

The Ctf18 RFC-like complex positions yeast telomeres but does not specify their replication time

Shin-ichiro Hiraga, E Douglas Robertson and Anne D Donaldson*

Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, UK

Chromosome ends in *Saccharomyces cerevisiae* are positioned in clusters at the nuclear rim. We report that Ctf18, Ctf8, and Dcc1, the subunits of a Replication Factor C (RFC)-like complex, are essential for the perinuclear positioning of telomeres. In both yeast and mammalian cells, peripheral nuclear positioning of chromatin during G1 phase correlates with late DNA replication. We find that the mislocalized telomeres of *ctf18* cells still replicate late, showing that late DNA replication does not require peripheral positioning during G1. The Ku and Sir complexes have been shown to act through separate pathways to position telomeres, but in the absence of Ctf18 neither pathway can act fully to maintain telomere position. Surprisingly *CTF18* is not required for Ku or Sir4-mediated peripheral tethering of a nontelomeric chromosome locus. Our results suggest that the Ctf18 RFC-like complex modifies telomeric chromatin to make it competent for normal localization to the nuclear periphery.

The EMBO Journal (2006) 25, 1505–1514. doi:10.1038/sj.emboj.7601038; Published online 9 March 2006

Subject Categories: chromatin & transcription; genome stability & dynamics

Keywords: Ctf8; Dcc1; Ku; SIR proteins

Introduction

The physical organization of DNA within the nucleus is related to chromatin function. Chromosomes of higher eukaryotes occupy specific nuclear ‘territories’, and the spatial territory of a chromosome frequently reflects its gene-density, with chromosomes containing a high proportion of nontranscribed sequence located close to the edge of the nucleus (Tanabe *et al*, 2002). Although it is clear that chromatin is organized and actively positioned within the nuclear space, the mechanisms determining physical organization of chromosomes within nuclei are not understood.

All eukaryotic cells replicate their DNA according to a reproducible temporal program, and spatial organization of the DNA is correlated with replication timing (reviewed in Taddei *et al*, 2004b). Replication foci are typically spread throughout the nuclear interior during early S phase, while

peripheral and perinucleolar DNA replicates in mid to late S phase. Nonexpressed heterochromatic DNA usually replicates late in the S phase (Gilbert *et al*, 2004; Woodfine *et al*, 2004). In general, the spatial organization, transcriptional activity and replication timing of chromatin are correlated, but causative relationships between these three properties are unclear.

The organization of the telomeres of *Saccharomyces cerevisiae* offers a useful model system for studying chromosome positioning, transcriptional activity, and replication timing. The 32 telomeres of haploid yeast cells associate in 3–6 clusters at the nuclear periphery (Gotta *et al*, 1996). *S. cerevisiae* subtelomeric sequences are subject to silencing of polymerase II-mediated transcription (Gottschling *et al*, 1990) and telomeres are replicated late during S phase (Ferguson and Fangman, 1992; Raghuraman *et al*, 2001).

Two partially redundant pathways have been identified that mediate tethering of telomeres to the nuclear rim. The first depends on the yeast Ku (yKu) protein complex (which consists of Yku70 and Yku80 proteins), and the second on the Sir4 and Esc1 proteins (Hediger *et al*, 2002; Taddei *et al*, 2004a). Different telomeres may differ somewhat in their dependence on these two pathways—for example, during G1, telomere VI-right (VIR) positioning depends primarily on the Ku pathway and telomere VI-left (VIL) primarily on the Sir4/Esc1 pathway (Bystricky *et al*, 2005). Some evidence suggests that the Ku-dependent pathway tends to dominate during G1, while the Sir4/Esc1-dependent tethering pathway is dominant in the S phase (Hediger *et al*, 2002). The telomeres lose their peripheral localization during G2 as cells prepare to enter mitosis, and perinuclear positioning is re-established in early G1 phase. However, the telomere positioning mechanism is not fully understood, and in particular the molecular components that act with Ku to mediate telomere positioning are not known. Identification of additional positioning components is complicated by the fact that Ku also plays a key role in other telomere-specific functions, including subtelomeric transcriptional silencing (Gravel *et al*, 1998; Laroche *et al*, 1998), telomerase recruitment (Stellwagen *et al*, 2003), and specification of late replication timing (Cosgrove *et al*, 2002).

Peripheral localization of DNA within the yeast nucleus has been shown to reinforce transcriptional silencing in a number of cases. For example, artificial localization to the periphery enhances transcriptional repression at a compromised silencer (Andrulis *et al*, 1998). Consistently, both Ku and *SIR4* are required for maximum silencing of subtelomeric genes (Palladino *et al*, 1993; Gravel *et al*, 1998; Laroche *et al*, 1998). However, under some circumstances, positioning and silencing can be separated. Repression can be maintained at an intact silencer that is released from the nuclear peripheral zone (Gartenberg *et al*, 2004), and at a modified version of telomere VIII there was no correlation between proportion of peripherally positioned telomeres and the efficiency of silencing (Tham *et al*, 2001).

*Corresponding author. Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK.
Tel.: +44 1224 550975; Fax: +44 1224 555844;
E-mail: a.d.donaldson@abdn.ac.uk

Received: 14 November 2005; accepted: 15 February 2006; published online: 9 March 2006

The relationship between peripheral localization and replication timing has been less investigated. The telomeres and ribosomal DNA are localized close to the nuclear envelope and replicate in the second half of S phase (Raghuraman *et al*, 2001; A Cosgrove and A Donaldson, unpublished), suggesting that peripheral localization may favor late replication. Moreover, removal of Ku function leads simultaneously to delocalization and abnormally early replication of telomeres (Cosgrove *et al*, 2002). Localization of the DNA during G1 phase has been proposed to be particularly crucial for correct timing control (Gilbert, 2002), since the *S. cerevisiae* telomere late replication program is pre-established during G1 (Raghuraman *et al*, 1997). The replication program in mammalian cells is also established during G1 coincident with re-positioning of DNA within the nucleus (Dimitrova and Gilbert, 1999).

Replication factor C (RFC) is a five subunit 'clamp-loading' complex consisting of the essential gene products Rfc1-5, all of which belong to the AAA + ATPase superfamily (Bowman *et al*, 2004). RFC loads the ring-shaped PCNA polymerase clamp component of replication forks. Three RFC-like complex have been identified in which the largest subunit (Rfc1) is replaced by either Rad24, Ctf18, or Elg1; *RAD24*, *CTF18*, and *ELG1* are nonessential genes with sequence similarity to *RFC1* (reviewed in Kim and MacNeill, 2003). The Elg1 and Rad24 RFC-like complexes (Elg1-RLC and Rad24-RLC) are important for genome stability and checkpoint responses, and Rad24-RLC has been shown to load the ring-shaped 9-1-1 complex onto damaged DNA. The Ctf18-RLC is a heptameric complex containing two extra subunits, Ctf8 and Dcc1, in addition to Rfc2-5 and Ctf18 itself. The function of Ctf18-RLC remains mysterious. Disruption of either *CTF18*, *CTF8*, or *DCC1* causes a sister chromatid cohesion defect, but no cohesin loading defect was detected in a *ctf8* mutant (Hanna *et al*, 2001; Mayer *et al*, 2001; Kenna and Skibbens, 2003). By analogy to RFC and the Rad24-RLC, the Ctf18-RLC is believed to act on a ring-shaped complex. Human Ctf18-RLC can load PCNA *in vitro*, although with reduced efficiency when compared to RFC itself (Ohta *et al*, 2002; Bermudez *et al*, 2003; Merkle *et al*, 2003). It has recently been demonstrated that yeast Ctf18-RLC efficiently unloads PCNA from DNA *in vitro* (Bylund and Burgers, 2005).

