***RLF2*, a subunit of yeast chromatin assembly factor-I, is required for telomeric chromatin function in vivo**

Shinichiro Enomoto,¹ Paul D. McCune-Zierath,² Maryam Gerami-Nejad,¹ Mark A. Sanders,³ and Judith Berman^{1,2,4}

¹Department of Plant Biology, ²Plant Biological Sciences Graduate Program, and ³College of Biological Sciences Imaging Center, University of Minnesota, Twin Cities Campus, St. Paul, Minnesota 55108 USA

In the yeast *Saccharomyces cerevisiae*, telomere repeat DNA is assembled into a specialized heterochromatin-like complex that silences the transcription of adjacent genes. The general DNA-binding protein Rap1p binds telomere DNA repeats, contributes to telomere length control and to telomeric silencing, and is a major component of telomeric chromatin. We identified Rap1p localization factor 2 (*RLF2*) in a screen for genes that alleviate antagonism between telomere and centromere sequences on plasmids. In *rlf2* mutants, telomeric chromatin is perturbed: Telomeric silencing is reduced and Rap1p localization is altered. In wild-type cells, Rap1p and telomeres localize to bright perinuclear foci. In *rlf2* strains, the number of Rap1p foci is increased, Rap1p staining is more diffuse throughout the nucleus, Rap1p foci are distributed in a much broader perinuclear domain, and nuclear volume is 50% larger. Despite the altered distribution of Rap1p in *rlf2* mutant cells, fluorescence in situ hybridization to subtelomeric repeats shows that the distribution of telomeric DNA is similar in wild-type and mutant cells. Thus in *rlf2* mutant cells, the distribution of Rap1p does not reflect the distribution of telomeric DNA. *RLF2* encodes a highly charged coiled-coil protein that has significant similarity to the p150 subunit of human chromatin assembly factor-I (hCAF-I), a complex that is required for the DNA replication-dependent assembly of nucleosomes from newly synthesized histones in vitro. Furthermore, *RLF2* is identical to *CAC1*, a subunit of yeast chromatin assembly factor-I (yCAF-I) which assembles nucleosomes in vitro. In wild-type cells, epitope-tagged Rlf2p expressed from the *GAL10* promoter localizes to the nucleus with a pattern distinct from that of Rap1p, suggesting that Rlf2p is not a component of telomeric chromatin. This study provides evidence that yCAF-I is required for the function and organization of telomeric chromatin in vivo. We propose that Rlf2p facilitates the efficient and timely assembly of histones into telomeric chromatin.

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In *Saccharomyces cerevisiae*, most genes replicate early in S phase and are in an open, potentially active state that consists largely of 10 nm chromatin fiber (Grunstein et al. 1995). Although the size of the yeast genome makes cytological detection of the chromatin condensation state very difficult, there is much evidence that yeast telomeres, the chromosomal termini, are packaged into domains with the properties of heterochromatin. Telomere proximal sequences replicate late in S phase (McCarroll and Fangman 1988) are largely inaccessible to methyltransferases (Gottschling 1992), are localized near the nuclear periphery (Gotta et al. 1996), and are subject to epigenetic repression (Gottschling et al. 1990). In addition, like the silent HM loci (Kimmerly and Rine

1987), telomere sequences are able to stabilize circular plasmids (Longtine et al. 1992).

Yeast telomeres are composed of long (300–500 bp) double-stranded tracts of telomere repeat sequence (TG_{1–3}/C_{1–3}A) that are bound by repressor/activator protein 1 (Rap1p) at a density of once per 18–40 bp (Longtine et al. 1989; Gilson et al. 1993). Rap1p binds to TG_{1–3}/C_{1–3}A with high affinity in vitro (Buchman et al. 1988; Longtine et al. 1989; Gilson et al. 1993) and in vivo (Conrad et al. 1990; Wright et al. 1992). Rap1p is important for telomere function: Mutations in *RAP1* cause alterations in telomere length (Lustig et al. 1990) and in the degree of telomeric silencing (Liu et al. 1994; Buck and Shore 1995).

Rap1p, together with the silent information regulator proteins Sir2p, Sir3p, and Sir4p, as well as histone H3 and histone H4, are required for the maintenance of telo-

⁴Corresponding author.

E-MAIL judith@biosci.cbs.umn.edu; FAX (612) 625-1738.

meric silencing (Aparicio et al. 1991). The fact that mutations in the histone H3 and H4 amino termini affect silencing demonstrates a link between silencing and chromatin structure (for review, see Grunstein et al. 1995). Like the heterochromatin of *Drosophila* and the inactive X chromosome in mammalian cells, transcriptional silencing in yeast is associated with reduced acetylation of the amino termini of nucleosomal histones (Braunstein et al. 1993). Genetic and biochemical studies indicate that Sir3p and Sir4p associate with histones H3 and H4 (Johnson et al. 1990; Chien et al. 1991; Hecht et al. 1995). In two-hybrid experiments Rap1p interacts with Sir3p, Sir4p, and Rif1p (Hardy et al. 1992; Moretti et al. 1994) and Sir3p and Sir4p interact with themselves and with one another (Chien et al. 1991; Moretti et al. 1994). Sir2p and Sir4p also interact in vitro (Moazed and Johnston 1996). Genetic studies provide additional evidence for the interaction of Rap1p and Sir3p (Liu et al. 1994; Cockell et al. 1995; Lustig et al. 1996).

In wild-type yeast cells, Rap1p localizes to punctate perinuclear foci that are thought to reflect the localization of telomeres (Klein et al. 1992). In wild-type cells, Sir3p and Sir4p localize to perinuclear foci that are similar in distribution to Rap1p foci. The punctate appearance of Rap1p is dependent on *SIR3* and *SIR4* (Palladino et al. 1993) and is partially disrupted by mutations in the amino termini of histone H4 and histone H3 (Hecht et al. 1995). The localization of Sir3p is dependent on *RAP1* and *SIR4* as well (Cockell et al. 1995). Immunofluorescence/fluorescence in situ hybridization (FISH) studies of Rap1p, Sir3p, Sir4p, and sub-telomeric Y' repeats are consistent with the idea that in wild-type cells Rap1p, Sir3p, and Sir4p are components of a subtelomeric DNA-protein complex (Gotta et al. 1996). Taken together, there is considerable evidence indicating that Rap1p, Sir2p, Sir3p, Sir4p, histones H3 and H4 are likely to be physically associated with one another in a large complex associated with TG₁₋₃/C₁₋₃A and/or telomere-adjacent DNA.

