The effect of Ku on telomere replication time is mediated by telomere length but is independent of histone tail acetylation


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ABSTRACT DNA replication in Saccharomyces cerevisiae proceeds according to a temporal program. We have investigated the role of the telomere-binding Ku complex in specifying late replication of telomere-proximal sequences. Genome-wide analysis shows that regions extending up to 80 kb from telomeres replicate abnormally early in a yku70 mutant. We find that Ku does not appear to regulate replication time by binding replication origins directly, nor is its effect on telomere replication timing mediated by histone tail acetylation. We show that Ku instead regulates replication timing through its effect on telomere length, because deletion of the telomerase regulator Pif1 largely reverses the short telomere defect of a yku70 mutant and simultaneously rescues its replication timing defect. Consistent with this conclusion, deleting the genome integrity component Elg1 partially rescued both length and replication timing of yku70 telomeres. Telomere length–mediated control of replication timing requires the TG1–3 repeat-counting component Rif1, because a rif1 mutant replicates telomeric regions early, despite having extended TG1–3 tracts. Overall, our results suggest that the effect of Ku on telomere replication timing results from its impact on TG1–3 repeat length and support a model in which Rif1 measures telomere repeat length to ensure that telomere replication timing is correctly programmed.

INTRODUCTION

DNA replication in eukaryotes initiates from multiple chromosomal loci called replication origins. In Saccharomyces cerevisiae, potential replication origin sites are termed autonomously replicating sequence (ARS) elements and are largely specified by DNA sequence (Nieduszynski et al., 2006). Replication initiation is tightly controlled to ensure that each origin initiates, or “fires,” no more than once during each S phase. Initiation of replication does not occur simultaneously at all origins but rather according to a temporal program. Some origins initiate replication at the onset of S phase, and others only later (Reynolds et al., 1989; Ferguson et al., 1991), as revealed by microarray-based genome-wide studies (Raghuraman et al., 2001; Yabuki et al., 2002; McCune et al., 2008). Such genome-wide studies facilitated the identification of replication origins as the earliest-replicating sequences in their chromosome localities.

Chromosomal context is important for determining the processional order of origin firing (Ferguson and Fangman, 1992; Friedman et al., 1996; Raghuraman et al., 1997). For example, regions within 35 kb of a telomere typically replicate later in S phase than the genomic average (Raghuraman et al., 2001), and proximity to a telomere is one of the chromosome contexts that tends to cause late origin initiation. Telomere-specified late replication is dependent on the presence of the chromosome end—that is, telomeric sequences placed in the context of a circular plasmid do not delay replication (Ferguson and Fangman, 1992). Two telomeric...
protein complexes have been implicated in specifying late replication close to telomere V-right—the Sir silencing complex (consisting of Sir2, Sir3, and Sir4 proteins) and the Ku complex (a heterodimer consisting of Yku70 and Yku80 subunits that binds to DNA ends, including double-stranded breaks and telomeres) (Stevenson and Gottschling, 1999; Cosgrove et al., 2002). Of these, the Ku complex has the greater effect, affecting the replication time of both subtelomeric sequences (such as Y′ elements) and telomere-proximal replication origins (such as ARS522, previously called ARS501, which is located 27 kb from chromosome V-right) (Cosgrove et al., 2002). The yku70 mutation causes abnormally early telomere replication, possibly through a combined effect on origin initiation time and efficiency (Cosgrove et al., 2002).

We do not understand in molecular terms how cells control the replication timing program, but there is evidence suggesting that chromatin organization or modification plays an important role. In particular, it was shown that increased histone H4 K12 N-terminal tail acetylation in an rpd3 mutant, which lacks a major cellular histone deacetylase, correlates with an advancement in origin replication time (Vogelauer et al., 2002; Aparicio et al., 2004; Knott et al., 2009). Moreover, tethering the histone acetyltransferase Gcn5 close to a normally late-initiating replication origin resulted in increased histone H3 lysine 18 acetylation and earlier initiation of the origin in S phase (Vogelauer et al., 2002). These experiments showed that changes in histone tail acetylation state can alter replication timing, suggesting a mechanism for determining the timing program. Perhaps surprisingly, however, there is no clear relationship in vivo between the level of histone tail acetylation at origins and their replication times (Nieduszynski et al., 2006).

Evidence is emerging for a link between the mechanisms that control telomere length maintenance and replication timing. A recent investigation showed that shortening a telomere early in the cell cycle, using a system in which telomere repeat sequences are recombinationally excised, causes it to replicate earlier in the subsequent S phase (Bianchi and Shore, 2007). This study established a connection between the length of a telomere and its time of replication, but provided no suggestion as to the mechanism by which telomere length can affect initiation of a replication origin tens of kilobases distant.

Here we examine the replication program of a yku70 mutant genome-wide. In the absence of Yku70 function, at most chromosome ends a region extending up to 80 kb from the telomere replicates earlier than in wild-type cells. In contrast, internal chromosome loci generally maintain their normal replication time. To test whether the effect of Ku on the replication timing of telomeric regions is mediated by histone N-terminal tail acetylation, we used chromatin immunoprecipitation (ChIP) to examine histone acetylation at replication origin sites that show an altered replication time. We observed no effect of the yku70 mutation on acetylation of the histone 4 tail or H3 lysine 18 at such telomere-proximal origins, suggesting that Ku affects origin replication time through a mechanism that is independent of these histone modifications. Next we tested whether the effect of Ku on replication timing is related to telomere length. Combining yku70 with the telomere-elongating mutations pif1 or elg1 led to rescue of both telomere length and replication timing defects, suggesting that the effect of the yku70 mutation on replication timing is mediated by telomere shortening. Analysis of the replication program of a rnf1 mutant suggests that telomere length measurement by the TG1-3 repeat binding protein Rif1 is critical for control of replication timing by telomere terminal repeat length. Our results support the idea of a close functional relation between telomere length maintenance and replication timing (Bianchi and Shore, 2007).