Here, we show that Ctf18-RLC mediates correct positioning of yeast telomeres at the nuclear periphery. Despite the disruption of telomere peripheral positioning, the telomeres of *ctf18* cells replicate late in the S phase, showing that peripheral positioning during G1 is not required for late replication of DNA. We propose that the Ctf18-RLC may act, through unloading of PCNA and/or exchange of PCNA-like ring-shaped complexes, to establish a chromatin structure that is required for telomere positioning.

Results

The Ctf18-RLC complex is required for perinuclear positioning of Rap1

To elucidate molecular mechanisms responsible for positioning chromatin within the nucleus, we screened for new gene products involved in localizing *S. cerevisiae* telomeres. The screen will be described in detail elsewhere; briefly, it is based on examining the subnuclear localization of the telo-

meric heterochromatin component Rap1. We transformed a series of disruption mutants in nonessential genes with a plasmid encoding GFP-Rap1, and screened for those mutants in which GFP-Rap1 localization appeared abnormal. Expression of GFP-Rap1 in wild-type (WT) cells (Hayashi *et al*, 1998) reveals several discrete dots corresponding to the telomere clusters (Figure 1). In unbudded and small-budded cells, these dots are predominantly localized at the nuclear rim, as expected since telomeres are localized to the nuclear periphery during the early part of the cell cycle. The Ku complex is required for correct localization of telomeres. As a control for the effect of telomere localization on Rap1 positioning, we confirmed that the Rap1 foci were largely dispersed in a *yku70* strain (Figure 1). On examination of a *ctf18* strain, we found that GFP-Rap1 foci were almost completely disrupted, with the Rap1-GFP signal dispersed throughout the nuclear interior (Figure 1). As described above, the Ctf18-RLC is a seven subunit RFC-like complex that includes the gene products Ctf8 and Dcc1. We found that Rap1 foci were also dispersed in *ctf8* and *dcc1* mutants (Figure 1). The fact that the *ctf18*, *ctf8*, and *dcc1* mutants all share the same Rap1 localization defect suggests that the Ctf18-RLC is essential for proper Rap1 localization to the nuclear periphery, rather than the effect being due to one of the gene products alone.

Rad24 and Elg1 are the largest subunits of the two other RLC complexes. Neither *rad24* nor *elg1* mutant showed a GFP-Rap1 localization defect (Figure 1), suggesting that the role in Rap1 localization within the nucleus is specific to Ctf18-RLC.

The Ctf18-RLC is required for telomere positioning

One possible interpretation of the results in Figure 1 is that the Ctf18-RLC is required for telomere positioning. To address this possibility, we tested the effects of deleting *CTF18*, *CTF8*, or *DCC1* on telomeres that were fluorescently tagged (Straight *et al*, 1996). We used a strain in which a single telomere is marked by GFP fused to the *lac* repressor and the nuclear envelope is simultaneously visualized by GFP tagging of a nuclear pore component, so that the telomere is visible as a bright dot within a circle corresponding to the nuclear envelope (Figure 2A). In the majority of interphase WT cells, the telomere dot appears to touch the nuclear envelope (corresponding to a distance of <230 nm). Telomeres VIR, VIIIIL, and XIVL were localized at the nuclear rim in a reduced proportion of *ctf18*, *ctf8*, and *dcc1* cells, with levels of localization similar to those of a *yku70* mutant (Figure 2B). The *CTF18*, *CTF8*, and *DCC1* gene products are therefore required for correct positioning of *S. cerevisiae* telomeres at the nuclear periphery.

To test whether another peripherally localized sequence is disrupted in *ctf18* cells, we examined the distribution of the ribosomal DNA that is normally packaged at the edge of the nucleus. Observation of the rDNA using a GFP-tagged Net1 protein (which binds the ribosomal DNA repeats) revealed no apparent mislocalization of the rDNA (data not shown). Nuclear structure therefore does not appear to be grossly disrupted in the *ctf18* mutant.

CTF18 is required for telomere localization in the G1 and S phase

To assess the cell cycle stages at which Ctf18 is important for telomere localization, we examined telomere position in

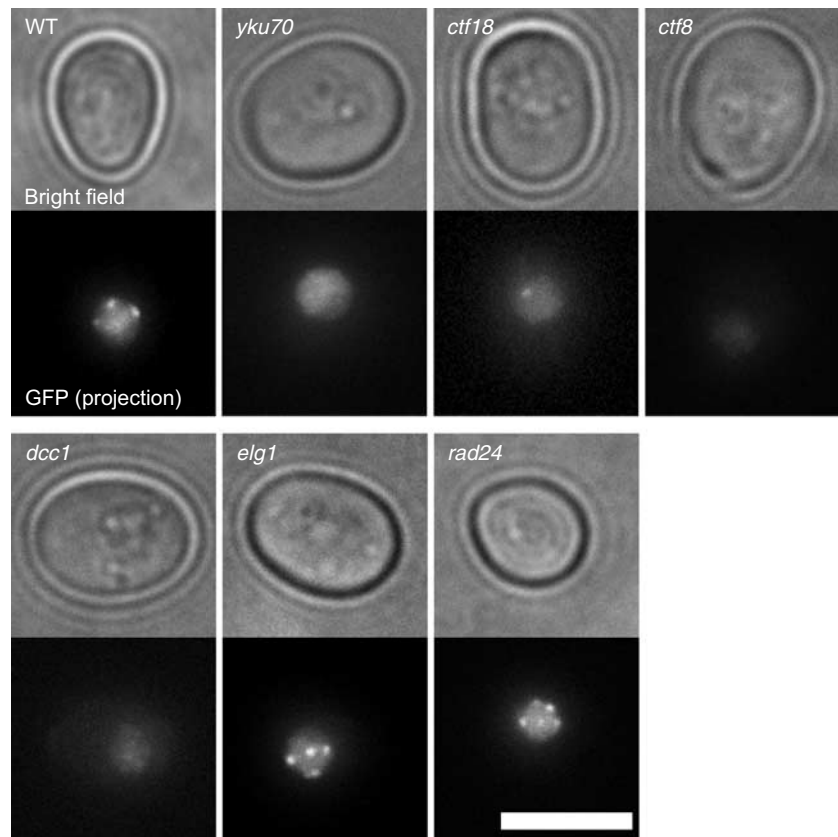


Figure 1 *CTF18*, *CTF8*, and *DCC1* are required for formation of Rap1 foci at the nuclear rim. WT, *yku70*, *ctf18*, *ctf8*, *dcc1*, *elg1*, and *rad24* strains were transformed with plasmid YCp-GFP-RAP1 and examined by fluorescence microscopy. The ‘whole-nucleus’ patterns of GFP fluorescence shown in the lower panels were created by capturing images of GFP fluorescence at 250 nm intervals and projecting the Z-stack series of images onto a single plane. Scale bar = 5 μ m.

cells scored for cell cycle position according to bud size. We quantified the position of telomere XIVL by dividing the nucleus into three concentric zones of equal area (Figure 3A) as described (Taddei *et al*, 2004a). In this assay, random telomere positioning would be represented by 33% of telomeres scored in each zone. In WT nuclei, telomere XIVL preferentially localizes to the outermost zone in both G1 and S phases (Figure 3B and Table I). In *ctf18* G1 phase nuclei, the same telomere was almost randomly positioned (39% of telomeres in Zone 1; Figure 3B and Table I). Once *ctf18* cells entered S phase, the telomere remained slightly delocalized from the periphery when compared to WT, although the effect was not as severe as in G1. χ^2 analysis confirmed that telomere position during S phase in *ctf18* is significantly different from that of WT and from random distribution. Very similar cell cycle effects were observed for telomere VIIIIL (localization to Zone 1 in 67% of WT G1 cells, 32% of *ctf18* G1 cells, 61% of WT S cells, and 49% of *ctf18* S cells). *P*-values assessing the statistical significance of these results are given in Table I. We conclude that telomere positioning is affected by *CTF18* deletion primarily in G1 phase cells, with the absence of Ctf18 being slightly deleterious in the S phase. Overall, the telomere delocalization phenotype of *ctf18* is reminiscent of that described for *yku70*, which has been reported to affect the positioning of some telomeres primarily during G1 (Hediger *et al*, 2002).