When a long TG₁₋₃/C₁₋₃A tract is inserted into circular replicating plasmids (TEL plasmids), the plasmids segregate more efficiently (Longtine et al. 1992). When a centromere sequence is added to TEL plasmids, the resulting TEL+CEN plasmids become very unstable, segregating as if neither the centromere sequence nor the telomere sequence is functional (Enomoto et al. 1994). The stability of TEL plasmids and the instability of TEL+CEN plasmids, termed TEL+CEN antagonism, requires genes such as *RAP1*, *SIR2*, *SIR3*, and *SIR4*, which act at chromosomal telomeres (Longtine et al. 1993; Enomoto et al. 1994). Our working hypothesis is that the double-stranded telomere sequences on these plasmids are assembled into a structure that resembles the chromosomal TG₁₋₃/C₁₋₃A tracts.

Here we describe *RLF2*, a gene identified in a screen for mutants that alleviate TEL+CEN antagonism. We demonstrate that Rlf2p is required for the formation of the telomeric heterochromatin-like state: *rlf2* mutants have reduced telomeric silencing and altered Rap1p localization. By comparing the localization of Rap1p by indirect

immunofluorescence microscopy and the localization of telomeric DNA by FISH in both wild-type and mutant strains, we demonstrate that Rap1p localization does not reflect the distribution of telomeric DNA in *rlf2* strains. In addition, we demonstrate that Rlf2p expressed from the *GAL10* promoter is a nuclear protein that localizes in a pattern distinct from Rap1p. Interestingly, *RLF2* encodes the large subunit of yeast chromatin assembly factor-I (yCAF-I), an activity that assembles nucleosomes onto replicated DNA in vitro (Kaufman et al., this issue). Human chromatin assembly factor-I (hCAF-I) catalyzes the assembly of newly synthesized histones H3 and H4 onto recently replicated DNA (Smith and Stillman 1989, 1991a,b; Kaufman et al. 1995; Verreault et al. 1996) and is localized to replication foci (Krude 1995). This study demonstrates that mutation of a subunit of yCAF-I perturbs the organization and function of telomeric chromatin in vivo.

Results

Isolation of the *rlf2-1* mutant

In an effort to identify genes that affect telomere structure and function, we screened for mutants that alleviate TEL+CEN antagonism (Enomoto et al. 1994). TEL+CEN antagonism can be detected visually by observing the colony sectoring pattern of an *ade2* yeast strain carrying

Figure 1. Colony-sectoring assay for TEL+CEN antagonism. All strains carrying TEL+CEN plasmid pSE49K. (*RLF2*), YJB203; (*rlf2-1*), YJB463; (*rlf2-1*+pRLF2) YJB463 (pSE58AJ); (*rlf2Δ1*), YJB469. (Bottom) YJB463(*rlf2-1*) carrying T7-RLF2 on pME370 pregrown in SDC(-Leu)+raffinose+galactose (Gal) or SDC(-Leu)+glucose (Glu) and then streaked on SDC+glucose. Red colonies represent plasmid loss that occurred during pregrowth.

a TEL+CEN plasmid with an *ADE2* marker [e.g., YJB203 (pSE49K)]. Colonies of wild-type cells produce many red sectors because TEL+CEN antagonism leads to loss of the TEL+CEN plasmid (Fig. 1). In the mutant, reduced TEL+CEN antagonism was visualized as a reduction in the red sectoring of colonies carrying a TEL+CEN plasmid (Fig. 1). Mutants of interest were identified as those carrying chromosomal mutations that did not affect CEN-plasmid maintenance (see Materials and Methods for details). Because genes required for TEL+CEN antagonism, such as *SIR3* and *SIR4*, are also required for normal Rap1p localization (Palladino et al. 1993), we examined the localization of Rap1p in these mutants by immunofluorescence microscopy (using antibodies raised against Rap1p). Mutant 14 (strain YJB463) appeared to have an altered distribution of Rap1p (see below). The mutated gene responsible for the phenotypes of mutant 14 is now called *RLF2* (Rap1 localization factor-2); The original mutant allele is *rlf2-1*.

Cloning RLF2

The *RLF2* gene was cloned from a genomic DNA library by its ability to restore TEL+CEN antagonism in YJB463. Clone pSE58A restored TEL+CEN antagonism to a level comparable to wild type when visualized as red/white sectoring (Fig. 1). pSE58A contained a 12.5-kb insert (Fig. 2A). A 2-kb *Bgl*III–*Bam*HI subclone (pSE58AJ) (Fig. 2A) was the minimal region required to restore TEL+CEN antagonism in YJB463. DNA sequencing of pSE58AJ identified one large open reading frame (ORF) of 606 amino acids. To test whether this 606-amino-acid ORF encoded the TEL+CEN antagonism function, we engineered frameshift mutations at amino acid 44 or amino acid 88 (*rlf2-2* and *rlf2-3*; Fig. 2A). Neither *rlf2-2* nor *rlf2-3* could restore TEL+CEN antagonism to YJB463, indicating that the 606-amino-acid ORF is responsible for complementation of the TEL+CEN antagonism in YJB463.

To test whether the 606-amino-acid ORF is allelic to the mutation in YJB463, we integrated *LEU2* adjacent to the intact genomic ORF. *LEU2* and TEL+CEN antagonism segregated 18:0:0 (PD:NPD:TT), indicating that the 606-amino-acid ORF cloned on pSE58AJ corresponds to *RLF2*. *RLF2* maps to the right arm of chromosome XVI between *DSS4* (Moya et al. 1993) and *CDC54* and has been submitted to the Saccharomyces Genome Database (accession no. S57552) and to GenBank (accession no. U14731).

To ask whether *RLF2* is essential for yeast cell viability, we constructed a null allele (*rlf2-Δ1::LEU2*) by replacing all but the carboxy-terminal 181 amino acids of the *RLF2* ORF with the *LEU2* gene (Fig. 2A). Strains carrying this null allele are viable and display a loss of TEL+CEN antagonism in both sectoring assays (Fig. 1) and plasmid loss rate assays (data not shown).

rlf2-1, the mutant allele in YJB463, was recovered by gap repair (Rothstein 1991). DNA sequence analysis of the mutant clone identified a single nucleotide alter-

ation (AAA → TAA) that produces a nonsense mutation at codon 314 of the predicted protein (Fig. 2B).

