The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities

Renata M. Polotnianka*, Jian Li† and Arthur J. Lustig*†

The Ku heterodimer, conserved in a wide range of eukaryotes, plays a multiplicity of roles in yeast. First, binding of Ku, which is composed of a 70 kDa (Hdf1p) and an 80 kDa (Hdf2p) subunit [1–3], to double-strand breaks promotes non-homologous end-to-end joining of DNA [3]. Second, Ku appears to participate in DNA replication, regulating both the number of rounds of replication permissible within the cell cycle and the structure of the initiation complex [3,4]. Furthermore, mutations in *HDF1* or *HDF2* rapidly reduce telomeric poly($TG_{1–3}$) tract size [1–3], hinting also at a possible telomeric function of Ku. We show here that the two subunits of the Ku heterodimer play a key role in maintaining the integrity of telomere structure. Mutations in either Ku subunit increased the single-strandedness of the telomere in a cell-cycle-independent fashion, unlike wild-type cells which form 3′ poly($TG_{1–3}$) overhangs exclusively in late S phase [5]. In addition, mutations enhanced the instability of elongated telomeres to degradation and recombination. Both Ku subunits genetically interacted with the putative single-stranded telomere-binding protein Cdc13p. We propose that Ku protects the telomere against nucleases and recombinases.

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Results and discussion

Levels of 3′ telomeric single-stranded overhangs are increased in Ku mutants

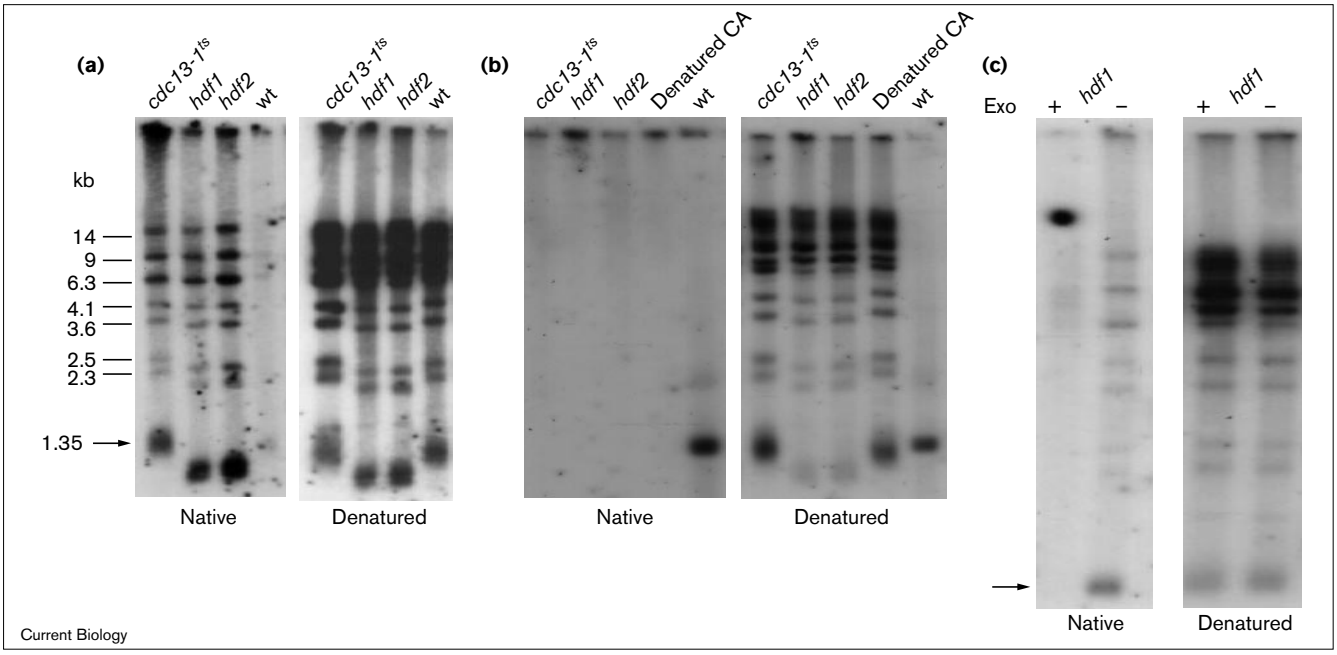
To test whether Ku acts to protect the telomere against exonucleases or endonucleases, the degree of single-strandedness at telomeric poly($TG_{1–3}$) tracts was analyzed in *hdf1* and *hdf2* null mutants. To do this, we used a non-denaturing *in situ* gel hybridization method [6] with end-labeled oligonucleotide probes homologous to either the G-rich or C-rich strand of telomeres (Figure 1a,b). DNA was isolated from wild-type, *hdf1*, *hdf2*, and *cdc13-1^{ts}* strains, digested with *Xho*I to release the Y′ class of telomeres [7], gel-fractionated, and probed initially under non-denaturing conditions with an oligonucleotide homologous to the G-rich strand (CA-*22* [6]; Figure 1a).