We performed time-lapse analysis to examine whether delocalized telomeres in *ctf18* nuclei can still visit the nuclear periphery. Tracings of movies showing the typical behavior of telomere XIVL in WT and *ctf18* cells are shown in Figure 3C (Movies in Supplementary data). In WT G1 phase cells (Sup_3.mpg), telomere XIVL remained confined within 0.2 μ m of the periphery for most of the analysis. Although the telomere did occasionally leave the nuclear envelope, it returned to the periphery after a short time and usually remained there. In the S phase, telomere movement became still more confined, suggesting even more stable localization (Sup_4.mpg). These data are consistent with previous observations in WT cells (Heun *et al*, 2001b). In *ctf18* cells in contrast, telomere XIVL was not confined to nuclear periphery but spent more time in the interior, particularly during G1 phase (Sup_5.mpg). The telomere was not excluded from the edge of the nucleus and did pay occasional visits to the periphery, but failed to become stably localized during those visits. Once *ctf18* cells entered S phase, brief periods of telomere positioning at the periphery were observed, although these were still not of the duration or stability seen in WT S phase cells (Sup_6.mpg).

We measured the duration of localization events (Figure 3D). In unbudded and small-budded *ctf18* mutant cells, periods of internal localization and brief visits to the periphery were increased at the expense of stable peripheral localization periods, to the extent that long-term (greater than

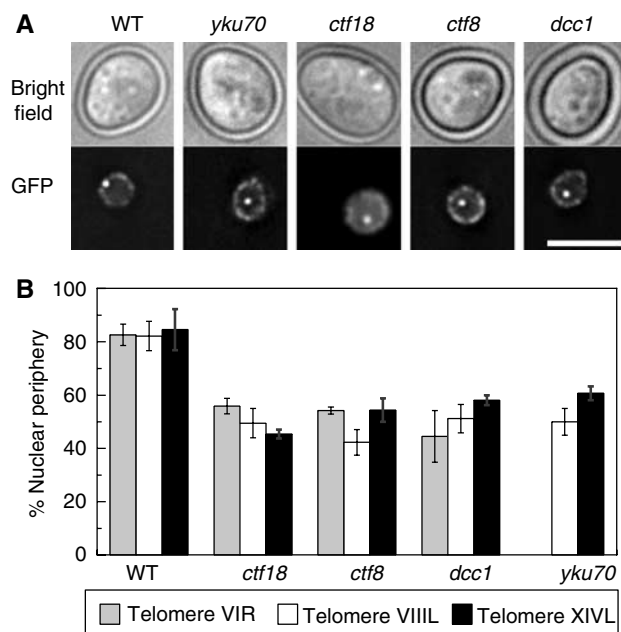


Figure 2 *CTF18*, *CTF8*, and *DCC1* are required for telomere peripheral positioning. (A) Typical images of telomere VIIIIL dot in WT (GA-1986), *yku70* (SHY120), *ctf18* (SHY117), *ctf8* (SHY118), and *dcc1* (SHY119) strains. Single bright dots in lower panels represent the left telomere of chromosome VIII. The encircling ring of dimmer fluorescence corresponds to the nuclear envelope marked by Nup49-GFP. Scale bar, 5 μ m. (B) Quantification of telomere position in asynchronous cultures. The position of telomeres VIR, VIIIIL, or XIVL was analyzed in WT, *ctf18*, *ctf8*, *dcc1*, and *yku70* strains. Cells were scored if the telomere dot was located in one of the equatorial Z sections. If the distance between telomeric dot and nuclear rim was <230 nm, it was scored as 'nuclear peripheral'. Error bars indicate standard deviations obtained from at least two independent cultures.

1 min) localization periods were almost never observed. We conclude that Ctf18-RLC is important for telomere positioning in both G1 and S phase, and that its primary role is to permit the establishment of stable localization at the nuclear periphery.

Telomeres replicate at the normal time in a *ctf18* mutant strain

S. cerevisiae telomeres are normally late-replicating and are localized at the nuclear periphery. Our discovery of a new effector of telomere localization enabled us to investigate whether peripheral localization of telomeres during G1 is a prerequisite for their late replication. We examined the replication program of a *ctf18* strain using the dense isotope transfer technique (Donaldson *et al*, 1998). Figure 4A shows the replication programs of WT, *ctf18* and *yku70* mutant cells analyzed by using this method. Markers for early and late replication in the S phase are provided by the early replication origin *ARS305* and a late-replicating sequence on chromosome XIV that lies far from either telomere (chr XIV-int). We examined the replication time of telomere VIIIIL, whose perinuclear localization depends on Ctf18 as shown in Figure 2B. We found that telomere VIIIIL replicated late in the *ctf18* mutant as in WT cells (Figure 4A, left and centre panels). This result contrasts with the situation in the *yku70* mutant in which telomere VIIIIL replicated much earlier in the

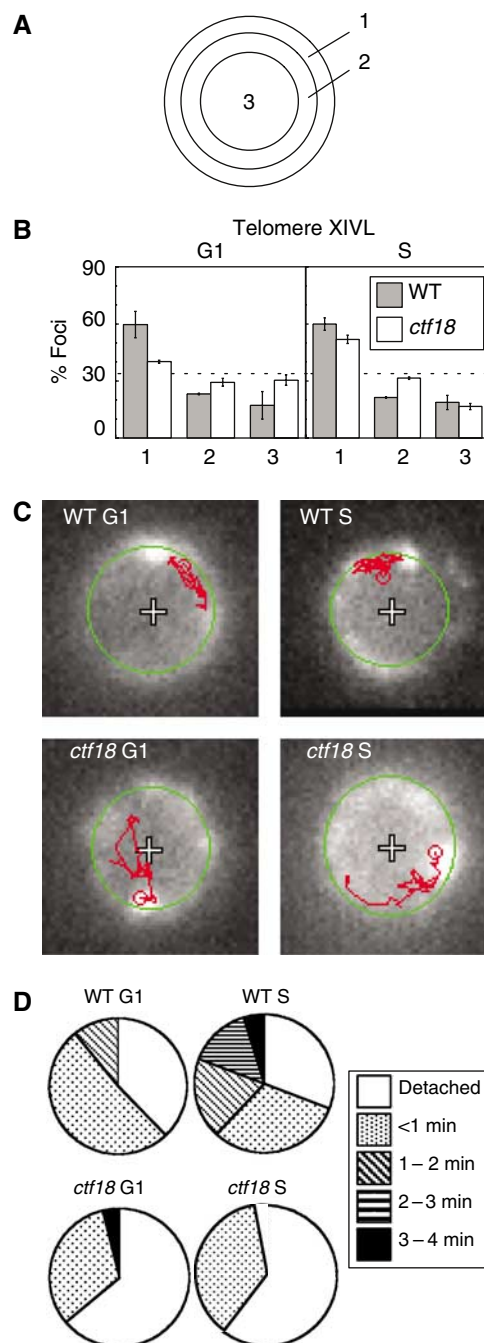


Figure 3 Effect of *CTF18* on telomere XIVL positioning in G1 and S phase. (A) Cartoon of zoning analysis. Nuclei with telomeres at the equatorial Z section were divided into three concentric zones with equal surface area as shown, and the position of the telomere dot was scored. (B) Analysis of position of telomere XIVL in WT (GA-1985) and *ctf18* (SHY114) strains. Histograms show the distribution of telomere dots to the three zones, with Zone 1 the most peripheral. The dotted line in each plot represents random distribution. (C) Telomere movement is less constrained in *ctf18* strain. The movement of telomere XIVL in WT and *ctf18* cells was analyzed during G1 and S phase. Red lines show track of telomere XIVL dots over a 6 min period. Green circles represent the nuclear rim. (D) The duration of telomere XIVL peripheral localization events was analyzed in WT and *ctf18* strains. The total number of scored time points was 37 for WT G1, 42 for WT S, 50 for *ctf18* G1, and 35 for *ctf18* S.