RLF2 is required for telomeric silencing but not for telomere length control

We asked whether *RLF2* is required for telomeric silencing by measuring the degree of repression of a *URA3* gene inserted adjacent to terminal telomere repeats at the left end of chromosome VII (*URA3-TEL*) (Gottschling et al. 1990) in strains carrying wild-type and mutant alleles of *RLF2* or *SIR4* (Fig. 3). The degree of *URA3-TEL* repression was measured as the proportion of cells able to grow on 5-fluoro-orotic acid (5-FOA), which kills Ura⁺ cells (Gottschling et al. 1990). Consistent with previous studies (Aparicio et al. 1991), in wild-type strains the *URA3-TEL* gene was repressed in ~50% of the cells in the population, whereas in a strain carrying *URA3* at an internal locus or a strain lacking *SIR3* or *SIR4*, *URA3* was expressed in virtually all cells in the population (Fig. 3 and data not shown). In both *rlf2-1* and *rlf2-Δ1::ΔLEU2* mutants, *URA3-TEL* was more derepressed than in wild-type cells. However, the derepression of *URA3-TEL* in the *rlf2* mutants was not as dramatic as in *sir4* mutants: In *rlf2* mutants, the telomeric *URA3* gene was derepressed 500- to 5000-fold more than wild type (Fig. 3). Furthermore, in *rlf2* mutants, the growth of 5-FOA-resistant colonies was highly variable in both size and frequency (Fig. 3). 5-FOA resistant colonies were highly heterogeneous in size: Large colonies, comparable to those observed in wild-type strains (visible within 2 days) appeared at a frequency of 10⁻⁴ to 10⁻⁵; microcolonies continued to appear upon prolonged incubation of the plate at a frequency ranging from 10⁻³ to 10⁻¹. Kaufman et al. (this issue) observe similar variability in the size of 5-FOA resistant colonies in *cac1/rlf2*, *cac2*, and *cac3/msi1* mutant strains. The frequency of appearance of both types of 5-FOA resistant colonies also was variable among different isolates of isogenic *rlf2* mutant strains (Fig. 3), yet within an individual isolate population, the range of 5-FOA resistance and the proportion of colony types produced was similar. Tetrad analysis of the different isolates suggests that this variability is stochastic and is not linked to one or two segregating genes. Cells that grew on the 5-FOA plates (Fig. 3) had not lost or mutated the *URA3* gene: Both types of colonies subsequently grew on medium lacking uracil.

Because many mutations that influence telomeric silencing also influence telomere length, we asked whether *RLF2* contributes to telomere length control. We compared the length of telomere DNA repeat tracts in sister spores from tetrads in which the *rlf2* and *RLF2* alleles are segregating. Minor variation in telomere length normally occurs in wild-type sister spores (Shampay and Blackburn 1988). There was no consistent difference in telomere length in either *rlf2-1* or *rlf2-Δ1::ΔLEU2* mutants relative to wild type (Fig. 4), indicating that *RLF2* is not required for telomere tract length regulation.

Figure 2. Structure of *RLF2* and comparison of Rlf2p and hCAF1 p150. (A) Restriction and domain map of *RLF2* and *rlf2* mutant alleles: (HA) Hemagglutinin antigen of influenza virus; (T7) T7 tag (Novagen) epitope tag. (B) Comparison of Rlf2p and hCAF1 p150. The acidic, p60-binding, and PEST box domains are from Kaufman et al. (1995). The coil domain is predicted to have >85% likelihood of forming a coiled-coil structure (Lupas et al. 1991). Comparison of Rlf2p and hCAF1 p150 at the primary amino acid sequence is indicated as % identical (% similar) amino acids within each domain demarked by the vertical broken lines.

RLF2 is required for *Rap1p* localization

To compare differences in Rap1p distribution between wild-type and *rlf2* mutant strains, we performed immunofluorescence experiments using a polyclonal antibody against full-length Rap1p (anti-Rap1p) and analyzed the results by confocal laser microscopy (Fig. 5). In wild-type cells, Rap1p localizes within the nucleus, primarily to brightly staining, well-defined punctate foci that often appear in regions near the periphery and rarely appear near the center of the nucleus. In this report, we use the term perinuclear to describe this distribution of Rap1p in

wild-type cells. Our results are consistent with those of Gasser and co-workers (Klein et al. 1992) and are similar to our previous observations using wide field microscopy and an antibody raised against an amino-terminal peptide of Rap1p (Konkel et al. 1995). In *rlf2* mutants, Rap1p localization is altered relative to wild type (Fig. 5). In *rlf2* mutants, there appear to be more Rap1p foci, many of which are more diffuse and have a more scattered distribution throughout the nucleus than the foci in wild-type cells.

In the majority of the experiments, cells were also labeled with MAB414 (which recognizes a number of

Figure 3. Analysis of the variability of telomeric silencing in *rlf2* isolates. Serial dilutions of YJB487 (WT), YJB1250 (Ura⁺), and five different isolates of strains carrying the *rlf2*Δ1::LEU2 allele all in the YJB203 strain background (1–5), were plated on 5-FOA (large panel) and on SDC (complete) and SDC-Ura (-URA) plates. Panels along the right are enlargements of the 10⁴ dilutions from the 5-FOA plate.

nuclear pore proteins including p62; Davis and Blobel 1986). We analyzed digital images from the confocal microscope using quantitative approaches.

The spatial distribution of Rap1p foci was determined relative to the XYZ center of the nucleus (Fig. 6). The theoretical XYZ center of the nucleus was determined from the minimum and maximum diameter of the nuclear pore staining. We then determined the distance between the center of the nucleus and center of the local maximum intensity regions of the Rap1p foci. In wild-type nuclei, the median radius of nuclear pore staining was 1.05 μm (arrow in Fig. 6). Of the Rap1p foci, 79% were distributed in a region of the nucleus between 0.80 and 0.95 μm from the center, which represents 37% of

Figure 4. Telomere tract length in *rlf2* mutants. Southern blots of yeast genomic DNA digested with *Pst*I and probed with G₁₋₃T telomeric sequence. The horizontal lines represent sets of tetrads segregating for *RLF2* (+) and *rlf2*Δ1::LEU2 (-). The broad band at ~800 bp (arrow) includes 520 bp of Y' TAS and ~250 bp of G₁₋₃T sequence (Louis and Borts 1995). Similar results were obtained with segregation of *RLF2* and *rlf2*-1 tetrad progeny.

the total nuclear volume. The distribution of Rap1p spots in wild-type cells is not random: The hypothetical uniform distribution of a protein throughout the total nuclear volume (Fig. 6, lower line) or throughout the volume occupied by Rap1p staining (Fig. 6, upper line) is different from the actual distribution of Rap1p foci (Fig. 6, bars).

