EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70Δ mutants

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We have examined the role of checkpoint pathways in responding to a yku70Δ defect in budding yeast. We show that CHK1, MEC1, and RAD9 checkpoint genes are required for efficient cell cycle arrest of yku70Δ mutants cultured at 37°C, whereas RAD17, RAD24, MEC3, DDC1, and DUN1 play insignificant roles. We establish that cell cycle arrest of yku70Δ mutants is associated with increasing levels of single-stranded DNA in subtelomeric Y′ regions, and find that the mismatch repair-associated EXO1 gene is required for both ssDNA generation and cell cycle arrest of yku70Δ mutants. In contrast, MRE11 is not required for ssDNA generation. The behavior of yku70Δ exo1Δ double mutants strongly indicates that ssDNA is an important component of the arrest signal in yku70Δ mutants and demonstrates a link between damaged telomeres and mismatch repair-associated exonucleases. This link is confirmed by our demonstration that EXO1 also plays a role in ssDNA generation in cdc13-1 mutants. We have also found that the MAD2 but not the BUB2 spindle checkpoint gene is required for efficient arrest of yku70Δ mutants. Therefore, subsets of both DNA-damage and spindle checkpoint pathways cooperate to regulate cell division of yku70Δ mutants.

[Key Words: Checkpoint; telomere; KU; EXO1; CDC13; ssDNA]

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The telomere is a DNA-protein complex at the end of eukaryotic chromosomes. If telomeric DNA, which has many properties of a double strand break (DSB), was perceived as a DSB by DNA repair machinery and underwent recombination, then harmful telomere fusions and dicentric chromosomes would be generated. Similarly, if telomeric DNA were perceived as a DSB by DNA damage checkpoint machinery, it would be harmful, because in budding yeast a single unrepaired DSB elsewhere in the genome can inhibit cell cycle progression for many generation times (Sandell and Zakian 1993). Therefore, it is essential for chromosome stability and cell cycle progression that telomeres hide the DSB-like structures that they contain. An important function of some of the large number of telomere binding proteins, such as Cdc13p [essential in budding yeast] and Ku70/Ku80 heterodimer [conserved from yeast to mammalian cells], is to hide telomeric DNA from repair and checkpoint pathways.

Budding yeast mutants defective in telomere binding proteins are useful tools to address the mechanisms by which checkpoint pathways recognize damaged DNA, because in these cells telomeres become potent activators of DNA damage checkpoint pathways in a conditional manner. For example, at 23°C, a permissive temperature for cdc13-1 mutants, telomeres are not recognized as damaged DNA, but at 36°C temperatures, they are potent activators of cell cycle arrest [Weinert and Hartwell 1993]. Cell cycle arrest of cdc13-1 mutants is associated with accumulation of single-stranded DNA (ssDNA) at telomeres [Garvik et al. 1995]. Furthermore, not only do checkpoint pathways recognize cdc13-1-induced damage, but they also affect the rate at which ssDNA arises [Lydall and Weinert 1995].

The Ku heterodimer is an evolutionarily conserved protein complex involved in the nonhomologous end-joining (NHEJ) pathway of DNA repair [Smith and Jackson 1999]. Interestingly and paradoxically, the Ku heterodimer is important for telomere stability. For example, there is evidence that the Ku heterodimer protects mammalian chromosomes from telomere fusions [Bailey et al. 1999; Hsu et al. 2000; Samper et al. 2000; d’Adda di Fagagna et al. 2001]. In one study, 62% of ku80Δ− mouse embryonal fibroblasts contained telomere fusions, a level 30 times higher than that seen in Ku-proficient fibroblasts [Hsu et al. 2000].

The budding yeast homolog of the Ku heterodimer is...
the Yku70p/Yku80p heterodimer. Mutants with deletions of *YKU70* or *YKU80* contain short telomeres [Boultong and Jackson 1996, 1998; Porter et al. 1996], have single-stranded DNA (ssDNA) in their repetitive telomeric TG sequences [Gravel et al. 1998, Polotnianka et al. 1998], display decreased telomeric silencing [Boulton and Jackson 1998; Mishra and Shore 1999, Pryde and Louis 1999], and altered telomere localization [Laroche et al. 1998]. Furthermore, there is evidence that the KU heterodimer is able to bind to the telomerase RNA directly [Peterson et al. 2001] and is localized at telomeres [Martin et al. 1999].