S phase (Figure 4A, right panel). To measure an ‘average’ telomere replication time, we examined the replication time of the *Y'* sequences. *Y'* is one of the repeated sequence elements found at more than half of yeast telomeres, so that examining *Y'* replication time gives a good view of overall telomere replication time. *Y'* sequences replicated late in the *ctf18* mutant as in WT cells. In the *yku70* mutant,

Y' sequences replicated much earlier in the S phase as shown previously (Cosgrove *et al*, 2002). A replication time can be assigned for a sequence as the time at which half the final level of replication has occurred, and the interval between the replication of the early and late marker sequences can be taken as a measure of S phase length. In the *ctf18* strain, this interval was 16.4 min for the experiment shown in Figure 4A, compared with 23.8 min in the WT and 19.7 min in the *yku70* mutant strain. The S phase program may therefore be slightly compressed in the *ctf18* strain. ‘Replication index’ (RI) values can be calculated to compare the replication programs in different strains and adjust for differences in the speed at which cultures release from synchrony. RI values express the time of replication of each sequence as a proportion of elapsed S phase. Figure 4B shows the replication programs of WT, *ctf18*, and *yku70* strains plotted according to RI. In this format, it is clear that there is no significant change in the relative replication time of *Y'* sequences in the *ctf18* strain when compared to WT. Analysis of two additional loci (sequences close to the left ends of chromosomes III and VI) also confirmed that the RI values in the *ctf18* S phase were very similar to WT, any slight differences observed lying within experimental error.

From these experiments, we conclude that the telomeric DNA of a *ctf18* mutant replicates at its normal, late time in the

Table I Significance of telomere localization

| | P-value | | |
|------------------------|--------------------------------|--|--------------------------------------|
| | WT against random ^a | <i>ctf18</i> against random ^a | <i>ctf18</i> against WT ^b |
| <i>Telomere XIVL</i> | | | |
| G1 | 2.43×10^{-16} | 0.08 | 5.34×10^{-12} |
| S | 2.22×10^{-21} | 1.09×10^{-7} | 2.39×10^{-3} |
| <i>Telomere VIIIIL</i> | | | |
| G1 | 2.65×10^{-19} | 0.98 | 6.21×10^{-33} |
| S | 5.92×10^{-13} | 8.37×10^{-4} | 2.29×10^{-4} |

^aP-values were calculated by χ^2 analysis in which actual distribution was compared to a hypothetical random distribution.

^bP-values were calculated by χ^2 analysis in which observed distribution for *ctf18* was compared to that for WT.

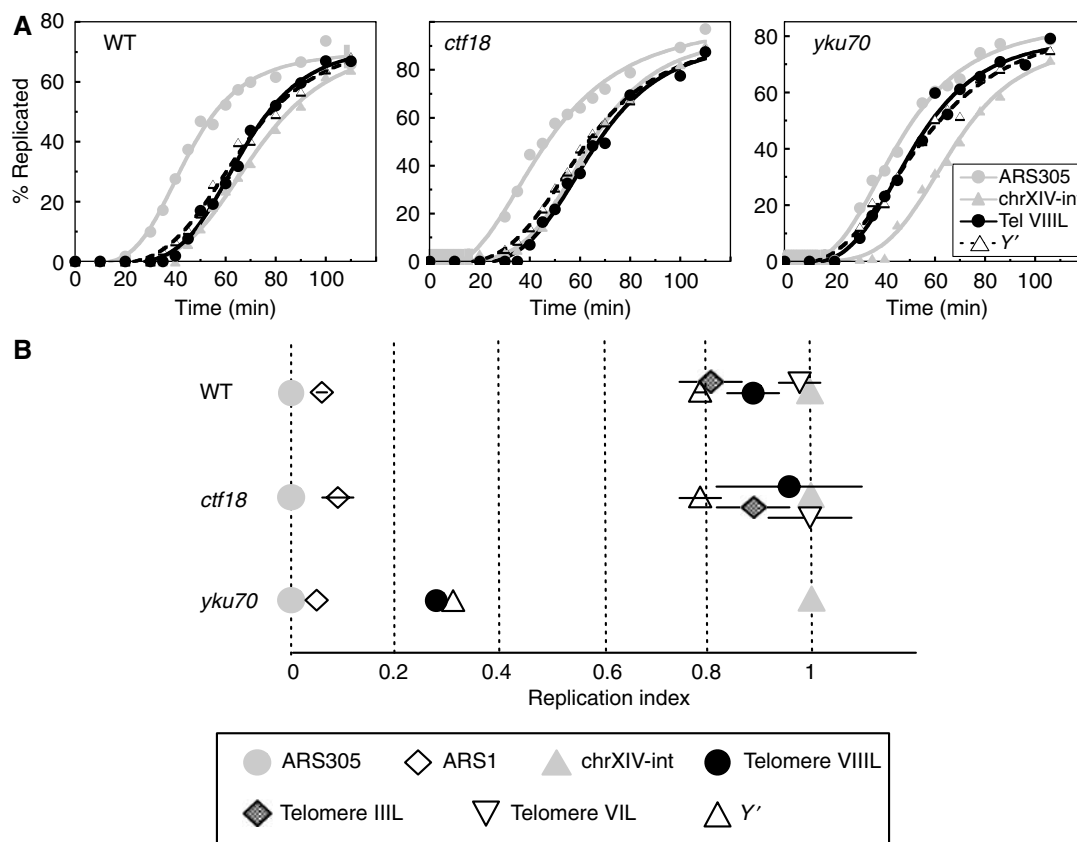


Figure 4 Telomeres replicate at the normal time in a *ctf18* strain. Replication timing programs of WT, *ctf18*, and *yku70* strains were analyzed using the density transfer technique. (A) Replication kinetics of ARS305, chromosome XIV-internal, telomere VIIIIL, and subtelomeric *Y'* sequences are shown for each strain. Percentage of cells that have replicated the various loci is plotted against time after release from α -factor. (B) Replication index values of internal (ARS305, chr XIV-internal, ARS1) and telomere-associated (*Y'*, telomeres VIIIIL, VII, and IIIIL) sequences. For WT and *ctf18* strains, the standard deviation obtained in two independent experiments is indicated by horizontal error bars. For *yku70*, the density-transfer procedure was carried out only once using this synchronization protocol to illustrate the effect on telomere replication timing described previously (Cosgrove *et al*, 2002).

S phase despite the aberrant subnuclear localization of the chromosome ends during the G1 phase. Our observation of telomere delocalization combined with normal replication timing in the *ctf18* strain shows that peripheral positioning of telomeres during G1 is not essential for their late replication.