Wild-type DNA hybridized only weakly with this probe, as expected for asynchronously grown cells [5]. In contrast, in *hdf1* and *hdf2* mutants, both single-copy and Y′ class telomeres hybridized strongly with CA-*22* (Figure 1a, native), demonstrating the presence of single-stranded DNA. DNA from *cdc13-1^{ts}* mutants, which have been shown to contain extensive telomere-specific single-stranded regions in a different assay [8], also hybridized to the probe. To test for a strand-specific bias, we probed the same set of DNAs on a separate gel with the complementary probe, GT-*22* [6] (Figure 1b, native). Distinct from the previous result, the GT-*22* probe did not hybridize, except to denatured control plasmid DNA. As a control, denaturation and rehybridization of both gels led to the expected array of telomeric, subtelomeric and internal fragments (Figure 1a,b; denatured). These results indicate a substantial increase in G-strand-specific single-stranded DNA in *hdf* cells.

Single-stranded regions may occur at either gapped telomeric sites or the extreme poly($TG_{1–3}$) 3′ overhang. To distinguish between these possibilities, we treated DNA with *Escherichia coli* exonuclease I before *in situ* gel hybridization (Figure 1c). Exonuclease I cleaves single-stranded DNA in a 3′ to 5′ direction [9], thereby degrading only 3′ overhang DNA. Brief treatment of genomic DNA with exonuclease I before *Xho*I digestion eliminated CA-*22* hybridization under non-denaturing conditions. In contrast, treated and untreated samples were indistinguishable from each other after denaturation and rehybridization, demonstrating a lack of overall degradation (Figure 1c). Hence, Ku mutations confer either increased single-stranded length or aberrant cell-cycle generation of telomeric 3′ overhangs.

To test the cell-cycle regulation of telomeric 3′ overhangs in *hdf* mutants, we isolated DNA from wild-type, *hdf1*, *hdf2* and *cdc13-1^{ts}* cells arrested at the G1–S boundary with α factor, and from untreated control cells, and conducted *in situ* gel hybridizations (Figure 2). If longer than wild-type telomeric single-stranded overhangs are formed in *hdf* mutant strains exclusively in late S phase, as observed in wild-type cells [5], α factor arrest should eliminate 3′ overhangs and, hence, hybridization to CA-*22*. Interestingly, telomeres from *hdf1* and *hdf2* cells hybridized to CA-*22* at intensities close to those observed in non-synchronized cells (Figure 2a). All DNAs hybridized to CA-*22* after gel