In *rlf2* nuclei, the median radius of nuclear pore staining was 1.25 μm (arrow in Fig. 6). In *rlf2* nuclei, 86% of the Rap1p spots occupy a much broader region (0.5 μm) of the nucleus, which corresponds to 64% of the nuclear volume.

In both wild-type and *rlf2* nuclei, there is a small zone of exclusion of Rap1p spots internal to the nuclear pore staining, suggesting that Rap1p is not tightly associated with the nuclear envelope. Consistent with this observation, immunoelectron microscopy of Rap1p indicates that it is not specifically associated with nuclear pores (M. Clark, unpubl.). Interestingly, recent observations of the distribution of the human telomere binding protein (TRF) demonstrated that clustered telomeres are not associated with the nuclear envelope (Ludérus et al. 1996).

Finally, comparison of wild-type and *rlf2* strains indicates that *rlf2* mutants have larger nuclei (1.87 μm vs. 2.17 μm, diam. of nuclear pore staining; 1.36 μm vs. 2.03

Figure 5. Immunofluorescence localization of Rap1p in wild-type and *rlf2* mutants. Confocal laser micrographs of the equatorial Z section of each nucleus. (*RLF2*), YJB213; (*rlf2*-1), YJB1213; (*rlf2*Δ1), YJB496.

Figure 6. Rap1p distribution in wild-type and *rlf2* strains. Histograms illustrate the number of Rap1p foci as a function of distance from the XYZ center of the nucleus. Arrows indicate the median radius of nuclear pore staining. Lower curves illustrate the theoretical distribution of Rap1p foci assuming uniform distribution throughout a sphere with radius 1.05 μm (wild type) and 1.25 μm (*rlf2*) mutants (the median radii of nuclear pore staining in these strains). Upper curves indicate the theoretical distribution of Rap1p foci assuming uniform distribution throughout a sphere with radius 0.75 μm (wild type) and 1.05 μm (*rlf2*) mutants (the mode of Rap1p distribution in these strains). The χ^2 goodness of fit values for Rap1p distribution relative to the upper line (232 and 107; wild type and *rlf2-1*, respectively) are all significantly different at $<1\%$ level, indicating that Rap1p foci are not distributed randomly. The χ^2 values can be regarded as a quantitative measure of fit to the model, indicating that *rlf2* mutants have Rap1p foci distributed more randomly than in wild-type nuclei. (Wild type) YJB213; (*rlf2-1*) YJB1213.

μm diam. of Rap1p staining). Slight differences between the radius measurements (above) and these diameter measurements are attributable to the different measurement approaches used (center of spots vs. edge of entire stained region). Similarly the nuclear DNA in living cells (stained with Syto14) had a larger diameter in *rlf2* than in wild-type cells (data not shown). Thus in both living and fixed cells, *rlf2* nuclei were larger than wild-type nuclei. The 15% increase in the diameter of *rlf2* nuclei represents a 52% increase in nuclear volume.

Rlf2p expressed from the *GAL10* promoter localizes to punctate nuclear foci that are distinct from Rap1p foci

To determine where Rlf2p is located in yeast cells, an epitope-tagged Rlf2p fusion protein (T7-Rlf2p) was overexpressed from the *GAL10* promoter (Johnston et al. 1994) because we could not detect T7-Rlf2p expressed from the native *RLF2* promoter (data not shown). Expression of T7-Rlf2p (on galactose) restored TEL+CEN antagonism to *rlf2-1* strain YJB463 (Fig. 1), demonstrating that T7-*RLF2* can functionally complement a *rlf2* mutant.

When wild-type cells carrying T7-*RLF2* [YJB278 (pME370)] were grown in galactose, we detected expression of T7-Rlf2p using T7-Tag antibody by immunoblotting (data not shown) and by immunofluorescence (Fig. 7A). T7-Rlf2p was intranuclear and clustered into a pattern distinctly different from the Rap1p localization pattern. We did not detect any staining in cells carrying the vector lacking insert or in samples in which either primary or secondary antibody was omitted (data not shown).

To detect the relative position of Rlf2p and Rap1p, we performed double-label immunofluorescence microscopy on YJB278 (pME370) using T7-Tag and anti-Rap1p-1 primary antibodies. In this double-labeling experiment, as in the single-label experiments, the majority of the T7-Rlf2p staining was intranuclear and Rap1p localized in foci as seen in wild type. Although the two patterns overlapped to some degree, the distribution of T7-Rlf2p and Rap1p appeared distinctly different (Fig. 7B). We do not know whether the localization of overexpressed T7-Rlf2p accurately reflects the localization of native Rlf2p. We note, however that a Rlf2p-LacZ fusion protein, which does not functionally complement *rlf2* mutations when overexpressed, appears uniformly diffuse throughout the nucleus. Thus the ability of overexpressed fusion proteins to localize with a punctate nuclear distribution correlates with the ability of the fusion protein to function in vivo.

Rlf2p is not required for perinuclear telomeric DNA clustering

Rap1p colocalizes with the majority of telomeric DNA in wild-type cells (Klein et al. 1992; Gotta et al. 1996). Because Rap1p localization is altered in *rlf2* mutants, we asked whether telomeric DNA localization was also altered in *rlf2* mutants when compared with wild-type

Figure 7. Localization of Rlf2p relative to Rap1p. (A) Indirect immunofluorescence staining of T7-Rlf2p. (B) Double-labeling of T7-Rlf2p (green) and Rap1p (red). Regions of overlap appear yellow. Both experiments were performed using YJB278 (pME370).

strains. We performed FISH to yeast telomeric DNA (Dernburg and Sedat 1997) using a probe derived from the Y' subtelomeric repeats (Fig. 8) and fixation conditions identical to those used for the immunolocalization of Rap1p. In the strains used, Y' repeats are present on at least 75% of the chromosome ends (data not shown). Qualitatively similar results were observed with a TG₁₋₃/C₁₋₃A probe, but use of the longer Y' repeat sequence gave more consistent, reproducible results (A. Dernburg, pers. comm.). In wild-type yeast cells, we observe a limited number of hybridizing spots (median of five spots per nucleus, range of four to seven in reconstructed nuclei). Consistent with the observations of Gotta et al. (1996), the majority of telomeric DNA staining is found in a limited number of discrete foci. Because diploid *S. cerevisiae* cells have 64 telomeres per nucleus, this result suggests that telomeres are clustered in these nuclei. The distribution of telomeric DNA and Rap1p are similar in wild-type yeast nuclei, implying that much of the Rap1p observed in wild-type cells reflects the localization of telomeres.