RESULTS

Yeast telomeres replicate earlier in the yku70 mutant

In the absence of Ku complex, certain telomeric regions replicate abnormally early in S phase (Cosgrove et al., 2002). We wished to examine the range of the effect of Ku on chromosome ends, whether all telomeres were affected, and whether any other regions of the genome alter their replication time in the absence of Ku complex. To address these questions, we used a microarray-based method previously used to examine S. cerevisiae genomic replication dynamics (Raghuraman et al., 2001). Our modification to the approach used isotopic labeling of newly replicated DNA to measure the proportion of each genomic sequence that has been replicated when a culture is in the middle of S phase. YKU70 ("wild type") or yku70 mutant cells were grown in medium containing heavy isotopes of carbon and nitrogen, arrested before S phase, and then released in isotopically light medium (Figure 1A). For each culture, a large sample was taken at a predetermined mid-S phase time point (25 min after release). Replicated heavy-light (HL) DNA was separated from unreplicated heavy-heavy (HH) DNA by cesium chloride gradient fractionation. Replicated and unreplicated DNA fractions were fluorescently labeled, pooled, and hybridized to genomic microarrays. The representation of each genomic sequence in the HL and HH DNA fractions depends on its time of replication: The earliest-replicating sequences will already have moved to the HL DNA fraction at mid-S phase, whereas late-replicating sequences remain in the HH fraction (Raghuraman et al., 2001). We found that the percentage replication of each sequence at mid-S phase shows an inverse linear relationship to its replication time (Supplemental Figure S1), allowing the array-based percentage replication measurements to be plotted as proxies for replication times of each sequence in S phase (see Materials and Methods). Specimen plots obtained for chromosomes III, V, and VI are shown in Figure 1B. A comparison with previous data sets is shown in Supplemental Figure S2, and wild-type and yku70 replication timing plots for the entire genome are presented in Supplemental Figure S3. To assemble these plots, the raw (%) replicated data were subjected to Fourier convolution and sliding window smoothing (Alvino et al., 2007) before further processing as described in Materials and Methods, including transformation to replication time (T_rep) based on the graphs in Supplemental Figure S1A.

The replication timing plots reveal that, in general, the replication dynamics of the yku70 mutant closely mirror those of wild-type cells throughout internal chromosomal regions. Most telomeric regions, however, replicate noticeably earlier in yku70 than in wild type (with the chromosome ends earlier by an average of 6.0 ± 1.3 min). For example, loci within 35 kb of the right end of chromosome III replicate between 6 and 10 min earlier in the yku70 mutant than in wild type (Figure 1B). Loci close to the right end of chromosome V replicate 6–7 min earlier in yku70 than in wild type, consistent with previous observations (Cosgrove et al., 2002). These differences are apparent even without correction for the ~3-min delay shown by yku70 mutants in entering S phase after release from α-factor (see Cosgrove et al., 2002, and axes in Figure 1B plots). The magnitude of the replication time advancement varies among telomeres (see Supplemental Table S2) and extends variable distances into the interior of the chromosome arms. Plotting the difference in replication time between wild type and yku70 against distance from the closest telomere for all genomic loci (Figure 2A) confirms that the largest differences in replication timing are observed at the chromosome ends. The effect on replication timing decreases gradually with increasing distance from telomeres, so that replication times of loci more than 80 kb from a telomere are almost unaffected by the yku70 mutation.
The variability seen in the effect of *yku70* on different chromosome ends may reflect the particular configuration of nearby replication origins and their time and efficiency of activation. In many cases, the change in replication timing could be explained by an effect of Ku on the X and/or Y ARS elements within the subtelomeric repeat sequences, which, because of their repetitive nature, are not represented in the microarray profiles. Analysis of a truncated chromosome V-right construct lacking the X and Y ARS elements demonstrated that the *yku70* mutation also advances the initiation time of the telomere-proximal origin ARS522 (Cosgrove et al., 2002). Supplemental Figure S3 timing profiles suggest that, in several other telomeric regions, origins at some distance from the chromosome end show advanced time of activation (in particular, at I-left, III-left and -right, IV-right, and XII-right)—indicating that in these cases at least, the effect of Ku extends tens of kilobases and probably affects multiple origins. Two early-replicating origin sites located fairly close to chromosome ends (37.5 kb from telomere III-left and 28 kb from telomere X-right) also displayed an advancement in replication time in *yku70*, although the effects on these origins were reduced in magnitude compared with late origins. Figure 2B plots the replication timing difference at all origin sites identified within 100 kb of a telomere. Most origins within 40 kb of a telomere showed an advancement in replication time in *yku70*, with the magnitude of the effect generally reflecting the proximity of the origin to the chromosome end. The one exception was an origin located 28 kb from the chromosome XIII-right telomere, the replication time of which was unaltered in *yku70*. At distances greater than 50 kb from telomeres, the effects of the *yku70* mutation on origin replication time appeared...
The replication dynamics of telomere-proximal regions: It causes an increase in their escape replication at a cdc7 block (as shown in Figure 1C and Supplemental Figure S4) and separately advances their replication time within S phase (as shown in Figure 1B and Supplemental Figure S3).