Figure 1



G-rich 3′ single-stranded overhangs are abundant in *hdf1* and *hdf2* mutants. **(a)** *Xho*I-digested wild-type (wt), *hdf1*, *hdf2* and *cdc13-1*^{ts} DNAs were hybridized *in situ* under native conditions with CA-22 (5′-CCCACCACACACACCCACACCC-3′) [6]. The gel was denatured and reprobed with CA-22. *In situ* gel hybridization, washing and denaturation steps were performed sequentially as described [6]. The arrow points to the major Y′ class of telomeres. The Y′ class in *hdf* mutants is reproducibly shorter than in wild-type cells. Single-copy telomeres and their sizes are marked to the left. All cells were grown at 30°C, with the exception of *cdc13-1*^{ts} cells which were grown at 23°C. **(b)** *In situ* hybridization of a single gel, containing the DNAs tested in (a), probed sequentially under native and denaturing conditions with GT-22 (5′-GGGTGGTGTGTGTGGGTGTGGG-3′) [6]. A control plasmid containing a telomeric tract sequence (pCA759) [6] was digested with *Pst*I and denatured prior to loading (denatured CA). **(c)** *In situ* hybridization of a single gel, probed sequentially under native and denaturing conditions with CA-22. The gel contained DNA isolated from *hdf1* cells, with (+) or without (–) prior treatment with 1 unit/μl of *E. coli* exonuclease I (Exo) before *Xho*I digestion. The arrow indicates the Y′ class of telomeres.

denaturation (Figure 2b). These data suggest a loss of cell-cycle-regulated formation of 3′ overhangs in *hdf* mutant cells.

Mutants defective in the Ku heterodimer decrease the resistance of telomeres to tract loss and recombination

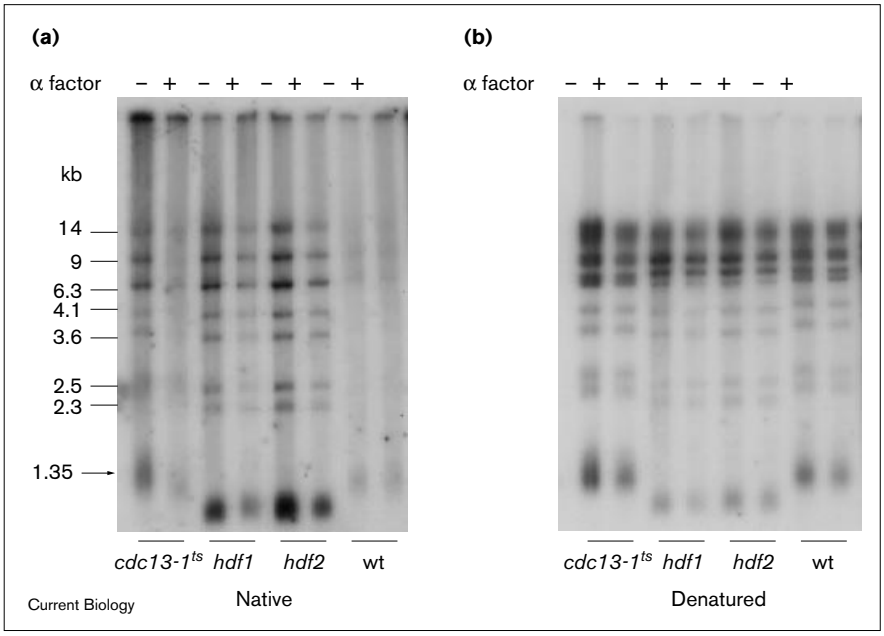
We also tested recombination and degradation of individual *ADE2*-marked elongated telomeres introduced into *hdf1* and *hdf2* mutants. We have previously demonstrated that elongated telomeres introduced into wild-type strains exhibit two effects: a slow continual loss of telomeric tract sequences, and/or a single-step intrachromatid deletion between poly($TG_{1–3}$) repeats which restores the elongated telomere to wild-type tract length, a process termed rapid deletion events (RDE) [7]. We reasoned that, if the Ku heterodimer serves to protect the termini from nucleases or recombinases, the stability of elongated telomeres may be significantly decreased when introduced into strains containing Ku mutations.