Ku complex remains bound to a telomere in a *ctf18* strain

The telomere positioning phenotype of *ctf18* resembles that reported for *yku70* and *yku80* mutants (Hediger *et al*, 2002). Normal telomeric replication timing in the *ctf18* mutant suggested that Ku binding to telomeres is intact, but we wished to test directly whether Ku is loaded onto telomeres in the *ctf18* strain. We examined binding of Myc-tagged Yku80 protein to two loci in the vicinity of telomere VIR. Yku80-Myc was bound at the telomeric sequence, but not to the locus 5 kb away from telomere (Figure 5A), consistent with previous studies (Martin *et al*, 1999; Roy *et al*, 2004). We observed no significant change in this telomere-specific bind-

ing in the *ctf18* mutant, showing that Ctf18 is not required for Ku binding to chromosome VIR. Yku70 was required for Yku80 to bind the telomere, as expected since Ku binds DNA as a heterodimer. Consistent with the observation that Ku still binds telomeres in *ctf18*, the *ctf18* mutant has only a slight defect in telomere length control (data not shown; Askree *et al*, 2004; Smolikov *et al*, 2004), while *yku70* mutant displays a severe telomere length defect (Boulton and Jackson, 1996; Porter *et al*, 1996).

We tested whether Ctf18 itself is localized at telomeres. Immunofluorescence and *in vivo* labeling experiments gave no suggestion that Ctf18 is specifically located at telomere clusters (data not shown). The higher sensitivity technique of chromatin immunoprecipitation also provided no evidence for Ctf18 binding specifically to telomere VIR (Figure 5B). It therefore seems unlikely that Ctf18 is a structural component of a telomere peripheral localization pathway, and we believe that it is more likely to play a regulatory role.

CTF18 is required for both Ku and Sir4-mediated telomere positioning pathways during G1

Two molecular pathways have been described that mediate localization of telomeres. Ku complex is believed to form a link between telomeres and the nuclear envelope by binding to an unidentified envelope-bound component. A second pathway involves interaction of the telomere-bound Sir4 protein with the nuclear envelope-bound protein Esc1. To clarify whether Ctf18-RLC affects telomere positioning through the Ku pathway, through the Sir4-Esc1 pathway, or through a previously unidentified pathway, we studied the localization of telomere XIVL in a set of double mutants. This telomere was chosen for analysis because it requires both Ku- and Sir4-dependent pathways for full positioning (Figure 6A); many other telomeres show more complete disruption of positioning on deletion of either of the known pathways (Hediger *et al*, 2002; Taddei *et al*, 2004a), which could obscure additional effects of further mutations.

Telomere XIVL positioning was random during G1 phase in the *sir4 yku70* mutant (Figure 6A), showing that during G1 the Ku and Sir positioning mechanisms are the only pathways involved in localizing this telomere. Either the *sir4* or *yku70* mutations alone resulted in significant peripheral positioning (Figure 6A), demonstrating that each pathway can mediate some positioning independent of the other—that is, in the absence of Sir4, the Ku pathway can position telomere XIVL to some extent and *vice versa*. However, introducing the *ctf18* mutation in the *sir4* background (Figure 6A, *ctf18 sir4*) resulted in completely random telomere positioning, showing that without Ctf18 the Ku pathway can no longer mediate any telomere positioning. Introducing the *ctf18* mutation into the *yku70* background also resulted in random telomere positioning, showing that Ctf18 is also required for the residual telomere positioning by the Sir pathway in the *yku70* mutant. These results suggest that, at least for telomere XIVL, both the Ku and Sir positioning pathways are largely dependent on Ctf18. This interpretation is consistent with the observation that disruption of *CTF18* leads to a more severe positioning defect than either the *sir4* and *yku70* mutations alone (Figure 6A).

We also examined the effects of the various mutations on telomere positioning during S phase. In this case, the *ctf18* mutant retained a significantly higher level of telomere XIVL

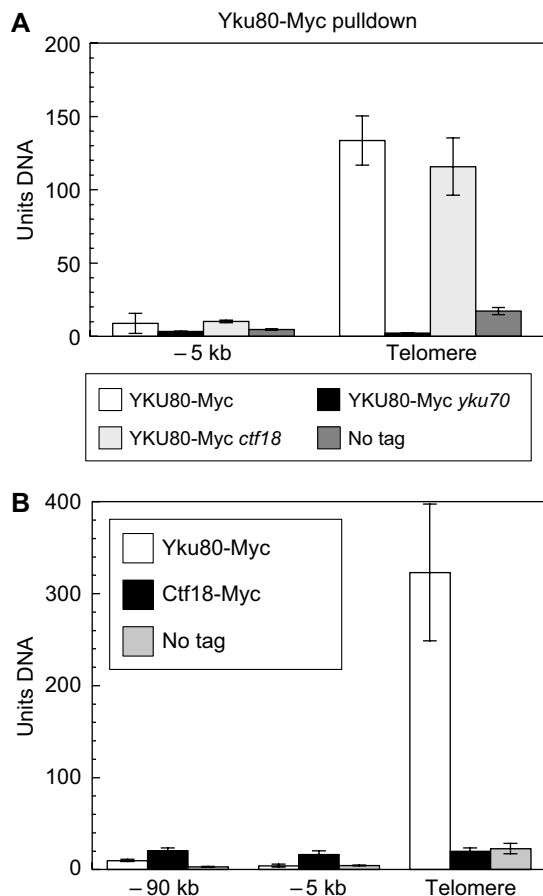


Figure 5 Ku binds telomere VIR in a *ctf18* mutant, and Ctf18 does not bind specifically to telomeres. Chromatin immunoprecipitation followed by quantitative PCR was used to examine binding of Yku80-Myc and Ctf18-Myc to loci in the vicinity of chromosome VIR. Histogram keys shows the tagged protein and any further mutation in each strain examined. (A) Telomere binding of Yku80 in a *ctf18* strain. Binding of Yku80 to telomere VIR appeared similar in WT and *ctf18* backgrounds. (B) Ctf18-Myc does not bind specifically to telomere VIR. Amounts of DNA precipitated from Yku80-Myc and non-tagged strains in the same experiments are shown as controls for telomere-specific binding.