The effect of the yku70 mutation appeared more extensive on chromosome I than on other chromosomes (Supplemental Figure S3). More detailed analysis of the replication dynamics of a nontelomeric origin site at ~70 kb on chromosome I (corresponding to ARS106; Nieduszynski et al., 2007) confirmed that this locus replicates earlier in the yku70 mutant strain and shows pronounced escape replication (Figure 3, A and C). ARS106 replication was, however, not advanced in yku70 when only α-factor (and not the cdc7 block) was used for synchronization (Figure 3, B and C), in contrast to telomeric sequences. It is unclear why chromosome I behaves differently from other chromosomes, but this observation suggests that the more extensive effects of yku70 on chromosome I replication dynamics may be related to use of the cdc7 block.

Ku complex is not present at telomere-proximal replication origins

Ku has been detected as binding only within 7.5 kb of telomeres (Martin et al., 1999) but can affect the replication time of origins up to 40 kb distant from a telomere (Figure 2 and Cosgrove et al., 2002). We investigated possible mechanisms by which Ku might exert this long-range effect on the replication timing of telomere-proximal replication origins. It has been suggested that the Ku heterodimer may bind replication origins in both yeast and mammalian cells (Shakibai et al., 1996; Novac et al., 2001), so we tested whether Ku affects telomere-proximal replication origins by binding them directly. ChIP was used to enrich for Yku80-bound DNA regions, and the sequences recovered were measured using quantitative PCR (qPCR). We first examined Yku80 binding at two control sequences close to telomere VI-right (Figure 4A). High levels of Yku80 binding to a sequence 300 bp from telomere VI-right were observed, as expected because Ku is known to bind telomeres. Slight, residual binding of Ku80 was observed at a sequence 5 kb from telomere VI-right (Figure 4A), but analysis of sites more distant from the chromosome end did not suggest any spreading of Ku farther from the telomere (unpublished data). A yku70 Yku80-Myc strain served as a “no binding” control, because Yku80 binds DNA only as an obligate heterodimer with Yku70 (Griffith et al., 1992; Ono et al., 1994). Yku80 binding to the telomere region was completely lost in this control strain, as expected.

We examined Ku binding at three telomere-proximal replication origins within regions showing advanced replication time in the absence of Ku function: ARS609 (14 kb from the chromosome VI-right telomere), ARS316 (43 kb from the chromosome III-right telomere), and ARS522 (27 kb from the chromosome V-right telomere). Our ChIP analysis revealed no significant enrichment of Yku80 at any of these telomere-proximal replication origins in log phase cell cultures (Figure 4A). The residual signal obtained at ARS609, ARS316, and minimal (Figure 2B). The fact that the effect of the yku70 mutation on replication timing propagates somewhat further than its effect on origins is as expected, because earlier origin initiation will cause an advancement in replication time of all sequences within that replicon, including those on the centromere-proximal side of the origin. To summarize, the results of our genome-wide replication timing analysis confirmed that the Ku complex causes telomeric regions to replicate late and showed that Ku has no general effect on the replication temporal program of internal chromosomal domains.

Effect of cdc7 synchronization on replication timing

In the microarray replication timing experiments, the cultures were synchronized using sequential α-factor and cdc7 temperature blocks. Low levels of “escape” replication are seen from certain very early-initiating replication origins (e.g., ARS306) in cells depleted of Cdc7 activity in both control and yku70 mutant strains (Reynolds et al., 1989; Donaldson et al., 1998a). Interestingly, microarray-based analysis of zero-time-point (i.e., cdc7 blocked) samples showed that telomeric regions in the yku70 strain are particularly prone to escape replication (Figure 1C and Supplemental Figure S4). The data plotted in Figure 1B, Supplemental Figure S3, and Figure 2 have been corrected (as described in Reynolds et al., 1989), to subtract the effects of escape replication from the proportion of replicated DNA in mid-S phase. The effect of yku70 on telomere replication time is apparent even after correcting for escape replication; therefore the yku70 mutation can be regarded as having two separate effects on replication dynamics of telomere-proximal regions: It causes an increase in their escape replication at a cdc7 block (as shown in Figure 1C and Supplemental Figure S4) and separately advances their replication time within S phase (as shown in Figure 1B and Supplemental Figure S3).

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ARS522 was similar to that found for nonorigin and internally located sequences (unpublished data). To test for a transient interaction of Ku with affected origins occurring only during replication, ChIP experiments were also carried out using synchronized cultures (Figure 4B). Even in mid-S phase cells, we observed no significant interaction of Ku80 with ARS522 (Figure 4C) or ARS609 (Supplemental Figure S5).

Overall, we find no evidence for binding of the Ku complex to replication origin sites. Although we cannot rule out the possibility of an interaction undetectable by ChIP, these results indicate that Ku is unlikely to influence the replication timing of telomeric regions by interacting directly with telomere-proximal origins.