Serial liquid subculturing of cells containing either *hdf1* (Figure 3) or *hdf2* (data not shown) mutations revealed two striking effects. First, in both *hdf1* and *hdf2* cells, the elongated telomeres became two-fold more heterogeneous than when present in wild-type cells. More remarkably, the average size of elongated telomeres decreased by 160–170 bp in only nine generations and telomeres were highly unstable until the lowered *hdf* telomere length was reached. These data suggest a rapid nucleolytic loss of telomeric sequences. Second, *hdf1* telomeres were also capable of an exceedingly high rate of RDE. Elongated telomeres were quantitatively truncated to *hdf1* telomere size within only 12 generations, about 50 times the percentage of RDE per cell generation observed in wild-type cells (0.2%; [7]). Curiously, this appears to be an epigenetic effect, as telomeres from identical spore colonies generated apparent nucleolytic loss, with or without RDE, in a stochastic fashion (Figure 3). These results argue for a loss of protection against both nucleolytic degradation and intrachromatid recombination in Ku mutants, possibly due to the high levels of 3′ overhangs.

Synthetic lethality between *cdc13* and Ku heterodimer mutants

Cdc13p is a putative telomere-binding protein [10,11], which, when defective, results in two distinct allele-specific

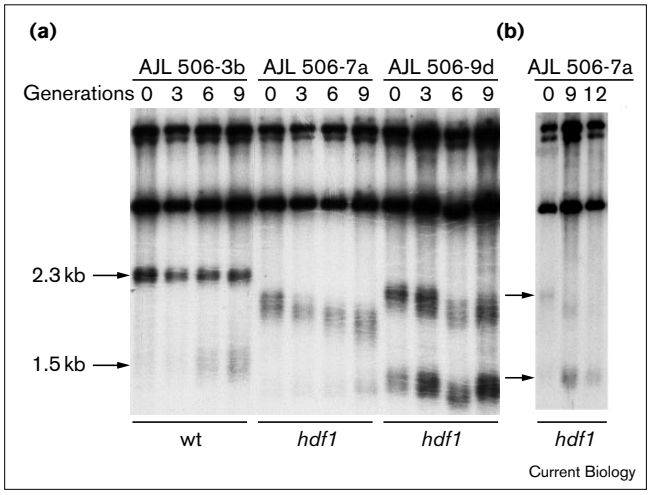
Figure 2



Single-strand overhangs are present in G1-arrested cells. Wild-type (wt), *hdf1*, *hdf2*, and *cdc13-1*^{ts} cells were grown at temperatures as described in the legend to Figure 1a to an OD_{600} of 0.15–0.2 and α factor added to a concentration of 25 μg/ml. Cells were maintained at their respective temperatures until > 90% of cells formed schmoo structures, which are indicative of G1 arrest (approximately 3.5 h). DNA of α-factor-arrested and untreated control cells were then isolated in parallel and treated as described in the legend to Figure 1. *In situ* gels were probed with CA-22 sequentially under **(a)** native and **(b)** denaturing conditions. Phosphoimager analysis revealed that the signals from G1-arrested *hdf1* and *hdf2* cells were about 60% of the wild-type signal in native gels, when corrected for the amount of DNA loaded.

phenotypes: an increase in 3′ single-stranded overhangs, exhibited by the *cdc13-1*^{ts} allele ([8]; Figure 1a), and a loss of telomeric tracts, leading to senescence, exhibited by the *cdc13-2*^{est4} allele [12]. To test whether *hdf* and *cdc13* alleles genetically interact, we conducted a pairwise combination of crosses between Ku subunit mutants and strains containing either *cdc13* allele.