In contrast, in *rlf2* strains, the distribution of Rap1p does not reflect the localization of telomeric DNA. In *rlf2* mutants, despite the fact that Rap1p localization is altered, the number of Y'-hybridizing spots (median five spots per nucleus, range four to eight) and their distribution in the nucleus was not significantly different from the pattern observed in wild-type cells. The pattern of hybridization to Y' repeats is not altered in *rlf2-1* or *rlf2-Δ1::LEU2* strains (Fig. 8). Thus, although Rlf2p is required for the normal localization of Rap1p, it is not required for the clustering of telomeric DNA. We assume that a subset of the Rap1p foci observed in *rlf2* cells represent telomeres, because telomere tract length is not altered in *rlf2* mutants (Fig. 4). Because the number of diffuse Rap1p foci per *rlf2* nucleus was significantly larger than the number of telomere DNA foci per nucleus, a subset of the Rap1p foci in *rlf2* cells may not be associated with telomeric DNA.

RLF2 predicts a highly charged coiled-coil protein with homology to hCAF-I p150

The 606-amino-acid ORF encoded by *RLF2* is predicted

to be a highly charged protein with a net isoelectric point of 6.62. The domain from amino acids 134 to 230 is very likely to form a coiled-coil structure ($P > 85\%$, window=28; Lupas et al. 1991). An acidic domain from amino acid 385 to 418 (Fig. 2B) is composed of 65% glutamic acid plus aspartic acid.

Similarity searches of the protein and translated DNA databases using FASTA and TFASTA identified the p150 subunit of human CAF-I (hCAF-I) as the protein most similar to Rlf2p (23.6% identical, 69.4% similar plus identical residues in 471 amino acids; (Fig. 2B). The BLASTP similarity score between Rlf2p and p150 was 2.8×10^{-18} .

hCAF-I is a replication-dependent chromatin assembly factor (Smith and Stillman 1989, 1991b) composed of three subunits, p150, p60, and p50. Three domains within p150 have been shown to be important for chromatin assembly in vitro: the KER-rich region, the acidic ED-rich region, and a carboxy-terminal region required for binding to hCAF-I p60 as well as for chromatin assembly (Kaufman et al. 1995). The region of similarity between Rlf2p and hCAF-I p150 extends through these three functional domains including the amino-terminal ~100 amino acids of the p60-binding domain of p150 (Fig. 2B).

Comparison of the phenotypes of *rlf2* mutants and the biochemical properties of hCAF-I p150 suggests that Rlf2p and hCAF-I have similar functions. The phenotypic consequences of the *rlf2-1* mutation, a nonsense mutation lacking the carboxy-terminal half of the protein, include altered localization of Rap1p, loss of TEL+CEN antagonism, and a reduction in telomeric silencing. In addition, two other alleles (*rlf2Δ1::lacZ-1* and *rlf2::lacZ-2*) that are missing the carboxyl terminus of Rlf2p were unable to complement the TEL+CEN antagonism phenotype of *rlf2* mutants (Fig. 2B), and did not localize to nuclear foci (data not shown). Thus, the carboxy-terminal half of Rlf2p, including the acidic/ED-box domain, is required for Rlf2p function.

Finally, Kaufman et al. (this issue) have determined that the largest component of yeast chromatin assembly factor I (CAC1) is encoded by *RLF2*. Thus, *RLF2* encodes a yeast protein that assembles newly synthesized histones onto recently replicated DNA in vitro.

Figure 8. Localization of subtelomeric DNA in wild type and *rlf2* mutants by FISH. Equatorial Z sections of nuclei from YJB213 (wild type), YJB1213 (*rlf2-1*), and YJB496 (*rlf2Δ1*), strains.

Discussion

Biochemical, structural, and functional similarities between Rlf2p and hCAF-I p150

hCAF-I is required for the DNA replication-dependent assembly of chromatin onto SV40 DNA in vitro. Similarly, yCAF-I preferentially assembles chromatin from cytoplasmic histones onto newly replicated DNA in vitro and yCAF-90 is encoded by *RLF2* (Kaufman et al., this issue). This paper provides evidence that Rlf2p/yCAF-90 is required for the function of telomeric chromatin in vivo.

Studies of hCAF-I p150 have revealed three domains important for chromatin assembly function. The sequence similarity between Rlf2p and p150 extends through these three regions (Fig. 2B). The amino termini of both proteins are not essential for function: T7-Rlf2p is missing the amino-terminal 10% of the protein and is able to complement *rlf2* mutants for TEL+CEN antagonism. Deletion of the amino terminus of p150 (including the PEST sequence) did not affect the in vitro chromatin assembly ability of the protein (Kaufman et al. 1995). The carboxyl termini of both proteins are required for function: Rlf2-1p is missing the carboxy-terminal half of the protein, including the regions homologous to the ED- and p60-binding domains of p150. Similarly, deletions of these regions eliminate p150 function in vitro (Kaufman et al. 1995). Rlf2-LACZ-1p, which is missing the carboxy-terminal 30% of the protein that corresponds to the p60-binding domain in p150, cannot restore TEL+CEN antagonism to a *rlf2* mutant (Fig. 2A). Deletion derivatives of p150 that cannot bind p60 cannot function in the in vitro assays. The homology between the two proteins is high in the region between the KER and ED domains. Insertion of the nine-amino-acid HA epitope tag within this region (Fig. 2A) eliminated the ability of the protein to complement TEL+CEN antagonism, indicating that the region between the KER and the ED domains is important for Rlf2p function.

The phenotypes of *rlf2* mutants point to a role for yCAF-I in the assembly of telomeric chromatin in vivo. First, mutations in the amino termini of histones H3 and H4 cause phenotypes like those seen for *rlf2* mutants (altered telomeric silencing and perturbed Rap1p localization; Aparicio et al. 1991; Thompson et al. 1994; Hecht et al. 1995), suggesting that telomeric silencing and Rap1p localization are influenced by chromatin. Second, mutations in *SIR2*, *SIR3*, *SIR4*, and *RAP1*, which encode proteins thought to be structural components of silent telomeric chromatin, cause phenotypes like those seen in *rlf2* mutants (altered telomeric silencing, reduced TEL+CEN antagonism, and perturbed Rap1p localization). Finally, mutations in the other two yCAF-I subunits, yCAF-50 (*MSI1*) and yCAF-60, also cause a reduction in TEL+CEN antagonism and Rap1p localization (data not shown), as well as in telomeric silencing (Kaufman et al. this issue), suggesting that the phenotypes of *rlf2* mutants are attributable to defects in the yCAF-I complex.