While both *yku70A* and *yku80A* mutants are viable at permissive temperatures such as 30°C, they are unable to form colonies at 37°C [Feldmann and Winnacker 1993; Barnes and Rio 1997]. This temperature-sensitive phenotype appears to be due specifically to a telomere defect, rather than a more generalized DNA repair defect, because the temperature-sensitive phenotype can be partially suppressed by overexpression of telomerase subunits [Nugent et al. 1998, Teo and Jackson 2001, Lewis et al. 2002] or rarely (1 × 10^-7/cell) by amplification of subtelomeric repeats [Fellerhoff et al. 2000]. By combining the *yku70A* mutation with checkpoint mutations and culturing the cells at high temperatures, we have been able to examine the role of different checkpoint genes in responding to (sub) telomeric defects in *yku70A* mutants.

Checkpoint pathways consist of proteins that interact with damaged DNA and signal transduction cascades that inhibit cell division [Lowndes and Murguia 2000; Caspari and Carr 2002]. Here we show that some, but not all DNA damage checkpoint genes contribute to the inhibition of cell division of *yku70A* mutants. Interestingly, a subset of spindle checkpoint pathways also contributes to arrest. Furthermore, there is a correlation between cell cycle arrest and the accumulation of ssDNA in subtelomeric sequences in *yku70A* mutants. Finally, we show that the mismatch repair-associated exonuclease EXO1 is essential for ssDNA generation in *yku70A* mutants, while *MRE11* is not, and that EXO1 is also required to generate ssDNA in *cdc13-1* mutants.

## Results

**CHK1, MEC1, and RAD9 are required for a yku70A-induced checkpoint**

To determine how checkpoint pathways interact with *yku70A*-induced damage in budding yeast, we generated a panel of double and triple mutants. Weinert and Hartwell showed previously that checkpoint mutations allow *cdc13-1* mutant strains, defective in a telomere binding protein, to form colonies at higher temperatures than checkpoint-proficient *cdc13-1* strains [Weinert and Hartwell 1993; Weinert et al. 1994]. This is presumably because loss of checkpoint control allows cells with non-lethal levels of DNA damage to divide and form colonies. Figure 1 shows the growth of serial dilutions of *yku70A* and checkpoint mutant cells at 28°C, 36°C, and 37°C. At 28°C, a permissive temperature for *yku70A* mutants, all strains grew at similar rates and formed similarly sized colonies. At the restrictive temperatures of 36°C and 37°C, different strains formed colonies with different efficiencies. A *chk1A* mutation had the most profound effect and significantly increased the ability of *yku70A* mutants to form colonies at both 36°C and 37°C (Fig. 1g–i). *rad9Δ* and *mec1Δ* also increased *yku70A* colony size, but the colonies were smaller than the *yku70A chk1Δ* colonies (Fig. 1d–f). In contrast, *mec3Δ, ddc1Δ, rad17Δ, rad24Δ*, and *dun1Δ* mutations had minor effects on the growth of *yku70A* mutants (Fig. 1a–i). The growth of *rad9Δ rad24Δ yku70Δ* triple mutants at 36°C and 37°C was most similar to that of *yku70A rad9Δ* mutants, indicating that the strong growth phenotype was epistatic (Fig. 1d–f). These experiments suggested that a *CHK1, MEC1, and RAD9*-dependent, but *DDC1, MEC3, RAD17, RAD24, and DUN1*-independent mechanism is responsible for the poor growth of *yku70A* mutants at 36°C and 37°C.

To determine whether checkpoint pathways are activated in *yku70A* mutants, we examined the growth and cell cycle distribution of *yku70A* and checkpoint mutants in liquid cultures (Fig. 2). In four separate experiments, the growth of *yku70A* mutants at 37°C was much slower than *YKU70* cells, such that by 8.5 h their cell number had increased about 4–8-fold, instead of 80–100-fold as observed in the *YKU70* cells [Fig. 2a–d]. In addition, in three of four experiments, the growth of *yku70A* cells began to plateau after about 6 h in liquid culture [Fig. 2a–d, data not shown]. The poor growth of *yku70A* mutants correlates with an increasing fraction of cells at the medial nuclear division stage of the cell cycle [Hartwell 1974], increasing from ~20% at the beginning of the experiments, to over 85% during 8.5 h culture at 37°C [Fig. 2e–h]. This accumulation of cells at medial nuclear division suggests they are accumulating before the metaphase/anaphase transition, and is consistent with an earlier study which showed that the large budding cells that accumulated in *yku70A* (*hfd1Δ*) mutant cultures at 37°C contained short mitotic spindles and a nucleus at the neck between the mother and daughter cells [Barnes and Rio 1997]. The slow kinetics of arrest of *yku70A* mutants is in contrast to the behavior of *cdc13-1* mutants, because 94% of *cdc13-1* mutant strains arrest in the first cell cycle (within 2 h) at restrictive temperature [Weinert and Hartwell 1993].