Figure 3



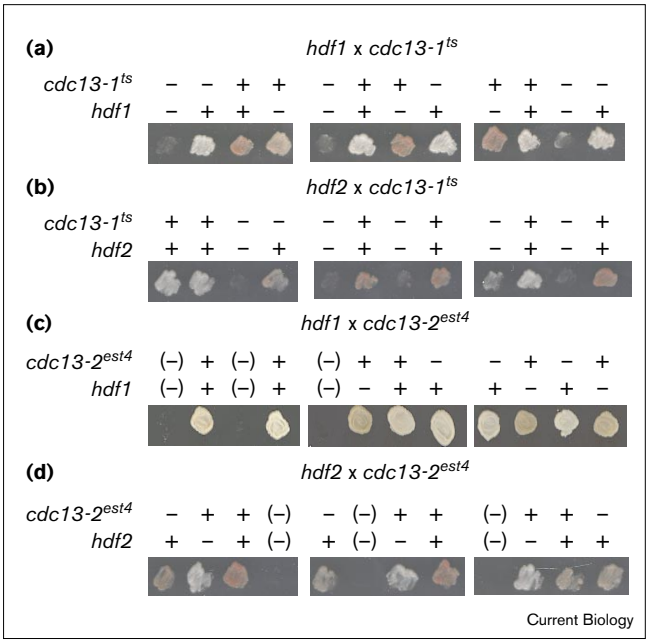
Rapid nucleolytic cleavage and deletion of elongated telomeres in *hdf1*-mutant cells. Wild-type (AJL 506-3b) and *hdf1* (AJL 506-7a and AJL 506-9d) cells, containing elongated *ADE2*-marked telomeres, were grown from individual colonies and subsequently subcultured for four rounds, each round consisting of three generations. DNAs were cleaved with *Nde*I, releasing the telomeric fragment, and probed with an *ADE2* probe [7]. Note that both phenotypes were observed in identical spore colonies as shown by a comparison of two independent experiments using AJL 506-7a **(a,b)**. RDE was monitored by the accumulation of the deleted form during continuous liquid subculturing. Arrows indicate the positions of the elongated and deleted forms of the telomere.

Intriguingly, each double mutant had synthetic lethal defects (Figure 4). The *cdc13-1*^{ts} allele has a maximally permissive temperature of 26°C, whereas *hdf1* and *hdf2* null alleles show only a weak temperature sensitivity at 37°C. In contrast, *hdf1* (or *hdf2*) *cdc13-1*^{ts} cells were fully permissive for growth at 20°C, semi-permissive at 23°C and incapable of growth at 25°C (Figure 4). This lethality may be explained either by the requirement of Ku for residual Cdc13p function, or by DNA damage produced in the absence of two redundant functions, possibly through the deregulation of 3′ end formation.

Crosses between *hdf* and *cdc13-2*^{est4} mutants generated a distinct synthetic lethal effect. Telomere loss in *cdc13-2*^{est4} strains normally leads to cell death after 50–75 generations [12]. In contrast, cells containing *hdf1* (or *hdf2*) and *cdc13-2*^{est4} mutations formed microcolonies that ceased growth after only approximately 10 generations. No growth of microcolonies was observed after subsequent plating onto YPD medium at either 23°C or 25°C (Figure 4).

One interpretation of the *hdf cdc13-2*^{est4} phenotypes is that the two mutants both diminish telomere size, increasing the rate of telomere loss and, hence, senescence. This interpretation is unlikely as double mutants containing a novel mutation, *trd1*, which also results in the rapid loss of

Figure 4



Synthetic lethality between *hdf* and *cdc13* alleles. **(a,b)** Diploids heterozygous for (a) *hdf1* and *cdc13-1^{ts}* or (b) *hdf2* and *cdc13-1^{ts}* were sporulated and dissected at the semi-permissive temperature of 23°C, and three representative tetrads were plated onto YPD media at 25°C. Double mutants displayed a cessation of growth within several generations. The identity of double mutants was confirmed by their ability to grow at 25°C in the presence, but not absence, of an exogenously introduced copy of the wild-type *HDF1* gene (pRS314–HDF1). The *hdf1* mutation was monitored by uracil prototrophy, whereas the presence of the *hdf2* allele was scored by adenine prototrophy and temperature sensitivity at 37°C. The presence of *cdc13-1^{ts}* was demonstrated by the inability of cells to grow at 30°C, but ability to grow at 25°C. Mutant alleles are indicated by –, and wild-type alleles are indicated by +. The white color is the consequence both of an *ADE2*-marked telomere and the *hdf2::ADE2* disruption. **(c,d)** Diploids heterozygous for (c) *hdf1* and *cdc13-2^{est4}* or (d) *hdf2* and *cdc13-2^{est4}* were sporulated and dissected at 23°C, and three representative tetrads were plated onto YPD media at 25°C. Double mutants on dissection plates displayed microcolonies that ceased growth following approximately 10 generations. No further growth of microcolonies was observed after plating onto YPD. The identity of double mutants was both inferred (as indicated by the parentheses) from the phenotypes of surviving colonies and by their absence in tetrads derived from *hdf1* × *cdc13-2^{est4}* diploids containing a wild-type *HDF1* on a centromeric plasmid (pRS314–HDF1). The presence of *hdf1* and *hdf2* mutations was determined as described above. The presence of *cdc13-2^{est4}* was demonstrated by either senescence or Southern blot analysis. The designations for mutant and wild-type phenotypes and the source of the *ADE2* genes in the cross are as described for (a,b).