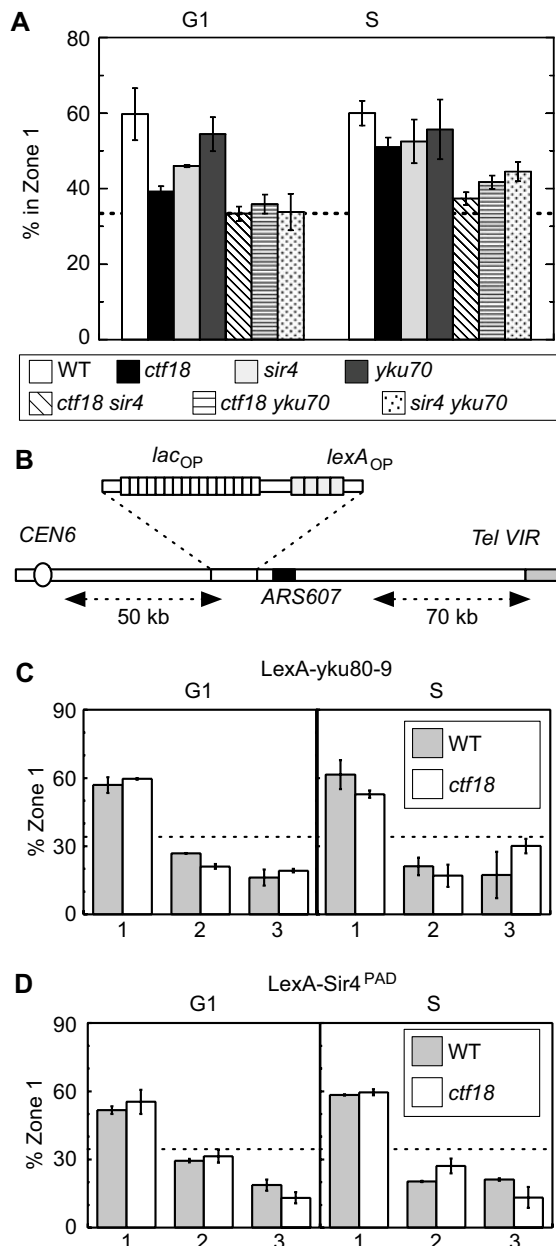


Figure 6 The role of Ctf18 in the telomere position machinery. (A) Localization of telomere XIVL was scored in the indicated mutants as in Figure 3B. The proportion of cells having the telomere positioned peripherally (in Zone 1) is plotted for each strain. Error bars indicate standard deviations obtained from at least two independent cultures. Dotted line represents random distribution. (B) The *ARS607* localization construct used to examine Ku- and Sir4-mediated tethering. Arrays of *lacOP* and *lexAOP* are integrated near *ARS607*. (C) Yku80-mediated tethering of *ARS607* to the nuclear periphery. The position of the *ARS607* locus in WT and *ctf18* strains expressing LexA-yku80-9 fusion protein was scored as in Figure 3. (D) Tethering of *ARS607* mediated by Sir4^{PAD} was examined as in panel (C).

positioning than the *sir4 yku70* double mutant (Figure 6A). One interpretation of this result might be that during S phase, the Ku and Sir positioning pathways are less dependent on Ctf18 than they are in G1. However, during S phase, significant telomere positioning remained in the *sir4 yku70* double mutant (Figure 6A), suggesting that an additional telomere positioning pathway plays a role at this cell cycle stage. It is

Table II Significance of LexA fusion-dependent tethering

| | P-value | | |
|--------------------------------|--------------------------------|--|--------------------------------------|
| | WT against random ^a | <i>ctf18</i> against random ^a | <i>ctf18</i> against WT ^b |
| <i>LexA-yku80-9</i> | | | |
| G1 | 5.28×10^{-10} | 2.17×10^{-12} | 0.18 |
| S | 3.15×10^{-6} | 6.72×10^{-6} | 0.13 |
| <i>LexA-Sir4^{PAD}</i> | | | |
| G1 | 5.88×10^{-5} | 2.01×10^{-6} | 0.19 |
| S | 7.26×10^{-6} | 9.07×10^{-7} | 0.10 |

^aP-values were calculated by χ^2 analysis in which actual distribution was compared to a hypothetical random distribution.

^bP-values were calculated by χ^2 analysis in which observed distribution for *ctf18* was compared to that for WT.

intriguing to speculate that this additional pathway could be related to S phase events such as telomere replication or telomerase extension. Because the components of this additional pathway are unidentified, it is not possible to assess from our results whether Ctf18 is required for this novel positioning mechanism, or whether instead the dependence of the Ku and Sir4 pathways on Ctf18 is altered during S phase. Further clarification will require the identification of components of the S phase-specific Ku/Sir-independent positioning pathway.

CTF18 is not required for Ku-mediated and Sir4-mediated linkage of an internal locus to the nuclear periphery

The double-mutant analysis showed that, at least during G1, the Ku and Sir-mediated telomere positioning pathways are largely dependent on Ctf18. Taddei *et al* (2004a) developed a system that allows artificial tethering of an internally located locus to the nuclear periphery. We wished to examine whether localization of an internal site requires Ctf18, or whether instead it can occur in the absence of Ctf18. We used a strain in which the early replication origin locus *ARS607* is flanked by *lexA* operators (to permit tethering by LexA-Sir4^{PAD} or by LexA-yku80-9) and by a series of *lac* operator sequences (to enable visualization using LacI-GFP) (Figure 6B). The *ARS607* locus is randomly located in a strain bearing this *lac_{op}-lexA_{op}-ARS607* construct if no LexA fusion protein is expressed. As described previously, expression of LexA-yku80-9 leads to significant localization of *ARS607* to the nuclear envelope (Figure 6C). The LexA-yku80-9 construct was still able to localize *ARS607* when *CTF18* was deleted. Similarly, the localization mediated by LexA-Sir4^{PAD} was not affected in the *ctf18* mutant (Figure 6D). χ^2 analysis confirmed that the *ctf18* mutation has no significant effect (Table II). Function of the Ctf18-RLC is therefore not required for Yku80 and Sir4 to bring about the peripheral localization of a chromosomal domain. We conclude that tethering of an ectopic locus to the nuclear periphery by either Yku80 or Sir4^{PAD} bypasses the need for Ctf18.

Discussion

We have found that the Ctf18-RFC-like Complex is critical for correct positioning of *S. cerevisiae* telomeres close to the nuclear periphery, particularly during G1 phase. Our findings

represent the discovery of a new molecular effector of chromosome localization, and the identification of a new role for the Ctf18-RLC in intranuclear organization. In mutants in any of the three of the subunits unique to the Ctf18-RLC (Ctf18, Dcc1, and Ctf8), we observed two phenotypes that indicate disrupted telomere organization—the dislodgement of individual chromosome ends from the nuclear periphery and the dispersal of Rap1 from its normal localization pattern in foci within the nucleus. The Ctf18-RLC is unique among the three known alternative RFC complexes in having this function in chromosome positioning.

The telomere localization defect in a *ctf18* mutant provided the opportunity to test one model for replication timing control. Several studies had suggested a close relationship between late replication and peripheral positioning of the DNA during G1 phase (Dimitrova and Gilbert, 1999; Heun *et al*, 2001a). In particular, deletion of the Yku70 subunit of the Ku heterodimer dislodges telomeres from the nuclear periphery, and simultaneously causes aberrantly early activation of telomere-proximal replication origins during S phase (Laroche *et al*, 1998; Cosgrove *et al*, 2002). Mutation of the Sir proteins causes a less dramatic but still noticeable disruption of telomere localization, and a slight but significant advancement in telomere replication timing (Stevenson and Gottschling, 1999). The *ctf18* mutation clearly abolishes telomere localization to the nuclear periphery during G1, but we found that the telomeres of *ctf18* cells replicate at their normal late time in the S phase, quite unlike the aberrant early telomere replication observed in a *yku70* mutant. The *ctf18* mutant phenotype therefore demonstrates that peripheral localization during G1 is not a prerequisite for the late replication of telomeres, and shows that the mechanisms of replication timing control must be distinct from those controlling G1 telomere intranuclear positioning.

By what mechanism does the Ctf18-RLC affect telomere positioning? This question is difficult to address while the precise molecular role of the Ctf18-RLC remains unclear. *ctf18* mutants are slightly compromised in mating-type and telomeric silencing (Suter *et al*, 2004), but the most prominent previously reported phenotype of *ctf18* is premature separation of sister chromatids (Hanna *et al*, 2001; Mayer *et al*, 2001; Naiki *et al*, 2001). We found that cohesin mutants show only a slight defect in telomere XIVL positioning under conditions where the sister chromatid separation defect is clear (data not shown). Moreover, not all mutants that affect sister chromatid cohesion compromise telomere positioning. For example, *CHL1* is required for cohesion (Petronczki *et al*, 2004; Skibbens, 2004), but the *chl1* mutation did not compromise telomere peripheral positioning as assessed by Rap1 localization and analysis of a tagged telomere (data not shown). Taking these results together, we have found no convincing evidence that the effect of *ctf18* on telomere positioning is a consequence of defective sister chromatid cohesion.