**No change in histone 4 or histone 3 N-terminal tail acetylation in yku70**

Alterations to histone tail acetylation levels in nucleosomes surrounding replication origins can affect their initiation time (Yabuki et al., 2002; Aparicio et al., 2004). We investigated whether altered histone tail acetylation close to telomere-proximal replication origins in the yku70 mutant might account for their altered replication timing. First, we tested whether the N-terminal tail of histone H4 shows increased acetylation in yku70 at telomere-proximal origins in ChIP experiments using an acetylated H4-specific antibody (raised against an H4 peptide acetylated at lysines 5, 8, 12, and 16). The antibody was first tested in Western blots to confirm its specificity (Figure 5A). As expected, mutating the four target lysine residues to glycine abolished antibody recognition of histone H4. Esa1 is an H4-specific acetylase and, in a temperature-sensitive esa1Δ-414 mutant, the level of histone H4 acetylation is reduced at the restrictive temperature (Clarke et al., 1999). The antibody did not recognize histone H4 in the esa1Δ-414 mutant, the level of histone H4 acetylation is reduced at the restrictive temperature of 37°C, confirming its specificity for the acetylated form of histone H4 (Figure 5A). Conversely, the Western blot signal was increased by deletion of the histone deacetylase Rpd3, further confirming specificity for acetylated forms of histone H4.

We used this acetylation-specific antibody to test for a change in histone H4 acetylation corresponding to the observed change in replication time. In yku70, H4 acetylation levels were unaffected (i.e., within experimental error) at origin ARS609 and at loci 500 bp on either side (Figure 5B). This result suggested that altered H4 tail acetylation in the ARS609 region does not account for the change in replication dynamics of the telomere VI-right region in yku70. Ku function is required for the establishment of telomeric silencing through Sir2-mediated histone deacetylation in regions close to (generally within ~4 kb of) telomeres (Robyr et al., 2002). We did observe approximately threefold increased recovery in the yku70 mutant of a sequence at the chromosome VI-right telomere (Figure 5B, 300 bp), confirming that we were able to detect changes in histone H4 acetylation state. The increased acetylation that we observed at the end of chromosome VI-right in yku70 is likely due to the reduced recruitment of the repressive Sir complex that contains the histone H4 lysine 16 deacetylase Sir2.

Examination of other telomere-proximal origins, ARS316 and ARS522, which showed precocious replication in the yku70 mutant,
Acetylated H3 Lys-18, we used this antibody in ChIP experiments to examine histone H3 Lys-18 acetylation levels at ARS316, ARS522, and ARS609 (Figure 5E). We observed no significant difference in acetylation of H3 Lys-18 at or close to any of these origins. On the basis of this result, we conclude that deacetylation of the histone H3 Lys-18 residue is not the mechanism by which Ku regulates the replication time of telomere-proximal origins.

In ChIP experiments to examine histone acetylation state (such as those shown in Figure 5), it is possible that high-density nucleosomes that have a low level of acetylation could produce a signal equal to that from lower density, more highly acetylated nucleosomes. To test whether changes in acetylation state at telomere-proximal origins might be “masked” by altered nucleosome occupancy, we examined histone H3 association with telomere-proximal replication origin loci using an antibody against unmodified histone 3 (Supplemental Figure S6A). We found no change in the level of histone H3 occupancy in the yku70 mutant close to ARS316 (Supplemental Figure S6B) or other telomere-proximal replication origins (unpublished data). The failure to observe increased histone acetylation at telomere-proximal origins in yku70 therefore does not result from reduced nucleosome occupancy.

Rescuing telomere length in the yku70 mutant restores late telomere replication

The yku70 mutant has short telomeres, caused by defects in telomerase recruitment and telomere capping (Peterson et al., 2001; Maringele and Lydall, 2002; Stellwagen et al., 2003; Fisher et al., 2004). Telomere shortening has been shown to lead to earlier telomere replication (Bianchi and Shore, 2007). To test whether the effect of yku70 on telomere replication time is caused by its shortened telomeres, we examined the effect of deleting the PIF1 gene. Pif1 is a 5′-directed helicase that negatively regulates telomere length. Pif1 limits telomerase processivity by unwinding of the RNA:DNA hybrid involved in TG1–3 tract extension (Zhou et al., 2000; Boule et al., 2005; Bochman et al., 2010). Loss of this helicase activity causes pif1 single mutants to have elongated telomeres due to their failure to control TG1–3 tract extension, but importantly, the telomere length sensing machinery is intact. To test whether shortened TG1–3 tracts cause the abnormal early replication of telomeric regions in yku70, we deleted PIF1—because the double yku70 pif1 mutant lacks Ku function, but is expected to have normal length telomeres and an intact telomere-length-sensing machinery.

