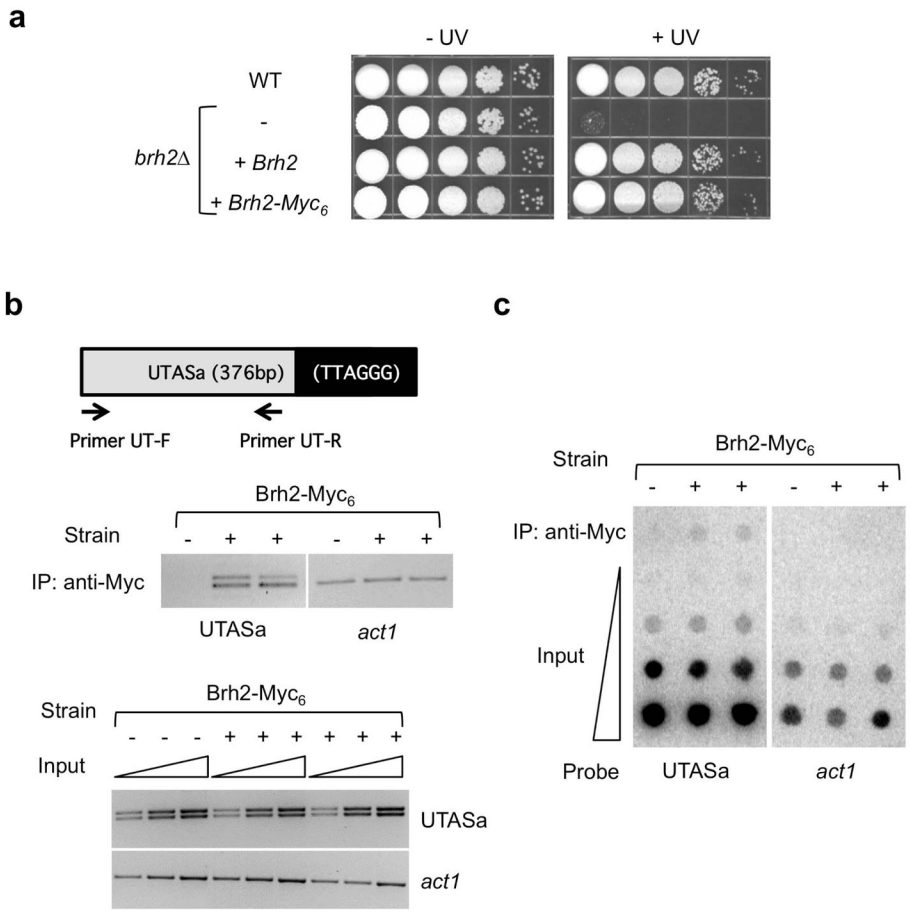

Fig. 3. Brh2 is localized to telomeres in vivo

a, UV sensitivity of the strains used in the ChIP analysis. **b,** PCR analysis of ChIP samples. Top, schematic outline for the amplification of UTASa in ChIP samples. Middle, PCR analysis of telomeric and control DNA levels in the IP samples. Bottom, titration of the levels of input DNA showing that similar quantities of telomere and control DNAs were present in the starting samples. **c**, Telomere dot blot. After the DNA samples were applied to a dot blot apparatus, the membrane was processed for standard telomere southern using the UTASa or *act1* fragment as the probes.