At 37°C, the growth and cell cycle distribution of *yku70A chk1Δ, yku70A rad9Δ*, and *yku70A meclΔ* mutants was most like *YKU70*-*RAD9*+ strains because they grew exponentially and did not accumulate at medial nuclear division [Fig. 2c,d,g,h, data not shown]. Therefore, it appears that the poor growth of *yku70A* mutants at 37°C and the accumulation in medial nuclear division is due to a *CHK1, MEC1*, and *RAD9*-dependent checkpoint pathway. In contrast, the growth and cell cycle distributions of *yku70A ddc1Δ, yku70A rad24Δ*, and *yku70A dun1Δ* mutants were most similar to *yku70A* strains, suggesting that *yku70A*-induced checkpoint pathways are intact in *rad24Δ, ddc1Δ*, and *dun1Δ* mutants (Fig. 2a,b,e,f). Both *yku70A meclΔ* and *yku70A*
rad1Δ mutants behaved similarly to yku70Δ rad24Δ mutants (data not shown). We found that rad53Δ sml1Δ single mutants, as well as yku70Δ rad53Δ sml1Δ triple mutants grew poorly in liquid culture at 37°C, which made it difficult to determine the role of RAD53 in cell cycle arrest (data not shown). This may be because RAD53 has an essential function at 37°C that is unrelated to checkpoint control (Gardner et al. 1999; Sanchez et al. 1999).

We noted that, despite their initial checkpoint defective phenotype, yku70Δ mec1Δ and yku70Δ chk1Δ mutants begin to slow cell division and start to accumulate at medial nuclear division, after long periods (8 h) at 37°C (Fig. 2, d,g,h). This suggests that another checkpoint pathway, independent of CHK1 and MEC1, can arrest cell division of yku70Δ mutants after long periods at 37°C. This arrest is due to the activation of spindle checkpoint pathways (see Fig. 4, below). Both yku70Δ rad24Δ rad9Δ and yku70Δ rad24Δ mec1Δ triple mutants showed the exponential growth phenotype of yku70Δ rad9Δ and yku70Δ mec1Δ mutants respectively, indicating that the exponential growth phenotype is epistatic to the poor growth phenotype (Fig. 2, d,g,h data not shown). In summary, these liquid culture experiments suggest that a checkpoint pathway that arrests yku70Δ mutants at medial nuclear division at 37°C is dependent on CHK1, RAD9, and MEC1, but independent of RAD17, RAD24, MECD, DDC1, and DUN1.