Some Rap1p remains at telomeres in rlf2 strains

The fact that telomeric silencing and Rap1p localization are altered in *rlf2* mutants implies that the organization of telomeric chromatin is altered in *rlf2* strains. To explain the different appearance of Rap1p in wild-type and *rlf2* cells, we suggest that in wild-type cells, Rap1p associates with telomeric regions by binding directly to the telomeric DNA and also by associating with a large Sir protein-DNA complex through protein-protein interactions. Because *RAP1* is required for the maintenance of telomere length, and because telomere length is not affected in *rlf2* strains, we assume that some Rap1p remains bound to telomeric repeats in *rlf2* mutants and appears as the less distinct foci, whereas the non-DNA-bound Rap1p dissociates from the telomeres and appears diffuse.

Rap1p localization by immunofluorescence reflects the state of telomeric chromatin, not the localization of telomeric DNA

It has been proposed that the distribution of Rap1p reflects the distribution of telomeric DNA, because there are large numbers of Rap1p binding sites at telomeres (one per 18–40 bp of telomeric repeats; [Longtine et al. 1989; Gilson et al. 1993]), and because Rap1p complexes are observed at the ends of condensed chromosomes in pachytene spreads of wild-type strains (Klein et al. 1992). Recent studies of wild-type cells confirm that Rap1p (detected by immunofluorescence) and subtelomeric repeats (detected by FISH) colocalize in ~75% of the detected foci (Gotta et al. 1996). Our work indicates that perturbation of Rap1p distribution (detected by immunofluorescence) is not necessarily accompanied by perturbation of the distribution of subtelomeric Y' repeats (detected by FISH). Similarly, in *sir3* and *sir4* mutant strains, Rap1p localization does not reflect the localization of subtelomeric DNA (Gotta et al. 1996). Because telomeric silencing is assayed using a telomere-adjacent gene, our results suggest that alterations in silent chromatin are not necessarily accompanied by alterations in the localization of the silenced DNA. This result implies that the factors required for telomere–telomere associations are different from the proteins required for the heterochromatin-like properties of telomere-adjacent genes.

Does Rlf2p have a general role in chromatin assembly in vivo?

CAF-I functions in vitro on nontelomeric substrates and it is likely that CAF-I acts at nontelomeric genomic loci in vivo as well. Consistent with this view, cells lacking Rlf2p/yCAF-90 are more sensitive to UV light (Kaufman et al., this issue), a phenotype unlikely to be a result of effects exclusively on silent regions of the genome. *rlf2* mutations cause a ~50% increase in nuclear volume, which may also reflect an effect on the state of chromatin in general. An increase in nuclear volume also was observed in *Tetrahymena* strains lacking histones H1

(Shen et al. 1995). Because cells lacking Rlf2p grow well, we assume that in *rlf2* strains functional chromatin is assembled at most loci by alternative mechanisms.

Does Rlf2p have a specific role at telomeres?

In *rlf2* mutants, telomeric silencing is reduced and Rap1p localization is altered. Telomeric silencing is thought to reflect the repressive, heterochromatin-like state of telomeric chromatin (for review, see Laurenson and Rine 1992; Sandell and Zakian 1992; Loo and Rine 1996). Because Rap1p is a major component of telomeric chromatin (Conrad et al. 1990; Wright et al. 1992), and in wild-type cells the majority of Rap1p foci colocalize with telomere DNA (Gotta et al. 1996), perturbation of Rap1p localization by *rlf2* mutations likely reflects alterations in the organization of telomeric chromatin.

The effect of *rlf2* mutations and deletion of other CAF-I components on the ability of strains to grow on 5-FOA is unusual. Although a small number of large colonies (similar in size to the wild-type colonies) appear after 2 days, there are a variable number of microcolonies that appear after 3 or more days. These microcolonies may be a result of low levels of constant expression of the telomeric *URA3* gene or fluctuation in the state of the telomeric *URA3* gene. Because the microcolonies, when streaked from 5-FOA to nonselective and then back to 5-FOA, give rise to populations with a higher proportion of large 5-FOA-resistant cells, it appears that there is a heritable component to this apparently epigenetic phenomenon.

The assembly of telomeric chromatin is sensitive in strains lacking any one of the three components of yCAF-I (Kaufman et al., this issue), suggesting that yCAF-I may assemble telomeric chromatin. hCAF-I recognizes newly synthesized (cytoplasmic) histones H3 and H4 (Kaufman et al. 1995; Verreault et al. 1996). In *S. cerevisiae*, cytoplasmic histone acetylases acetylate histone H4 primarily on Lys 12 (Kleff et al. 1995). Interestingly, this pattern of nascent histone acetylation is retained in silent chromatin (Braunstein et al. 1996), perhaps because access to the nucleosomes deposited by yCAF-I is restricted by the silencing complex.

Telomeres replicate late in S phase (Friedman et al. 1995), suggesting that telomeric chromatin is assembled during a short, critical period in late S phase or early G₂. Telomeric silencing is enhanced when the S/G₂ phase of the cell cycle is lengthened (Laman et al. 1995), indicating that the time spent in S/G₂ is limiting the extent of silent chromatin assembly. The series of events by which CAF-I may improve the efficiency of telomeric silencing is illustrated in Figure 9. We propose that the silencing complex factors dissociate from the DNA just prior to DNA replication, resulting in high local concentrations of silencing factors immediately following replication. CAF-I facilitates the efficient assembly of nascent nucleosomes (using specific acetylated forms of histones H3 and H4) to allow the rapid association of nucleosomes with the available silencing factors (e.g., Sir proteins and Rap1p). Association with the silencing com-

Figure 9. Model of CAF-I-facilitated assembly of silent telomeric chromatin.

plex protects the nucleosomes from enzymes (including histone acetylases, histone deacetylases, and transcription factors). In the absence of CAF-I, nucleosome assembly is less efficient and does not use the same types of histones H3 and H4. Furthermore, the telomeric region that was derepressed in the previous cell cycle will have fewer silencing factors in the region and this local concentration will continue to decrease with the increasing time of chromatin assembly. The result is chromatin that is much more frequently accessible to enzymes that activate transcription.

