Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres

Thierry Laroche*, Sophie G. Martin*, Monica Gotta*, Hazel C. Gorham†, Fiona E. Pryde†, Edward J. Louis† and Susan M. Gasser*

The mammalian Ku70 and Ku86 proteins form a heterodimer that binds to the ends of double-stranded DNA *in vitro* and is required for repair of radiation-induced strand breaks and V(D)J recombination [1,2]. Deletion of the *Saccharomyces cerevisiae* genes *HDF1* and *HDF2* – encoding yKu70p and yKu80p, respectively – enhances radiation sensitivity in a *rad52* background [3,4]. In addition to repair defects, the length of the TG-rich repeat on yeast telomere ends shortens dramatically [5,6]. We have shown previously that in yeast interphase nuclei, telomeres are clustered in a limited number of foci near the nuclear periphery [7], but the elements that mediate this localization remained unknown. We report here that deletion of the genes encoding yKu70p or its partner yKu80p altered the positioning of telomeric DNA in the yeast nucleus. These are the first mutants shown to affect the subnuclear localization of telomeres. Strains deficient for either yKu70p or yKu80p lost telomeric silencing, although they maintained repression at the silent mating-type loci. In addition, the telomere-associated silencing factors Sir3p and Sir4p and the TG-repeat-binding protein Rap1p lost their punctate pattern of staining and became dispersed throughout the nucleoplasm. Our results implicate the yeast Ku proteins directly in aspects of telomere organization, which in turn affects the repression of telomere-proximal genes.

Addresses: *Swiss Institute for Experimental Cancer Research, Chemin des Boveresses 155, CH-1066 Epalinges/Lausanne, Switzerland. †Yeast Genetics, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, UK.

Correspondence: Susan M. Gasser
E-mail: sgasser@eliot.unil.ch

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Results and discussion

Ku proteins are required for efficient telomeric silencing

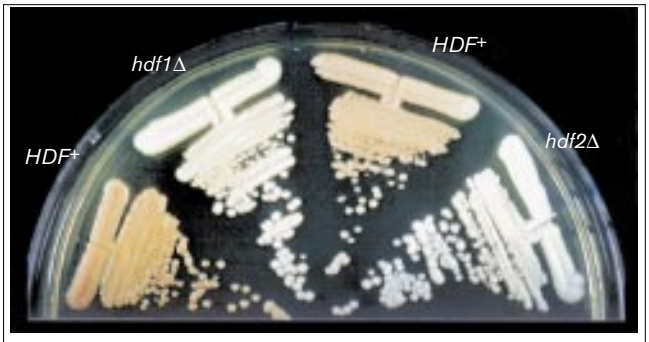
In the budding yeast *S. cerevisiae*, genes inserted near the repetitive TG-rich telomeric repeat succumb to position-dependent repression of transcription (called telomere position effect, TPE) [8]. To assay telomere-proximal silencing, we monitored the expression of an *ADE2* gene inserted near the telomeric repeat of chromosome V-R. In cells with wild-type copies of *HDF1* and *HDF2*, *ADE2* was repressed and cells accumulated a red pigment. If either *HDF1* or *HDF2* was deleted (*hdf1*Δ or *hdf2*Δ), colonies were white, indicating extensive disruption of the chromatin structure that represses *ADE2* at the marked telomere (Figure 1). This phenomenon could be assessed quantitatively by monitoring the growth of strains carrying *URA3* adjacent to the telomeric repeat on medium containing 5-fluoro-orotic acid (5-FOA), a compound that is toxic for cells expressing *URA3*. The *hdf1*::*kanMX4* strain grown at 30°C showed a 7×10^3-fold drop in repression, which was only three times less than the maximal derepression observed in a strain deleted for *SIR3* (2×10^4-fold; Figure 1). To see whether the derepression simply reflects the shortened telomeric repeat found in *hdf* strains, we monitored TPE in a *tel1* mutant, which shortens telomeres to nearly the same extent as *hdf* mutants [9]. As previously reported, mutation of *tel1* only slightly derepresses TPE ([8,10] and data not shown).

Surprisingly, the severe derepression brought about by *hdf* deletion was specific for telomeric silencing. Using a quantitative mating assay, we monitored expression of the homologous mating-type locus *HMLα*, which in wild-type cells is silenced by the same Sir-dependent mechanism that represses telomeres [8]. In isogenic *HDF1*$^+$ and *hdf1*::*kanMX4* strains, the latter mate with an efficiency 76% of that of the congenic wild-type rate, compared with the efficiency of < 0.01% detected in a *sir3*-deficient strain (Figure 1). Similarly, strains carrying either an *hdf2* or a *tel1* disruption mated with near wild-type efficiency (data not shown). Thus, despite the profound effect of *hdf* mutation on TPE, yKu70p and yKu80p are not general components of silent chromatin.