We constructed a pif1 yku70 mutant and measured telomere length using Southern blotting. Deletion of PIF1 largely rescued the short telomere defect of the yku70 mutant (Figure 6A); telomeres in the yku70 pif1 strain have an average length only slightly shorter than wild type. To test whether rescuing the telomere length defect of yku70 also leads to recovery of the normal late telomere replication time, we used the dense isotope transfer
FIGURE 5: Histone tail acetylation is unchanged at telomere-proximal replication origins in the yku70 mutant. (A) Western blots analyzing specificity of anti-tetra-acetylated histone H4 primary antibody (06–598; Upstate). In control "wild-type" (wt) extracts, the presence of a single band at ~10 kDa represents acetylated histone H4. The antibody fails to recognize histone H4 in a strain in which the four target lysines residues (K5, 8, 12, and 16) are mutated to glycine (left panel). The right panel shows the reactivity of the antibody to extracts from esa1ts and rpd3 strains, in comparison to their respective wild-type parents. From left to right, strains are: WY130, WY129, LPY02991, LPY03291, DR3, and BY4741. Equivalent loading was confirmed by Coomassie staining (unpublished data). (B) Chromosome cartoon shows an ~20 kb region at telomere VI-right, with the location of the subtelomeric X element shown as a gray box. Locations of sequences analyzed are marked by vertical black bars. Graph shows qPCR analysis of chromatin sequences immunoprecipitated from wild type (RM14–3a) and yku70 mutant (AW101) cells using anti-tetra-acetylated histone H4 primary antibody. Primer pairs for ARS609, 5 kb and 300 bp loci are those used in Figure 3, with additional pairs used to measure recovery of sequences 500 bp either side of ARS609. Results are expressed relative to the amplification obtained for a standard internal locus. Error bars indicate the range of values between two ChIP experiments. (C) As B, except measuring the recovery of sequences at or flanking the telomere-proximal replication origins ARS316 and ARS522. (D) Western blots to examine specificity of anti-acetyl histone H3K18 primary antibody (ab1191; Abcam). In extracts from wild-type cells, a band at ~15 kDa detects acetylated histone H3. The left panel shows Western blot signal obtained from rpd3 mutant extract (DR3), compared with the corresponding wild type (BY4741). The right-hand panel shows antibody reactivity against strains carrying the single lysine mutants K9R, K14R, K18R, K23R, or K27R (strains NSY119, NSY120, NSY130, NSY131, NSY132, respectively), compared with the corresponding wild-type strain (NSY115). Equivalent loading was confirmed by Coomassie staining (unpublished data). (E) qPCR analysis of sequences immunoprecipitated using the anti-acetyl histone H3K18 antibody from wild-type (RM14–3a) and yku70 mutant (AW101) cells. Primer pairs are as used in parts B and C, and results are expressed in the same way. Error bars indicate the range of values between two ChIP experiments.
method to examine the yku70 pif1 replication program (Figure 6B). We measured the replication time of telomere-proximal origins ARS522 and proARS1202 (which lies 31 kb from telomere XII-left), as well as the average replication time of the Y' subtelomeric repeat sequences. In a YKU70‘ PIF1‘ (“wild-type”) strain, ARS522, proARS1202, and Y' sequences all replicate at a similar time to the internal late origin ARS1412, but in a yku70 mutant these telomere-proximal sequences replicate much earlier (compare with early origin ARS1; Figure 6B). In the yku70 pif1 mutant, we found that all three telomere-proximal sequences reverted to a late replication time similar to that of ARS1412, and close to their normal time of replication. Telomeric sequences in a pif1 single mutant replicated at close to their normal time in late S phase.

The replication time of a sequence can be assigned as the time at which replication has achieved half of its final level. Figure 6C shows the relative replication times (Replication Indices; Friedman et al., 1996) of various sequences in wild type, yku70, pif1, and yku70 pif1 strains. The replication times of telomeric markers proARS1202, ARS522, and Y' are largely (60–80%) recovered in the yku70 pif1 strain when compared with yku70, comparable with 76% recovery of telomere length (the average length of the terminal Xho1 fragment being 1280 bp in wild type, 1144 bp in yku70, and 1247 bp in yku70 pif1).

To examine further whether telomere replication timing in yku70 is linked to telomere length, we tested the effects of combining yku70 with other telomere-lengthening mutations (Askree et al., 2004; Gabbott et al., 2006). Most of the mutations tested (such as mlp2, rad5, and rad27) failed to cause any elongation of the shortened telomeres of a yku70 mutant. Likewise, expression of Cdc13-Est1 or Cdc13-Est2 proteins lengthened telomeres in wild type (Evans and Lundblad, 1999), but not in a yku70 background (unpublished data). We did however identify one mutation, elg1, which caused mild (~20%) rescue of yku70 short telomeres (Figure 7A). ELG1 encodes the largest subunit of a Replication Factor C-like complex (Parnas et al., 2010). Analyzing the replication program of the yku70 elg1 strain revealed that the replication times of telomeric marker sequences also showed partial rescue (25% recovery of Y', 19% of proARS1202, and 39% of ARS522) replication timing; Figure 7B), consistent with the suggestion that the replication time of chromosome ends in yku70 is linked to telomere length. Interestingly, replication of Y' sequences showed increased asynchrony in the yku70 elg1 mutant (Supplemental Figure S7; note shallow slope of Y' curve), possibly reflecting the greater spread of telomere lengths observed in this mutant (Figure 7A). In conclusion, the phenotypes of both the yku70 pif1 and yku70 elg1 mutants suggest that telomere shortening in the yku70 strain can account for its replication timing defect.

Rif1 is required for late telomere replication

Analysis of the yku70 pif1 mutant suggested that the effect of the yku70 mutation on telomere replication timing is due to shortened...
telomeres. To investigate further the idea that telomere length and TG1–3 repeat-sensing machinery are important to regulate telomere replication timing, we tested a mutant in which telomere length sensing is lost. Wild-type cells use a TG1–3 terminal repeat-counting mechanism that controls telomerase-mediated elongation, such that short telomeres are preferentially lengthened. This counting mechanism involves TG1–3 tract binding by Rap1, followed by recruitment of its interacting factors Rif1 and Rif2. Rif1 and Rif2 “measure” telomere length and down-regulate telomerase activity at chromosome ends with adequate TG1–3 repeat length (reviewed by Bianchi and Shore, 2008). In a rif1 mutant, this telomere length–sensing machinery is defective, and a rif1 mutant has long telomeres due to failure to control telomerase and consequent uncontrolled TG1–3 tract elongation (Figure 8A) (Hardy et al., 1992; Wotton and Shore, 1997).