Although we favor the idea that yCAF-I is directly involved in the efficient spreading of the silent chromatin complex, we cannot rule out the possibility that yCAF-I directly affects the binding of Rap1p to telomere repeats or that it affects heterochromatin assembly indirectly.

Materials and methods

Escherichia coli and S. cerevisiae strains and plasmids

Escherichia coli strains XL1-blue (Stratagene, Inc.) and MC1066 (Casadaban et al. 1983) were used for all standard plasmid preparations and manipulations (Ausubel et al. 1989). Yeast strains used in this study are listed in Table 1.

pSE49K and pRACUT1 are TEL+CEN plasmids that each include the *ADE2* marker; pRACUT also includes a *URA3* marker (Enomoto et al. 1994). pSE58A is an original isolate from the YCp50 library (Rose et al. 1987) including a 12-kb *Sau3A* fragment from chromosome XVI (Fig. 2). pSE58AJ is a 2-kb *Bgl*III–*Bam*HI fragment including *RLF2* derived from pSE58A (Fig. 2A), inserted in YCplac33 (Gietz and Sugino 1988). pSE193 carries the *RLF2* as a 3-kb *Eco*RI–*Bam*HI fragment with the *Xba*I site (amino acid 44) filled in to create a frame shift mutation (Fig. 2; *rlf2-2*); pSE58AT carries the *RLF2* as a 3-kb *Eco*RI–*Bam*HI fragment with the *Hind*III site (amino acid 88) filled in to create a frame shift mutation (Fig. 2; *rlf2-3*). pSE58AO is a 3-kb *Eco*RI–*Bam*HI subclone of pSE58A in YIplac128 (Gietz and Sugino 1988) lacking the *Hind*III site in the polylinker.

The *rlf2-Δ1::LEU2* allele was constructed by inserting the *Eco*RI–*Hind*III fragment 5' to the *RLF2* gene and the *Xba*I–*Bam*HI fragment from the carboxyl terminus of *RLF2* into

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
YJB203	<i>MATa</i> ; <i>leu2-3,112</i> ; <i>ura3-52</i> [<i>trp1-289</i>] <i>his3Δ</i> ; <i>ade2Δ</i> ; [<i>cir0</i>]	Longtine et al. (1993)
YJB212	YJB203 <i>MATa</i>	Longtine et al. (1993)
YJB252	YJB212 <i>MATa cyh2</i>	Konkel et al. (1995)
YJB463	YJB203 <i>rlf2-1</i>	this study
YJB469	YJB203 <i>rlf2-Δ1::LEU2</i>	this study
YJB470	YJB212 <i>rlf2-Δ1::LEU2</i>	this study
YJB472	YJB203 <i>rlf2-1 adh4::URA3-TEL</i>	this study
YJB487	YJB203 <i>adh4::URA3-TEL</i>	Enomoto et al. (1994)
YJB850	YJB203 <i>rlf2-Δ1::LEU2 adh4::URA3-TEL his⁺</i>	this study
YJB278	<i>MATa/MATα leu2-3,112/leu2-3,-112; ura3-52/ura3-52; trp1-289/trp1-289 his3Δ/his3Δ ade2Δ/ade2Δ; [cir⁺]</i>	this study
YJB1250	YJB203 <i>trp1::URA3 HIS3</i>	this study
YJB213	YJB203 × YJB212	Enomoto et al. (1994)
YJB496	YJB469 × YJB470	this study
YJB1213	<i>MATa/MATα leu2-3,112/leu2-3,-112; ura3-52/ura3-52; trp1-289/trp1-289; his3Δ/HIS3; ade2Δ/ade2Δ; adh4::URA3-TEL/ADH4; rlf2-1/rlf2-1; [cir⁺]</i>	this study
YJB464	YJB252 <i>RLF2-LEU2</i>	this study

YIPlac128 in inverted order relative to their genomic organization (pSE58AQ) and using this for gamma disruption of the genomic copy of *RLF2* (Sikorski and Hieter 1989). pSE58W includes *RLF2* on the 3-kb *EcoRI*–*Bam*HI genomic fragment inserted into YCPlac33 that is missing the *Bam*HI–*Sal*I fragment in the polylinker.

Plasmid pME370 was used to express the T7-Tag-Rlf2 fusion protein. It is composed of the *RLF2* gene encoding amino acids 86–606 from pSE58A inserted behind the *GAL10* promoter in pSE351. pSE351 was constructed from the *GAL1-10* promoter in pBM272 (Johnston and Davis 1984), the T7-tag leader peptide from pRSET-C (Kroll et al. 1993), and *CENIV*, *ARS1*, *LEU2*, and pUC19 sequences essentially from YCPlac111 (Gietz and Sugino 1988).

Mutant screen and cloning of *RLF2*

Mutants affecting TEL+CEN antagonism were isolated by screening for reduced red sectoring of colonies in YJB203 carrying pRACUT1 on SDC plates as described previously (Enomoto et al. 1994). Approximately 40,000 mutagenized cells were screened and to date at least 10 complementation groups have been identified. Putative mutants were cured of pRACUT1 and then independently retransformed with a TEL+CEN plasmid (pSE49K) to confirm that the loss of TEL+CEN antagonism was

attributable to a chromosomal mutation and with a CEN plasmid (pASZ11; Stotz and Linder 1990) to identify mutant strains in which the segregation and replication of a CEN plasmid was not affected. Dominance tests were performed by comparing the sectoring of pRACUT1 in a backcross [(YJB463 × YJB212 [*rlf2-1/RLF2*])] to that of wild-type diploid strain YJB213; *rlf2-1* is recessive to wild-type for TEL+CEN antagonism by this criterion. The backcross diploid (YJB463 × YJB212) was sporulated. Because pRACUT1 does not segregate to all the tetrad progeny, not all complete tetrads could be analyzed for TEL+CEN antagonism. *rlf2-1* segregated 2:2 in five complete tetrads and in 10 tetrads no more than two spores displayed the mutant TEL+CEN antagonism phenotype, suggesting that the loss of TEL+CEN antagonism was attributable to a single nuclear gene.

The *RLF2* gene was cloned from a yeast genomic library (YCP50; Rose 1987) transformed in YJB463 (pSE49K). Complementation of TEL+CEN antagonism was detected as colonies with increased red sectoring (because of increased loss of pSE49K) on SDC-URA medium (which selects for the library plasmid and permits loss of TEL+CEN plasmid). Candidate clones were rescued into *E. coli* strain MC1066 by selecting for uracil prototrophy. Plasmid DNA was then retransformed into YJB463 carrying pSE49K to verify the complementation of TEL+CEN antagonism. One clone, pSE58A, consistently complemented the TEL+CEN antagonism defect. Subclones of pSE58A were tested for complementation of TEL+CEN antagonism in a similar manner.