The perinuclear arrangement and clustering of telomeres are affected in Ku-deficient strains

We have shown previously that in wild-type cells, Rap1p and the Sir proteins are found in six to eight immunoreactive foci that co-localize with subtelomeric DNA, as detected by fluorescence *in situ* hybridization (FISH) with a probe containing both TG-rich repeats and the subtelomeric Y′ repeat (Y′ FISH) [7]. These foci reflect the clustering of the 64 telomeres present in diploid interphase nuclei [11,12], and about 70% of the foci are found within a perinuclear rim measuring one-tenth the nuclear radius [11]. In confocal images this perinuclear organization of clustered telomeres appeared as a ring-like distribution of bright spots (Figure 2a).

Figure 1



	Mat **a** mating efficiency	*Tel::URA3* 5-FOA resistance
HDF+	1.0	0.7
hdf1Δ	0.76	0.00016
sir3Δ	0.001	0.00004

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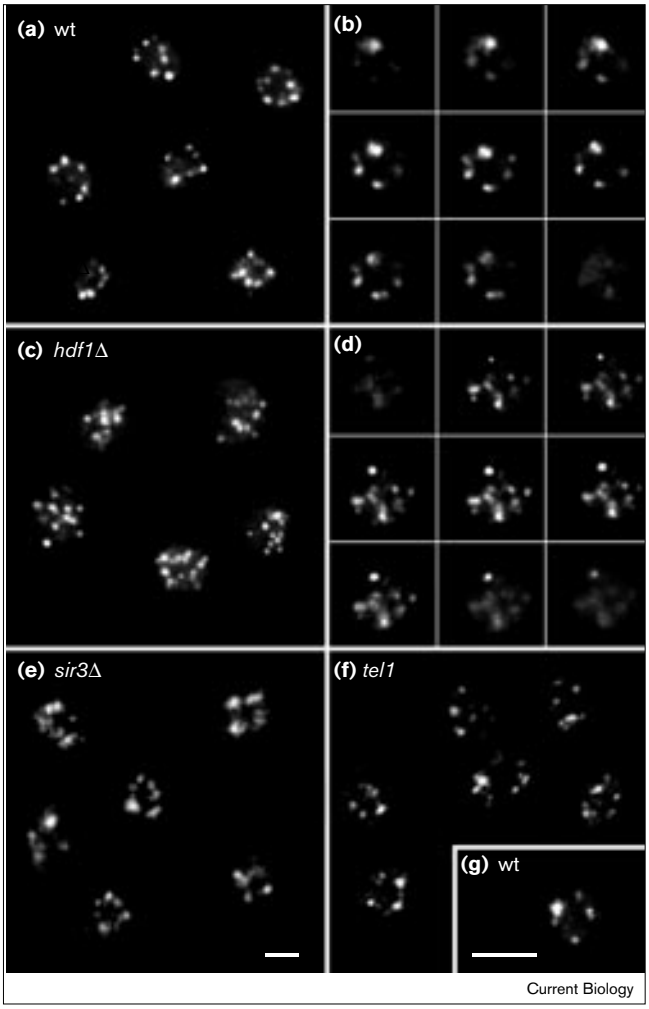
Silencing at telomeres, but not at the mating-type loci, is lost in *hdf* mutants. A haploid *HDF+* strain (GA426) and the isogenic *hdf1::kanMX4* (*hdf1Δ*, GA911) and *hdf2*::*URA3* (*hdf2Δ*, GA912) strains carrying *ADE2* adjacent to the telomere of chromosome V-R [8] were streaked onto YPD medium and allowed to grow for three days at 30°C. Transcriptional repression of *URA3* integrated at the VIIL telomere (*Tel*::*URA3*) was determined by measuring resistance to 5-FOA from a minimum of four colonies [8]. An isogenic *sir3::kanMX4* (*sir3Δ*) strain was used to measure full derepression. Mating efficiencies were determined by a quantitative mating assay [25] using the wild-type (*HDF1+*, GA180) strain and its congenic *hdf1*::*kanMX4* (*hdf1Δ*, GA852) strain with an α tester strain, PT2.