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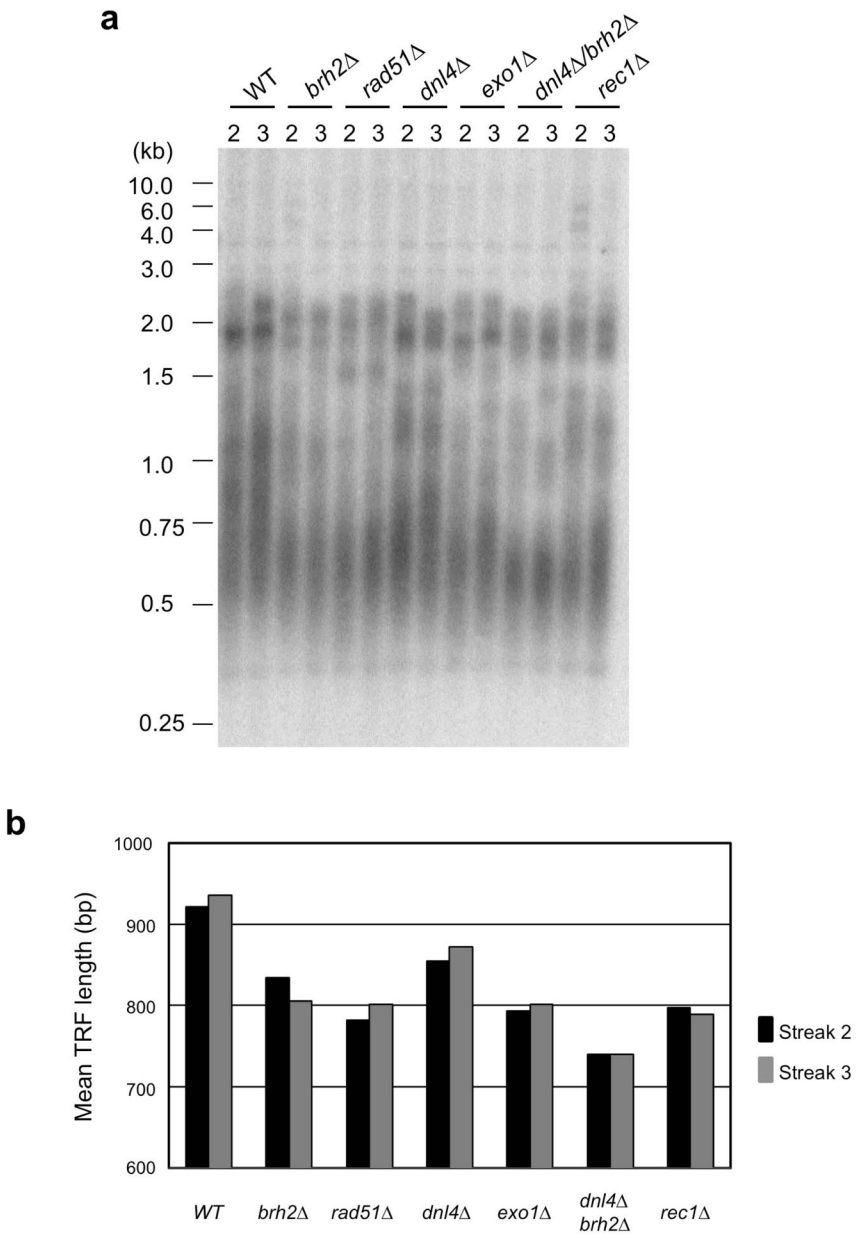

Fig. 4. Many U. maydis DNA repair mutants have shorter than normal telomeres

a and **b**, TRF analysis of DNA repair mutants and the estimated mean TRF lengths in all the samples.

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Table 1

U. maydis strains

Alias (Haploids)	*Relevant Genotype*	Reference
UCM350[a]	WT	Kojic et al., 2002
UCM565	*brh2Δ::nat*	Kojic et al., 2002
UCM565-pBrh2	*brh2Δ::nat/pBrh2*[b]	This work
UCM565-pBrh2-Myc$_6$	*brh2Δ ::nat/pBrh2-Myc$_6$*[b]	This work
UCM666[c]	*rec1Δ::nat*	This work
UCM705	*exo1Δ::hph*	Ninghui et al., 2009
UCM750[d]	*dnl4Δ::hph*	This work
UCM751	*brh2Δ::nat dnl4Δ::hph*	This work
UCM770[e]	*brh2Δ::neo*	This work
MK75[e]	*brh2Δ::neo*	This work
MK76[d]	*rad51Δ::neo*	This work
MK77[d]	*rad51Δ::neo*	This work

[a] Genotype of UCM350 *is nar1-6 pan1-1 alb1. nar*, *pan*, and *ab* indicate inability to reduce nitrate, auxotrophic requirement for pantothenate, and mating type loci, respectively. All other listed strains are derivatives of UCM350.

[b] Brh2 or Myc$_6$-tagged Brh2 was expressed in *brh2* null strain.

[c] rec1 allele is disrupted by insertion of *nat* cassette expressing resistance to nourseothricin (Nat^R).

[d] *dnl4* and *rad51* mutant alleles are generated by insertion of *hph* cassette expressing hygromycin resistance (Hyg^R).

[e] *brh2* allele is disrupted by insertion of *neo* cassette expressing resistance to geneticin (G418^R).

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Table 2

Presumed Telomere Proteins in the *U. maydis* genome

Proteins	*U. maydis* locus	size	Domain detected
Shelterin			
Pot1	UM05117	1050 aa	OB
Trf1	UM02326	1127 aa	Myb
Tpp1	UM11538.2	530 aa	OB
Rap1	UM04676	1156 aa	BRCT, SANT
Tin2	n.f. *		
CST complex			
Cdc13 or CTC1	N.F.		
Stn1	UM11687	517 aa	OB, winged helix
Ten1	UM11842	223 aa	OB
Telomerase			
TERT	UM11198	1317 aa	
TER	n.f. *		

* n.f. : not found