To assess whether telomere length measurement for replication timing control involves the same length-sensing machinery, we tested whether telomere replication time responds correctly to telomere length when TG1–3 tract sensing is defective—that is, in a rif1 mutant. If Rif1 is required to detect long telomeres and in response specify late initiation of nearby origins, then we would predict that rif1 telomeric regions would replicate early, because cells will be unable to detect their elongated TG1–3 tracts. If, in contrast, Rif1 is not involved in controlling telomere replication timing, then the expectation is that the elongated telomeres of a rif1 mutant would cause its chromosome ends to replicate at the normal late time (or possibly even later than normal).

We tested the replication-timing program of a rif1 mutant (Figure 8B). We found that telomere-proximal origins ARS522 and proARS1202 both replicate substantially earlier than normal, at approximately the same time as the early origin ARS1 (Figure 8B and C). The average replication time of Y′ sequences is also much earlier in rif1 than in the wild-type control. In a rif1 mutant, telomeric and telomere-proximal sequences therefore replicate abnormally early, despite their excessively long TG1–3 tracts. We conclude that Rif1 is part of the pathway that senses TG1–3 tract length and relays that information to telomere-proximal origins to specify their replication time. A rif1 yku70 double mutant also replicated its telomeres early despite their almost normal length, consistent with loss of the length-sensing machinery.

**DISCUSSION**

In this study we have examined the effect of deleting Ku on the *S. cerevisiae* temporal program of DNA replication. We discovered that Ku can influence replication timing of chromosome ends over a long range, affecting the replication time of sequences up to 80 kb from telomeres. Our genome-wide study showed that the yku70 mutation affects the replication timing of all telomeres to some extent. The effect on some telomeres extends over tens of kilobases, but on others (e.g., XIII-right, XV-right) the effect is restricted to the chromosome end. The extent of the effect on replication timing may reflect the number, configuration, and normal initiation time of replication origins close to each telomere.

One caveat in interpreting microarray-based replication timing data arises from the presence of nonunique sequences within the terminal 20–30 kb of many chromosomes arms (23 of the 32 telomeres falling into groups sharing some degree of homology; Louis, 1995). Such similarity compromises the uniqueness of some microarray probes, so that the replication timing data for some chromosome ends must be interpreted with caution. We focused on chromosome VI-right in several experiments because it contains unique sequence to within ~500 bp of the chromosome end, permitting reliable probe and primer design.

We tested whether Ku modulates telomere replication dynamics by affecting nucleosome modifications that have previously been implicated in control of replication timing—namely, acetylation of the histone N-terminal tails at residues H4K5, 8, 12, and 16 or H3K18. We did not detect changes in acetylation of these residues at telomere-proximal replication origins that account for the effects of yku70 on replication initiation, suggesting that the advancement in replication time of telomere regions in yku70 is independent of histone tail acetylation. It remains possible that a different chromatin modification might be involved in mediating the effect of Ku on initiation time of telomere-proximal origins (Pryde et al., 2009).
Loss of Ku function has several effects on yeast telomere organization that could potentially be related to the replication timing defect. Specifically, deletion of YKU70 causes loss of normal telomere positioning at the nuclear periphery, failure of subtelomeric transcriptional silencing, and abnormally short telomeres (Boulton and Jackson, 1998; Laroche et al., 1998; Stellwagen et al., 2003). deregulated telomere replication timing in yku70 does not appear to be due to defective telomere subnuclear positioning (because other mutants that derail telomere positioning do not affect replication timing; Hiraga et al., 2006) or to the telomeric silencing defect (because deleting Sir components has a milder effect than yku70 on replication timing; Stevenson and Gottschling, 1999; Cosgrove et al., 2002). We created a pif1 yku70 mutant to examine whether the replication timing defect of cells lacking Ku can be its greatly extended terminal TG\textsubscript{1-3} tract length, suggesting that rit1 cells lack the ability to transmit telomere length information to nearby replication origins (as well as failing to repress telomerase activity at long telomeres). Figure 9 illustrates our model for the role of Rif1 in telomere length–controlled replication timing in wild-type and mutant strains. The most likely explanation for the early replication of rit1 telomeres is that, without Rif1 protein, cells lack the machinery required to detect their long telomeres and program the initiation time of nearby replication origins accordingly (Figure 9D). In other words, in the absence of Rif1 the cells constitutively inter-
that may (like ARS522) lie tens of kilobases distant from the chromosome end. Our results suggest that the effect of yku70 (and telomere length) on replication timing is not mediated by altered acetylation state of the histone H3 or H4 tails. Although changes in histone tail acetylation clearly can affect replication origin initiation (Vogelauer et al., 2002), they do not appear to mediate the long-range effect of telomeres on replication timing. In their study of the effects of deacetylase Rpd3 and HAT Gcn5 on replication dynamics, Vogelauer et al. concentrated on internal late replication domains and did not examine telomere-proximal origins. One possibility therefore is that histone tail acetylation state mediates origin initiation time within internal late replication domains (distant from telomeres), but a different mechanism (not acting through histone tail acetylation) mediates the effect of telomeres on replication timing. We propose that the programming of replication time close to chromosome ends occurs through a Rif1-dependent mechanism in which TG repeat length is measured, and the information is fed into an unidentified pathway that controls replication initiation time. We are investigating the possibility that, as well as measuring TG repeat length, an activated form of Rif1 itself transmits the telomere-length signal to replication origins, regulating replication initiation through an effect on the Cdc7 kinase replication initiation factor. Such a role for S. cerevisiae Rif1 in replication control could be related to the reported function of the human Rif1 homologue, which is involved in regulating DNA replication of heterochromatic domains (Buonomo et al., 2009). Understanding the pathways by which yeast telomeres affect the replication program of nearby sequences will elucidate the links between telomere biology and replication and may help uncover general mechanisms that regulate DNA replication.