Figure 2



Current Biology

Perinuclear clustering of telomeres is affected in an *hdf1Δ* strain as detected by Y′ FISH, whereas it is maintained in *sir3Δ* and *tel1* strains. Diploid strains that are **(a,b)** *HDF+* (wt; GA229), **(c,d)** *hdf1*::*kanMX4* (*hdf1Δ*; GA773) and **(e)** *sir3*::*HIS3* (*sir3Δ*; GA192), as well as haploid **(f)** *tel1-1* (*tel1*; KR75-2A) and **(g)** *TEL1+* (wt; AHFA8L) cells were hybridized with a digoxigenin–dUTP-labeled Y′ probe [7], detected by fluorescein-coupled anti-digoxigenin F(ab) fragments. (a,c,e–g) Optical sections (0.3 μm) at the mid-section of the nuclei. (b,d) Nine focal sections of 0.25 μm, from the bottom to the top of a single nucleus. Nuclear integrity was monitored by three-dimensional optical sectioning (b,d), total nuclear volume, total detectable FISH signal per cell (see Table 1) and nuclear pore staining [7]. Quantification of the number and localization of Y′ spots is shown in Table 1. Scale bars indicate 2 μm.

FISH was performed with telomeric probes on intact yeast spheroplasts from a homozygous diploid *HDF1+* strain and an isogenic *hdf1*::*kanMX4* mutant. In contrast to the typical ring of telomeric foci detected in wild-type cells, in the *hdf1Δ* mutant we observed a striking redistribution of the telomeric signals to a more random or dispersed organization throughout the nucleoplasm (Figure 2c). Optical sectioning confirmed a shift from a ring-like distribution of telomeric foci in wild-type cells (Figure 2b) to a more dispersed distribution in *hdf1Δ* cells (Figure 2d). Similar observations have been made for a haploid *hdf2*::*HIS3* mutant (data not shown), confirming that telomere clustering requires the intact Ku heterodimer.

We have scored the number of cells that have all their telomeric foci in a ring-like arrangement at the nuclear periphery, as opposed to cells that have both internal and peripheral foci, after double-labeling with nuclear pore immunostaining and Y′ FISH (Table 1). As shown in Table 1, only 22% of the *hdf1*::*kanMX4* nuclei had telomeric signals exclusively in a perinuclear ring, as opposed to 44% of the wild-type nuclei. Besides an altered positioning of telomeres, the number of discrete Y′-labeled foci in the *hdf1*::*kanMX4* strain increased by roughly 40% over the wild-type strain, showing on average 9.0 ± 0.3 foci, rather than 6.5 ± 0.4 foci per midnuclear section (Table 1). Individual telomeric signals in the mutant strains were often weaker and of smaller surface area, consistent with the partial dispersion of telomere clusters upon loss of yKu70p. We do not know what confers the residual degree of telomere clustering.

Significantly, neither an increase in the absolute number of foci nor foci delocalization was observed in a *sir3*-deficient strain, although individual foci became larger and

more diffuse (Figure 2e). In a *tel1*-deficient strain, despite a loss of TG repeats, telomeres remained in discrete clusters at the nuclear periphery (Figure 2f,g). In all FISH assays, we monitored the integrity of the nuclei by three-dimensional confocal sectioning, nuclear pore staining and measurement of the nuclear diameter. Because these measurements and the average intensity of the Y′ signal per nucleus were similar in both mutant and wild-type strains (Table 1, Figure 2b,d), we conclude that the absence of yKu70p or yKu80p alters the spatial distribution of telomeres within yeast nuclei. This is not due to the loss of TPE nor to loss of TG-repeat length, because telomeres remained clustered in *sir3* and *tel1* mutants.

Sir proteins are redistributed in the absence of Ku

Consistent with the loss of telomeric silencing and the reduction of telomere clustering, Rap1p, Sir3p and Sir4p proteins were delocalized in the *hdf1*::*kanMX4* mutant strain, showing a diffuse nuclear staining pattern (Figure 3d–f) distinct from the discrete foci observed in repression-competent strains (Figure 3a–c). Intriguingly, the diffuse staining pattern in these mutant strains was occasionally interrupted by one or two residual bright foci (Figure 3d–f), the chromosomal identity of which remains unclear. An identical loss of the focal staining pattern for Rap1p and Sir3p was obtained in *hdf2*, but not in *tel1*, mutants (data not shown).

Telomeric silencing is exquisitely sensitive to the dosage of Sir proteins, which are targeted to telomeric clusters through interaction with the Rap1p carboxy-terminal domain [13–16]. As in *hdf* mutants, Sir proteins become dispersed throughout the nucleoplasm in Sir-deficient strains, although telomeres remain clustered [7,12,14,17] (Figure 2e). Thus, although insufficient in itself to confer repression, telomere clustering is thought to promote TPE by creating a subcompartment within the nucleus that maintains high concentrations of silencing factors [18, 19].