To verify that the cloned *RLF2* gene was allelic to the gene mutated in YJB463, we integrated a copy of *LEU2* adjacent to the *RLF2* genomic locus by transforming YJB212 with pSE58AO linearized at the *Hind*III site. The integration was confirmed by Southern blot hybridization and the resulting *RLF2-LEU2* strain (YJB464) was crossed to YJB463 carrying pRACUT1 and was sporulated. Of 50 tetrads dissected, 18 tetrads carried pRACUT1 in all four segregants. In all 18 tetrads, the *LEU2* marker and wild-type TEL+CEN antagonism cosegregated. In addition, the spores from the remaining tetrads in which pRACUT1 was retained, the *LEU2* marker, and TEL+CEN antagonism always cosegregated, indicating tight linkage of the cloned gene and the *rlf2-1* mutant allele.

The *rlf2-1* mutant allele was cloned from YJB463 × YJB212 strain, by gap repair (Rothstein 1991) of plasmid pSE58W digested with *Xba*I. Two isolates with the expected restriction map were unable to complement TEL+CEN antagonism; DNA sequencing of both clones revealed the same single nucleotide change.

Plasmid loss rate, telomeric silencing, and telomere tract length

Plasmid loss rate is defined as frequency of loss events per division and was measured as described (Longtine et al. 1992). Telomere position effect (TPE) was measured in strains containing *URA3* inserted at the *ADH4* locus on the left end of chromosome VII (Gottschling et al. 1990). Telomere tract length was measured on Southern blots (1% agarose gels) of yeast genomic DNA digested with *Pst*I, hybridized with a 75-bp probe from pCA75+ (Wellinger et al. 1993), and detected with the Genius System (Boehringer Mannheim, Inc.).

Antisera

Anti-Rap1p was prepared in New Zealand white rabbits (BAbCo, Inc., Berkeley, CA) with highly purified *E. coli*-expressed Rap1p (generous gift of Anne Cassidy-Stone and Steve Schultz, University of Colorado, Boulder). Both the primary and

the secondary antisera were preadsorbed with fixed yeast extracts made from strain YJB937, which carries the *rap1-6* allele (Moretti et al. 1994). T7 tag antibody (Novagen) was used at 0.01–0.02 mg/ml. FITC-anti-mouse, FITC-anti-rabbit and rhodamine-anti-rabbit antisera were purchased from Cappel Organon Teknika Corp. (Durham, NC) and were used at 1:50, 1:50, and 1:500 dilutions, respectively. MAB414 antibody was purchased from BAbCo, Inc. (Berkeley, CA).

Indirect immunofluorescence

Indirect immunofluorescence was performed essentially as described previously (Konkel et al. 1995). For any given experiment, wild-type and mutant strains were treated identically. Briefly, log phase cells were fixed in paraformaldehyde [4% (vol./vol.) final concentration] for 75 min at room temperature. After two washes with buffer A (1.2 M sorbitol, 100 mM potassium phosphate at pH 7.5), cells were digested with lyticase (ICN) for 15 min at 37°C, followed by three washes in buffer A. Spheroplasts were placed on polylysine-treated microscope slides and incubated in blocking buffer (10 mg/ml of bovine serum albumin, 0.05% Tween 20, 5 mM Tris-HCl at pH 8, 75 mM NaCl) for 10 min at 23°C. Spheroplasts were incubated in appropriate dilutions of the first primary antibody (see above) and incubated for 1 hr at 37°C or overnight at 4°C. The slides were then washed with buffer A. In double-labeling experiments, cells were incubated in a second primary antibody overnight at 4°C and then washed with buffer A. Cells were then incubated in the appropriate secondary antibody for 45 min at 23°C and washed with buffer B (buffer A plus 300 mM NaCl). DNA was counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (1 µg/ml), and cells were mounted in Vectashield (Vector Laboratories, Inc.) antifading medium.

FISH

The telomeric probe for in situ hybridization was the Y' subtelomeric repeat sequence on YRp131S (Chan and Tye 1983), which includes a short TG₁₋₃/C₁₋₃A tract, was digested into small fragments with *AluI*, *HaeIII*, *RsaI*, and *HpaII* and end-labeled with rhodamine-4-dUTP (Amersham, Inc.) using Terminal Deoxynucleotidyl Transferase (Ratcliff Biochemicals or Promega, Inc.) according to supplier's instructions. Cells were fixed and permeabilized under conditions identical to those used for immunofluorescence (above) and hybridized with the telomeric DNA probe using conditions modified for use with yeast nuclei (Dernburg and Sedat 1997). DNA was counterstained with 0.5 µg/ml DAPI in 2× SSC and samples were mounted in Vectashield mounting medium.

Image collection and analysis

Serial 0.5 µm optical Z sections of the labeled cells were collected with a Bio-Rad MRC1000 laser confocal microscope equipped with a 15 mW krypton-Argon laser. Gain and offset settings on the confocal PMTs were held constant between strains to best compare the relative intensities of the signals. Images were collected using Kalman functions and stored to disk for analysis.

Image analysis was performed using either a Dell 120 Pentium IBM compatible computer utilizing Media Cybernetics Image Pro Plus version 1.3 or an Apple Macintosh Power PC 8500 utilizing NIH Image version 1.60.

To determine the three-dimensional distribution of Rap1p spots, 360° projections of double-labeled nuclei were made using NIH Image Version 1.60. MAB414 staining of nuclear pores

was used to identify the nuclear boundary. The center of the 360° projection of the nuclear boundary was determined using the max./min. diameter function. The image was thresholded and an outline of the nuclear boundary was saved. The resulting outlines were overlaid on the matching Rap1p data set. Measurements from the data were performed by identifying local maximum intensity regions associated with the Rap1p spots and measuring their distance from the center. Approximately 80–100 nuclei for each genotype were scored in this manner. Resulting data were plotted using Excel 5.0.

Color overlays and final plates were generated in Adobe Photoshop using identical settings for each set of images. Images were printed to a Tektronix Phaser IISdx dye-sublimation printer (Tektronix, Wilsonville, OR).

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RLF2, a subunit of yeast chromatin assembly factor-I, is required for telomeric chromatin function in vivo.

S Enomoto, P D McCune-Zierath, M Gerami-Nejad, et al.

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