MATERIALS AND METHODS
Yeast strains
Genotypes and sources of the strains used are listed in Supplemental Table S1. Mutated alleles were created by targeted PCR-based gene knockout, with correct transfectants verified by PCR according to standard procedures.

Replication timing analysis
The replication time of specific sequences was measured using the dense isotope transfer procedure (McCarroll and Fangman, 1988; Donaldson et al., 1998b), probing for specific genomic EcoRI fragments as described previously (Cosgrove et al., 2002). ARS106 replication time was measured by probing for a 4956 bp EcoRI restriction fragment (coordinates 66359–71315) on chromosome I.

Analysis of percent replication and replication time
Genome-wide replication timing analysis was performed in a MATa cdc7- strain background using an adaptation of the procedure described by Raghuraman et al. (2001). yku70 (AW101) and control YKU70 (KK14–3a) strains grown in isotopically heavy medium were presynchronized using α-factor, transferred into isotopically light medium containing α-factor, then released from α-factor at 37°C and allowed to proceed to a cdc7 block. Cultures were released into S phase by transferring to a 23°C shaker. Two series of small-volume samples were taken throughout S phase for flow cytometry analysis and to allow measurement of replication kinetics of specific sequences using standard slot blot hybridization as previously described (McCarroll and Fangman, 1988; Cosgrove et al., 2002). From each culture two large samples were taken for array analysis, the first at the cdc7 block (0 min) and the second at a mid-S phase point (25 min after release). From these samples genomic DNA was prepared and digested with EcoRI, after which HL (replicated) DNA was separated from HH (unreplicated) by cesium chloride gradient centrifugation. The replicated and unreplicated DNA fractions were recovered and separately labeled with Cy5-dUTP (2′-deoxyuridine, 5′-triphosphate) or Cy3-dUTP (Amersham Biosciences, Piscataway, NJ) before pooling and hybridizing to glass slide microarrays, on which each open reading frame was represented by a PCR fragment (GEO platform accession number GPL1914; Fred Hutchinson Cancer Research Center, Seattle, WA). After quality control and removal of unreliable points from the data files, the raw %HL values were assembled into chromosome plots and subjected to normalization and smoothing using a 10-kb sliding window as described (Raghuraman et al., 2001; Alvino et al., 2007).

Approximately equal amounts of HH and HL DNA were used in each array hybridization. For culture samples in which the actual percentage of replicated DNA lay outside the range of 40–60% of total DNA (i.e., in the zero-time-point samples, in which the actual percentage of replicated DNA was typically 10–20%), data processing included a global normalization step to adjust the total proportion of signal generated by HL DNA to the experimentally determined value, obtained by hybridizing fractions from the appropriate cesium

FIGURE 9: Model of replication timing control by Rif1-mediated telomere length measurement. (A) In wild-type cells, terminal TG repeat length is “counted” by activated Rif1 molecules (gray hexagons). If TG repeat length is sufficient, Rif1 relays a signal to nearby origins (such as telomere-proximal Y or ARS522 origins) specifying their late replication time (black circle). (B) In yku70, TG1–3 tract length is short due to compromised telomerase recruitment and telomere uncapping. TG1–3 repeat length is insufficient for activated Rif1 to cause late origin programming, so a nearby origin initiates early (white circle). (C) In pif1 yku70 TG1–3 repeat length is restored, activating sufficient Rif1 to allow late origin programming. (D) A rif1 mutant lacks the Rif1-mediated “count-and-delay” mechanism, with the result that nearby origins initiate replication early despite their extended TG1–3 repeat length.
chloride gradient with a probe containing whole genomic DNA. All plots were additionally corrected for “noncycling” cells (i.e., for the proportion of HH DNA contributed by cells that failed to release from the cdc7 block, determined by analyzing final replication levels in each experiment) (Alvino et al., 2007). Next the smoothed “%HL” curves were converted into “% replicated” curves (i.e., corrected for the doubling of each sequence when it replicates) using the equation:

%replication = [50x%HL]/[100 – (%HL/2)]

This analysis produced a series of plots showing the percentage of cycling cells that had replicated each sequence in that sample. For the zero-time-point samples, these plots of “% replicated” are shown in Figure 1C and Supplemental Figure S4.

The elevated levels of replication at the zero time point in extended telomere-proximal domains in the yku70 mutant (Supplemental Figure S4) caused a relative overrepresentation of the latest-replicating sequences in the HL DNA fraction in the mid-S phase samples (unpublished data). This overrepresentation in turn affected the gradients of the yku70 plots at very-late-replicating loci within telomere-proximal regions, causing a misleading apparent “acceleration” of replication forks toward replication termination zones. To correct for this effect, and to allow separate assessment of the effects of Ku complex on “escape” (zero time point) replication and S phase replication time, the % replication values at mid-S phase were corrected for zero-time-point replication as described (Reynolds et al., 1989).