We propose two mutually non-exclusive ways in which the yeast Ku proteins might influence telomeric silencing. First, it was shown that the Ku heterodimer can bind free DNA ends and bring these together, forming DNA loops *in vitro* [20]. Thus, if the yeast Ku heterodimer were bound directly to chromosomal ends, it might mediate the clustering of telomeres either by binding a component of the nuclear envelope or by binding itself. The resulting cluster of TG repeats would increase the local concentration of Rap1p-binding sites, which in turn would enhance the local concentration of Sir proteins necessary for silencing. Our data suggest that telomere–telomere interactions can occur in the absence of assembled Sir complexes, but that the interactions are compromised in the absence of yeast Ku (Figure 2) [7].

Recent results from two-hybrid assays indicate that yKu70p interacts with a coiled-coil region in the carboxyl terminus of Sir4p [21]. Thus, a second function of the yeast Ku heterodimer might be to help recruit Sir4p to telomeres by protein–protein interaction. It is important to note that even if yKu70p can bind Sir4p, its ability to do so is not sufficient to promote TPE, as telomeric repression requires the Sir-binding domain of Rap1 [13,14,19]. In support of a direct role for yKu70p in Sir protein recruitment, however, we observed that the overexpression of *SIR3* and *SIR4* does not restore TPE to wild-type levels in the *hdf1*::*kanMX4* mutant, although it does improve silencing at an internal silencing cassette ([18] and data not shown). Moreover, data from the Jackson laboratory [10] show that the loss of TPE associated with *hdf* mutation does not apply to other mutants in the double-strand break repair pathway.

Consistent with our suggestion that the yeast Ku heterodimer is implicated in nuclear organization, previous reports have noted abnormal nuclear morphology in Ku86-deficient Chinese hamster ovary cells, and irregular interactions

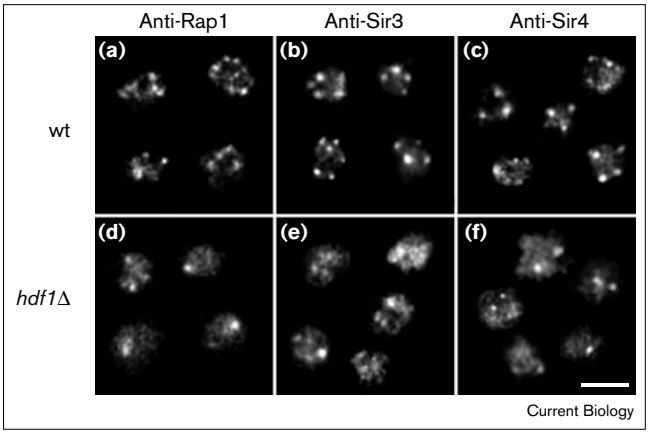
Table 1

Telomeric foci increase in number and are less peripheral in an *hdf1Δ* strain.

Strain	Nuclear diameter (μm)	Intensity of Y′ FISH per nucleus	Y′ FISH foci per nucleus	Nuclei with only peripheral foci (%)	Nuclei with both internal and peripheral foci (%)
HDF+	2.3 ± 0.3	26.2	6.5 ± 0.4	44	56
hdf1Δ	2.3 ± 0.4	24.2	9.0 ± 0.3	22	78

Isogenic diploid strains that are *HDF*+ (GA229) or *hdf1Δ* (*hdf1*::*kanMX4*; GA773) were hybridized with a digoxigenin–dUTP-labeled Y′ probe as in Figure 2. The average diameter (in μm) of propidium-iodide-stained nuclei was averaged from 24 measurements. The average Y′ FISH signal per nucleus was calculated from untreated signals of over 100 nuclei under identical scanning settings and is given in arbitrary units. Quantitation of the number of foci per nucleus was done in quadruplicate on over 100 cells of each cell type, after normalization of the signal for each individual nucleus and subtraction of a 15% threshold value. Quantitation of the fraction of cells containing Y′ foci exclusively in a perinuclear ring included over 100 nuclei of each cell type and is presented as the percentage of total nuclei scored. The distribution of the telomeric foci was assessed on cells double-stained for Y′ foci and for nuclear pores to define the nuclear periphery. From three independent experiments, the distribution of individual Y′ FISH foci between the nuclear periphery (one-tenth of the diameter of the nucleus) and the interior was analysed, revealing 68 ± 5% peripheral foci and 32 ± 5% internal for the wild-type diploid, and 51 ± 2% peripheral foci and 49 ± 2% internal for the *hdf1*::*kanMX4* diploid.