Two independently acting telomere positioning mechanisms have been characterized in budding yeast—the Ku-dependent and Sir4/Esc1-dependent pathways. Ctf18 is required for the full activity of both pathways (Figure 6A). Ctf18-RLC is not required to load the Ku complex (Figure 5A), and chromatin fractionation experiments (not shown) gave no suggestion that the association of Ku with chromatin is altered in a *ctf18* mutant strain. *ctf18* and *dcc1* strains retain significant telomeric silencing (Suter *et al*, 2004), implying

that Sir and Rap1 loading onto telomeres (which is essential for telomeric silencing) is not severely compromised. Since it does not seem to be required to load Ku or Sir complexes, Ctf18-RLC must presumably play a regulatory role to activate telomere positioning by the Ku- and Sir-dependent pathways. Ku or Sir fragments tethered to an internal locus are capable of re-positioning that chromosome domain in a *ctf18* mutant, showing that isolated fragments can bypass the need for Ctf18-RLC to activate peripheral positioning (Figure 6). These results are consistent with models in which Ctf18-RLC ‘unmasks’ the inherent positioning capability of telomeric chromatin. The requirement for Ctf18 for localization of telomeres therefore probably reflects a particular characteristic of telomeric chromatin, such as the need for a regulatory modification to telomeric chromatin to establish its competence for linkage to the periphery. For example, a post-translational modification of another telomeric protein (such as Rap1) might be required to allow linkage of telomeric heterochromatin to the nuclear periphery by Ku and Sir proteins.

The role of Ctf18 in regulating telomere positioning is doubly mysterious since the Ctf18-RLC is proposed to act at replication forks during S phase (see below) whereas the *ctf18* mutation is most deleterious to telomere positioning during G1 phase. It would be informative to test whether the presence of Ctf18-RLC during DNA replication is required for telomere positioning in the subsequent G1 and S phase.

What is the relationship between the regulatory role of Ctf18-RLC in telomere positioning and the molecular function of RFC-like complexes? RFC itself loads the ring-shaped sliding clamp PCNA onto replication forks, while Rad24-RLC loads the ring-shaped ‘9-1-1 complex’. By analogy, Ctf18-RLC is believed to load or unload a ring complex. Yeast Ctf18-RLC can unload PCNA from DNA very efficiently *in vitro* (Bylund and Burgers, 2005), and PCNA unloading after DNA synthesis is the clearest suggestion for the biochemical function of Ctf18-RLC (Bylund and Burgers, 2005). However, it is not obvious why compromised PCNA unloading should lead to either telomere de-positioning or defective sister chromatid cohesion. It is noteworthy that the Ctf8 and Dcc1 subunits of Ctf18-RLC are required for telomere positioning (Figure 2) and establishment of cohesion (Hanna *et al*, 2001; Mayer *et al*, 2001), but dispensable for *in vitro* PCNA unloading. Perhaps, Ctf18-RLC has two activities, and the apparently unrelated *in vitro* and *in vivo* observations of its properties reflect different aspects of its function. We suggest that PCNA unloading by Ctf18-RLC might be coupled *in vivo* to another chromatin modification that is required to activate the Ku and Sir telomere positioning pathways in the subsequent G1 phase. Unlike the PCNA-unloading step, the second, coupled step would be expected to require Ctf8 and Dcc1 since these subunits are required for telomere positioning. One possibility is that the second activity of Ctf18-RLC involves loading of another ring-shaped complex.

Ctf18-RLC could conceivably play a related role at other chromosomal loci, for example, to establish cohesin loading sites as competent for sister chromatid attachment. If so, Ctf18-RLC might be envisaged as having a general involvement in activating particular properties of specialized chromatin sites following DNA replication. It will be of interest to investigate whether the complex is involved in regulating chromosome organization within mammalian nuclei.

Materials and methods

Yeast strains

Gene deletion collections were purchased from EUROSCARF. Other strains are described in Supplementary data.

Plasmids

Plasmid pAT4-yku80-9 (encoding Yku80-9 fused to LexA) and pAT4-Sir4^{PAD} (encoding Sir4^{PAD} fused to LexA) were as described (Taddei *et al*, 2004a). Additional plasmids are described in Supplementary data.

Microscopic techniques

Cell cycle classification was as follows: unbudded cells = G1 phase; bud size less than 2 μm = S phase; bud larger than 2 μm with round nucleus not at the bud neck = G2; bud larger than 2 μm with elongated nucleus at the bud neck = M phase. Microscopic techniques are described in Supplementary data.

Chromatin immunoprecipitation

Chromatin immunoprecipitation of Myc-tagged Yku80 and Ctf18 proteins was performed as described (Strahl-Bolsinger *et al*, 1997;

Tanaka *et al*, 1997), using monoclonal anti-Myc antibody (9E11) (Abcam). Units of DNA in each PCR reaction were calculated relative to amplification of a dilution series of whole-genomic standard DNA by the same primer pair. Details of primer pairs used are described in Supplementary data.

Analysis of replication timing program

Dense isotope transfer experiments were carried out as described previously (Donaldson *et al*, 1998), using α -factor synchronization and release at 30°C in light medium. Probes are described in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

Thanks to Luis Aragon, Aki Hayashi-Hagihara, Yasushi Hiraoka, Susan Gasser and Virginia Zakian for materials, and to Tomoyuki Tanaka and Kozo Tanaka for technical advice. Berndt Müller and Conrad Nieduszynski provided comments on the manuscript.