We assessed the relationship between percent replication as measured from the mid-S phase (25 min) samples and replication time in S phase, using replication timing values obtained for individual sequences by conventional slot blot analysis (Supplemental Figure S1). A linear relationship was observed for both wild-type and yku70 experiments, allowing the equations describing the lines in Supplemental Figure S1A to be used to transform the 25-min percent replication values into replication times, facilitating comparison of the curves with each other and with previous data sets. The resulting plots of replication time in S phase are presented in Supplemental Figure S3 and Figure 1B.

Plots show the mean of two hybridizations. The terminal 5 kb was deleted from each end of all chromosome plots to remove end-related artifacts in the data processing and analysis. The results obtained in this study were highly consistent with those from previous analyses that used slightly different strategies to measure replication dynamics (Raghuraman et al., 2001; Alvino et al., 2007; see comparative plot in Supplemental Figure S2).

Origin sites were assigned using criteria similar to those described (McCune et al., 2008). SD values in the microarray-derived data were estimated based on the zero-time-point percent replicated curves in wild type, converted to their equivalent replication times. In this case, to be called an origin site, a peak was required to rise 2.5 SD values above the surrounding valleys in both wild-type and yku70 mutant plots. Peaks were required to lie within 7.5 kb to be taken to correspond to the same origin (based on the estimated resolution for this type of study; Raghuraman et al., 2001; Nieduszynski et al., 2007). These fairly strict criteria strengthened the likelihood that differences observed reflect initiation timing differences (as opposed to efficiency differences) but exclude some likely origin sites, particularly those close to chromosome ends where the data set may terminate close to the origin. Several apparent origins the initiation times of which appear to be affected by the yku70 mutation (e.g., XII-left –33 kb and XII-right –1060 kb) were not called because they failed to meet the criteria in either wild-type or mutant curves, and are therefore omitted from the plot in Figure 2B. In two cases (at VIII-left and X-left) probable origins failing to meet these criteria were called, based on a falling curve at the terminal data point displaying a drop of greater than 1.5 SD values from the origin value.

Western blotting
Proteins were extracted as described (Stirling et al., 1994). Ten or fifteen percent polyacrylamide gels were run, and proteins were transferred to a Hybond-P membrane (GE Healthcare Life Sciences, Piscataway, NJ), according to standard methods. For Western blot analysis, membranes were blocked and then incubated for 3 h with primary antibody—anti-acetyl histone H4 K5,8,12,16 (06–598 Lot 29867; Upstate Biotechnology, Lake Placid, NY), anti–acetyl histone H3 K18 (07–354 Lot 27132; Upstate), anti–histone H3 (ab1791 Lot 80051; Abcam, Cambridge, MA), or anti–c-Myc [9E11] (ab56 Lot 191919; Abcam)—then washed, and incubated for 2 h with secondary antibody (either HRP-conjugated anti–mouse [ab6729 Lot 131853; Abcam] or HRP-conjugated anti–rabbit [12–348 Lot 29902; Upstate]) before enhanced chemiluminescence visualization.

Flow cytometry
Flow cytometry analysis was performed as described by McCune et al. (2008).

ChiP
ChiP was performed as described (Hecht and Grunstein, 1999; Hiraga et al., 2006). Briefly, samples were fixed with formaldehyde for 30 min at room temperature before sonication to an average size of 500 bp. Samples were immunoprecipitated using Dynabeads M-280 sheep anti–rabbit immunoglobulin (Ig)G-112.04 (Invitrogen, Carlsbad, CA) and 10.8 µl of anti–acetyl histone H4 K5,8,12,16 (06–598; Upstate), 1.8 µl of anti–acetyl histone H3 K18 (07–354; Upstate), or 3.6 µl of anti–histone H3 (ab1791; Abcam). Alternatively, 125 µl of Dynabeads Pan Mouse IgG-110.41 (Invitrogen) was used with 10 µl of anti–c-Myc [9E11] (ab56 Lot 191919; Abcam). Precipitated DNA was resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA for use in real-time qPCR.

Real-Time qPCR
Reactions were run using Finnzymes F-400L qPCR mix on a Roche LightCycler 480 or a DNA Engine Opticon 2 real-time PCR machine, and results were analyzed using Opticon Monitor software (version 3.1; Bio-Rad, Hercules, CA). For each primer pair in each reaction, a threefold dilution series of standard DNA (whole genomic DNA) was run in duplicate. Genomic standard DNA was prepared as for ChiP but omitted the immunoprecipitation step.

For the experiments shown in Figure 5, samples were run as a 1 in 3 dilution series, performed in duplicate. Results were obtained in “DNA units” in comparison to the standard dilution series, where the top dilution “standard” sample was assigned as containing 100 DNA units. Results were then expressed relative to the signal obtained from an internal locus (PPH3, which showed consistently similar levels of immunoprecipitation from wild-type and yku70 mutant chromatin). Error bars in Figure 5 and Supplemental Figure S6 show the range of values between two ChiP experiments. The data shown in Figure 4 and Supplemental Figure S5 plot “raw DNA units” in relation to the standard curves, with the data presented as an average of qPCR duplicates. Primer sequences used in the PCRs are listed in Supplemental Table S3.

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