Figure 3



Peripheral focal staining of Rap1p, Sir3p and Sir4p is lost in a *hdf1*::*kanMX4 (hdf1Δ)* strain. **(a–c)** Isogenic wild-type (wt; GA229) and **(d–f)** *hdf1*::*kanMX4* (*hdf1Δ*; GA773) diploid cells were stained with affinity-purified (a,d) anti-Rap1, (b,e) anti-Sir3 or (c,f) anti-Sir4 antibodies, which were detected by Texas-red-conjugated secondary antibodies. Scale bar indicates 2 μm.

between the nuclear lamina and chromatin in nuclei assembled in Ku70-depleted *Xenopus* egg extracts [22,23]. Immunolocalization of vertebrate Ku70 suggests a perinuclear staining pattern [24]. Although yeast has no detectable nuclear lamina, analogous coiled-coil interactions may exist between Sir4p, the yeast Ku heterodimer, and unidentified nuclear envelope components. An independent indication that yeast Ku proteins influence telomere organization within the nucleus comes from the recovery of an *hdf2* mutant in a screen for increased recombination between subtelomeric and internal chromosomal regions (F.E.P., H.C.G. and E.J.L., unpublished observations). Future studies will examine the possibility that the yeast Ku heterodimer also serves to localize double-strand breaks to sites of efficient repair [21], which may also be found at the nuclear periphery.

Supplementary material

All yeast genotypes and additional methodological detail are published with this paper on the internet.

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Supplementary material

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Materials and methods

Yeast strains

Yeast strains were GA192 (*MAT***a**/*MAT*α, *ade2-1/ADE2, trp1-1/TRP1, his3-11,15/his3, ura3-1/ura3-52, leu2-3,112/LEU2, LYS2/lys2-6, can1-100/CAN1, sir3*::*TRP1/sir3*::*LYS2*), GA229 (*MAT***a**/*MAT*α, *ade2-1/ade2-1, trp1/trp1, his3/HIS3, ura3-1/ura3-1, leu2-3,112/leu2-3,112*), GA773 (isogenic to GA229 but *hdf1*::*kanMX4/hdf1*::*kanMX4*), GA426 (MAT**a**, *ade2Δ*::*hisG*, *can1Δ*::*hisG*, *trp1Δ*, *his3-11*, *ura3-52*, *leu2*, *VR*::*ADE2-Tel*), GA911 (isogenic to GA426 but *hdf1*::*kanMX4*), GA912 (isogenic to GA426 but *hdf2*::*URA3)*, GA822 (isogenic to GA426 but *sir3*::*TRP1*), GA180 (*MAT***a**, *ade2-1*, *trp1-1*, *his3-11*,*15*, *can1-100*, *ura3-1*, *leu2-3*, *112*), the congenic GA852 (*MAT***a**, *ade2-1*, *trp1-1*, *his3-11*,*15*, *ura3-1*, *leu2-3*, *112, hdf1*::*kanMX4),* PT2 (*MAT*α, *hom3*), AHFA8L (*MAT***a**, *ade2, ade1*, *ade8Δ-SX, tyr1*, *his7*, *ura3*, *leu2Δ-RC, lys2, gal1)*, and KR75-2A (isogenic to AHFA8L, but *tel1-1*, *cdc27*).

Repression assays

For each repression assay, several different colonies were tested individually and the mean and variance calculated using the standard fluctuation test. The frequency of 5-FOA-resistant cells due to repression of the telomeric *URA3* gene is calculated after 3–4 days' growth at 30°C [8]. Individual colonies from an *ade2-1* strain carrying an intact *ADE2* gene at the chromosome V-R telomere [8] were grown on YPD medium to score for pigment accumulation.

Immunofluoresence and FISH

Both immunofluorescence and Y′ FISH were performed as previously described [7,10,11,17]. Cells were grown at 30°C except for the *TEL1*$^+$ and *tel1-1* strains, which were grown at 24°C, due to the linked *cdc27* allele. An anti-nuclear pore monoclonal was used (Mab144, Berkeley Antibody) to monitor nuclear integrity and the nuclear periphery. Standardized conditions for image capture and subtraction of a background value using a Zeiss Axiovert 100 microscope with Zeiss Laser Scanning Microscope 410, with a 63 × Plan-Apochromat objective (1.4 oil) was previously described [7].