telomeric sequences (data not shown), and *cdc13-2^{est4}* do not confer a synthetic lethal phenotype. This suggests that Ku interacts uniquely with Cdc13p. A second possibility is that loss of Ku may result in a synergistic loss of telomeric sequences, possibly driven by increased rates of RDE in conjunction with decreasing telomere size [7]. Interestingly, survivors of synthetic lethality, representing intragenic suppressors of *cdc13-1^{ts}*, arise at a high frequency and invariably contain telomeres that are several kilobases larger than wild type (data not shown), raising the intriguing possibility that increased telomere length abrogates synthetic lethality.

The phenotypes of both *hdf* alleles argue that Ku acts at the telomere, perhaps targeted by the association of Hdf1p with the telomere-associated silencing factor Sir4p [13]. The heterodimer, possibly in conjunction with Cdc13p, is then likely to protect the telomere against recombinogenic and nucleolytic activities, thus serving to maintain the integrity of the telomere.

Supplementary material

A detailed Materials and methods section is published with this paper on the internet.

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Supplementary material

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Materials and methods

Plasmid constructions

The plasmid pRS314–HDF1 was produced by cloning a PCR product of *HDF1* sequences doubly digested with *Xho*I and *Eco*RI, followed by ligation into the *Xho*I/*Eco*RI-cleaved polylinker of pRS314. Functionality of the gene was ascertained by the ability of the plasmid to complement both the temperature sensitivity and telomere-shortening defects of *hdf1* strains. The plasmid pRS314–HDF2 was produced by cloning a PCR product of *HDF2* sequences digested with *Spe*I, and ligated into the *Spe*I-cleaved polylinker of pRS314. Functionality was confirmed as described for pRS314–HDF1. To derive the disruption allele *hdf2::ADE2*, pRS314–HDF2 was cleaved with *Bcl*I, which retains a total of 600 bp distributed at the 5′ and 3′ ends of the gene. This cleaved vector was then ligated to a 3.6 kb *Bam*HI fragment carrying the *ADE2* gene, generating the plasmid pRS314–*hdf2::ADE2*.

Yeast strains

All yeast strains were isogenic to W303. The *hdf1* strain, containing an *hdf1::URA3* disruption (W303a/hdf1), was a gift of Feldmann and Winnacker [1]. To construct the *hdf2* strain (W303a/hdf2), the plasmid pRS314–*hdf2::ADE2* was digested with *Not*I and *Pst*I, releasing the disrupted *hdf2* gene, and the fragment was transformed into W303a. Transformants were confirmed both by Southern analysis and by the generation of temperature sensitivity and short telomere size.

Strain AJL 506 was derived from a cross between W303a/hdf1 and BL26-6a, the latter carrying an elongated *ADE2*-marked telomere at the left end of chromosome VII [16]. Wild-type and *hdf1* spore colonies containing an elongated *ADE2*-marked telomere were used for the experiments described in Figure 3 of the paper.

Strains containing single-copy alleles of *cdc13-1*^{ts} (W303a/cdc13-1^{ts}) and *cdc13-2*^{est4} (W303a/cdc13-2^{est4}) were generated by standard methods of two-step gene replacement using the plasmids pVL451 and pVL437 (kind gifts of Victoria Lundblad), respectively. Integrants were confirmed by phenotypic and Southern analyses. To prevent any rearrangements associated with the senescence phenotype, only *cdc13-2*^{est4} cells that had not been subcultured were used for crosses. Standard crosses between *hdf* and *cdc13* strains were used to generate double mutant strains.