References

- Andrulis ED, Neiman OM, Zappulla DC, Sternglanz R (1998) Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature* **394**: 592–595
- Askree SH, Yehuda T, Smolnikov S, Gurevich R, Hawk J, Coker C, Krauskopf O, Kupiec M, McEachern MJ (2004) A genome-wide screen for *Saccharomyces cerevisiae* deletion mutants that affect telomere length. *Proc Natl Acad Sci USA* **101**: 8658–8663
- Bermudez VP, Maniwa Y, Tappin I, Ozato K, Yokomori K, Hurwitz J (2003) The alternative Ctf18-Dcc1-Ctf8-replication factor C complex required for sister chromatid cohesion loads proliferating cell nuclear antigen onto DNA. *Proc Natl Acad Sci USA* **100**: 10237–10242
- Boulton SJ, Jackson SP (1996) Identification of a *Saccharomyces cerevisiae* Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. *Nucleic Acids Res* **24**: 4639–4648
- Bowman GD, O'Donnell M, Kuriyan J (2004) Structural analysis of a eukaryotic sliding DNA clamp-clamp loader complex. *Nature* **429**: 724–730
- Bylund GO, Burgers PM (2005) Replication protein A-directed unloading of PCNA by the Ctf18 cohesion establishment complex. *Mol Cell Biol* **25**: 5445–5455
- Bystricky K, Laroche T, van Houwe G, Blaszczyk M, Gasser SM (2005) Chromosome looping in yeast: telomere pairing and coordinated movement reflect anchoring efficiency and territorial organization. *J Cell Biol* **168**: 375–387
- Cosgrove AJ, Nieduszynski CA, Donaldson AD (2002) Ku complex controls the replication time of DNA in telomere regions. *Genes Dev* **16**: 2485–2490
- Dimitrova DS, Gilbert DM (1999) The spatial position and replication timing of chromosomal domains are both established in early G1 phase. *Mol Cell* **4**: 983–993
- Donaldson AD, Raghuraman MK, Friedman KL, Cross FR, Brewer BJ, Fangman WL (1998) *CLB5*-dependent activation of late replication origins in *S. cerevisiae*. *Mol Cell* **2**: 173–182
- Ferguson BM, Fangman WL (1992) A position effect on the time of replication origin activation in yeast. *Cell* **68**: 333–339
- Gartenberg MR, Neumann FR, Laroche T, Blaszczyk M, Gasser SM (2004) Sir-mediated repression can occur independently of chromosomal and subnuclear contexts. *Cell* **119**: 955–967
- Gilbert DM (2002) Replication timing and transcriptional control: beyond cause and effect. *Curr Opin Cell Biol* **14**: 377–383
- Gilbert N, Boyle S, Fiegler H, Woodfine K, Carter NP, Bickmore WA (2004) Chromatin architecture of the human genome: gene-rich domains are enriched in open chromatin fibers. *Cell* **118**: 555–566
- Gotta M, Laroche T, Formenton A, Maillat L, Scherthan H, Gasser SM (1996) The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type *Saccharomyces cerevisiae*. *J Cell Biol* **134**: 1349–1363
- Gottschling DE, Aparicio OM, Billington BL, Zakian VA (1990) Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell* **63**: 751–762
- Gravel S, Larrivee M, Labrecque P, Wellinger RJ (1998) Yeast Ku as a regulator of chromosomal DNA end structure. *Science* **280**: 741–744
- Hanna JS, Kroll ES, Lundblad V, Spencer FA (2001) *Saccharomyces cerevisiae* CTF18 and CTF4 are required for sister chromatid cohesion. *Mol Cell Biol* **21**: 3144–3158
- Hayashi A, Ogawa H, Kohno K, Gasser SM, Hiraoka Y (1998) Meiotic behaviours of chromosomes and microtubules in budding yeast: relocalization of centromeres and telomeres during meiotic prophase. *Genes Cells* **3**: 587–601
- Hediger F, Neumann FR, Van Houwe G, Dubrana K, Gasser SM (2002) Live imaging of telomeres: yKu and Sir proteins define redundant telomere-anchoring pathways in yeast. *Curr Biol* **12**: 2076–2089
- Heun P, Laroche T, Raghuraman MK, Gasser SM (2001a) The positioning and dynamics of origins of replication in the budding yeast nucleus. *J Cell Biol* **152**: 385–400
- Heun P, Laroche T, Shimada K, Furrer P, Gasser SM (2001b) Chromosome dynamics in the yeast interphase nucleus. *Science* **294**: 2181–2186
- Kenna MA, Skibbens RV (2003) Mechanical link between cohesion establishment and DNA replication: Ctf7p/Eco1p, a cohesion establishment factor, associates with three different replication factor C complexes. *Mol Cell Biol* **23**: 2999–3007
- Kim J, MacNeill SA (2003) Genome stability: a new member of the RFC family. *Curr Biol* **13**: R873–R875
- Laroche T, Martin SG, Gotta M, Gorham HC, Pryde FE, Louis EJ, Gasser SM (1998) Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres. *Curr Biol* **8**: 653–656
- Martin SG, Laroche T, Suka N, Grunstein M, Gasser SM (1999) Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. *Cell* **97**: 621–633
- Mayer ML, Gygi SP, Aebersold R, Hieter P (2001) Identification of RFC(Ctf18p, Ctf8p, Dcc1p): an alternative RFC complex required for sister chromatid cohesion in *S. cerevisiae*. *Mol Cell* **7**: 959–970
- Merkle CJ, Karnitz LM, Henry-Sanchez JT, Chen J (2003) Cloning and characterization of hCTF18, hCTF8, and hDCC1. Human homologs of a *Saccharomyces cerevisiae* complex involved in sister chromatid cohesion establishment. *J Biol Chem* **278**: 30051–30056
- Naiki T, Kondo T, Nakada D, Matsumoto K, Sugimoto K (2001) Chl12 (Ctf18) forms a novel replication factor C-related complex and functions redundantly with Rad24 in the DNA replication checkpoint pathway. *Mol Cell Biol* **21**: 5838–5845
- Ohta S, Shiomi Y, Sugimoto K, Obuse C, Tsurimoto T (2002) A proteomics approach to identify proliferating cell nuclear antigen

- (PCNA)-binding proteins in human cell lysates. Identification of the human CHL12/RFCs2-5 complex as a novel PCNA-binding protein. *J Biol Chem* **277**: 40362–40367
- Palladino F, Laroche T, Gilson E, Axelrod A, Pillus L, Gasser SM (1993) SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. *Cell* **75**: 543–555
- Petronczki M, Chwalla B, Siomos MF, Yokobayashi S, Helmhart W, Deutschbauer AM, Davis RW, Watanabe Y, Nasmyth K (2004) Sister-chromatid cohesion mediated by the alternative RF-C^{Ctf18/Dcc1/Ctf8}, the helicase Chl1 and the polymerase-alpha-associated protein Ctf4 is essential for chromatid disjunction during meiosis II. *J Cell Sci* **117**: 3547–3559
- Porter SE, Greenwell PW, Ritchie KB, Petes TD (1996) The DNA-binding protein Hdf1p (a putative Ku homologue) is required for maintaining normal telomere length in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **24**: 582–585
- Raghuraman MK, Brewer BJ, Fangman WL (1997) Cell cycle-dependent establishment of a late replication program. *Science* **276**: 806–809
- Raghuraman MK, Winzeler EA, Collingwood D, Hunt S, Wodicka L, Conway A, Lockhart DJ, Davis RW, Brewer BJ, Fangman WL (2001) Replication dynamics of the yeast genome. *Science* **294**: 115–121
- Roy R, Meier B, McAinsh AD, Feldmann HM, Jackson SP (2004) Separation-of-function mutants of yeast Ku80 reveal a Yku80p-Sir4p interaction involved in telomeric silencing. *J Biol Chem* **279**: 86–94
- Skibbens RV (2004) Chl1p, a DNA helicase-like protein in budding yeast, functions in sister-chromatid cohesion. *Genetics* **166**: 33–42
- Smolnikov S, Mazor Y, Krauskopf A (2004) *ELG1*, a regulator of genome stability, has a role in telomere length regulation and in silencing. *Proc Natl Acad Sci USA* **101**: 1656–1661
- Stellwagen AE, Haimberger ZW, Veatch JR, Gottschling DE (2003) Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. *Genes Dev* **17**: 2384–2395
- Stevenson JB, Gottschling DE (1999) Telomeric chromatin modulates replication timing near chromosome ends. *Genes Dev* **13**: 146–151
- Strahl-Bolsinger S, Hecht A, Luo K, Grunstein M (1997) SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev* **11**: 83–93
- Straight AF, Belmont AS, Robinett CC, Murray AW (1996) GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. *Curr Biol* **6**: 1599–1608
- Suter B, Tong A, Chang M, Yu L, Brown GW, Boone C, Rine J (2004) The origin recognition complex links replication, sister chromatid cohesion and transcriptional silencing in *Saccharomyces cerevisiae*. *Genetics* **167**: 579–591
- Taddei A, Hediger F, Neumann FR, Bauer C, Gasser SM (2004a) Separation of silencing from perinuclear anchoring functions in yeast Ku80, Sir4 and Esc1 proteins. *EMBO J* **23**: 1301–1312
- Taddei A, Hediger F, Neumann FR, Gasser SM (2004b) The function of nuclear architecture: a genetic approach. *Annu Rev Genet* **38**: 305–345
- Tanabe H, Habermann FA, Solovei I, Cremer M, Cremer T (2002) Non-random radial arrangements of interphase chromosome territories: evolutionary considerations and functional implications. *Mutat Res* **504**: 37–45
- Tanaka T, Knapp D, Nasmyth K (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90**: 649–660
- Tham WH, Wyithe JS, Ko Ferrigno P, Silver PA, Zakian VA (2001) Localization of yeast telomeres to the nuclear periphery is separable from transcriptional repression and telomere stability functions. *Mol Cell* **8**: 189–199
- Woodfine K, Fiegler H, Beare DM, Collins JE, McCann OT, Young BD, Debernardi S, Mott R, Dunham I, Carter NP (2004) Replication timing of the human genome. *Hum Mol Genet* **13**: 191–202