Microcolony assays were used to confirm that yku70Δ mutants are able to divide several times before ceasing growth at 37°C, and that rad9Δ and rad24Δ mutations had different effects on cell division. MATα cells were first arrested in G1 using the mating pheromone alpha factor, the pheromone was removed, and single cells incubated on plates for 20 h at 37°C. After 20 h at 37°C, YKU70Δ-induced damage is that effective checkpoint response in budding yeast. We noted that, despite their initial checkpoint defective phenotype, yku70Δ mec1Δ and yku70Δ chk1Δ mutants begin to slow cell division and start to accumulate at medial nuclear division, after long periods (8 h) at 37°C (Fig. 2, d,g,h). This suggests that another checkpoint pathway, independent of CHK1 and MEC1, can arrest cell division of yku70Δ mutants after long periods at 37°C. This arrest is due to the activation of spindle checkpoint pathways (see Fig. 4, below). Both yku70Δ rad24Δ rad9Δ and yku70Δ rad24Δ mec1Δ triple mutants showed the exponential growth phenotype of yku70Δ rad9Δ and yku70Δ mec1Δ mutants respectively, indicating that the exponential growth phenotype is epistatic to the poor growth phenotype (Fig. 2, d,g,h data not shown). In summary, these liquid culture experiments suggest that a checkpoint pathway that arrests yku70Δ mutants at medial nuclear division at 37°C is dependent on CHK1,
damage becomes more extensive. If true, this could explain why cdc13-1 mutants cultured at 36°C, 10°C higher than their maximum permissive temperature, depend on both RAD9 and RAD24 for cell cycle arrest (Lydall and Weinert 1995), whereas yku70Δ rad24Δ mutants, cultured at 37°C, 2°C higher than their maximum permissive temperature, depend on RAD9, but not on RAD24. Wild-type yeast strains do not form colonies above 38°C, and so it was not possible to test whether arrest of yku70Δ mutants at higher temperatures depends on RAD24 as well as RAD9. However, it was possible to test whether arrest of cdc13-1 mutants at marginally permissive temperatures depended more on RAD9 than RAD24. We used the microcolony assay to test whether RAD9 was required for the primary checkpoint pathways in cdc13-1 strains cultured at the moderately restrictive temperature of 28°C. At this temperature, cdc13-1 cells formed colonies in the range of 2 to 20 cells, compared with 2–6 cells at 36°C (Lydall and Weinert 1997), cdc13-1 rad9Δ cells formed medium-sized colonies (20–200 cells), whereas cdc13-1 rad24Δ and cdc13-1 cells formed large-sized colonies (1000–3000 cells) (Fig. 3e–h). Therefore, at both moderately (28°C) and strongly (36°C) restrictive temperatures, cdc13-1 rad9Δ mutants form smaller colonies than cdc13-1 rad24Δ mutants. In contrast, yku70Δ rad9Δ mutants form larger colonies than yku70Δ rad24Δ mutants at 37°C. Therefore, we conclude that RAD9- and RAD24-dependent checkpoint pathways play different roles in responding to yku70Δ or cdc13-1-induced DNA damage.

MAD2 contributes to the arrest of yku70Δ mutants and BUB2 to the arrest of cdc13-1 mutants

Despite their initial checkpoint-defective phenotype, yku70Δ mec1Δ and yku70Δ chk1Δ mutants began to
slow cell division, and started to accumulate at medial nuclear division, after long periods [8 h] at 37°C [Fig. 2g,h]. The MAD2-dependent spindle checkpoint arrests cells at a stage of cell division similar to the RAD9-dependent DNA damage checkpoint, just prior to the metaphase/anaphase transition, when the APC (anaphase promoting complex) is activated. The MAD2-dependent checkpoint inhibits APC activation by inhibiting Cdc20p, an essential factor for APC activation [Hwang et al. 1998]. To determine whether spindle checkpoint pathways might be responsible for the residual cell cycle arrest observed in yku70Δ chk1Δ mutants, we examined the effect of mad2Δ and bub2Δ mutations on the growth of yku70Δ strains at 37°C (MAD2 and BUB2 belong to different arms of the spindle checkpoint pathways [Gardner and Burke 2000]). Whereas a mad2Δ mutation increased the growth of yku70Δ mutants [Fig. 4a–c], a bub2Δ mutation had no effect on the growth of yku70Δ mutants [Fig. 4d–f]. Interestingly, simultaneous disruption of both RAD24 and MAD2 increased the growth of yku70Δ mutants more than either single mutation [Fig. 4b,c], suggesting that perhaps RAD24 plays a small role in the arrest of yku70Δ mutants, a role that can be unmasked by deletion of MAD2. A yku70Δ chk1Δ mad2Δ triple mutant grew nearly as well as YKU70+ cells [Fig. 4c].

To determine whether the MAD2 spindle checkpoint also contributes to inhibiting the growth of cdc13-1 mutants, we combined mad2Δ and bub2Δ mutations with cdc13-1. Curiously, and once again, cdc13-1 and yku70Δ mutants showed different, almost opposite interactions with checkpoint pathways. A bub2Δ deletion had a moderate effect on the growth of cdc13-1 mutants [Fig. 4i–l], whereas a mad2Δ deletion had less effect [Fig. 4g–l]. The effect of the bub2Δ mutation was not as strong as a rad9Δ DNA damage checkpoint mutation, and the rad9Δ bub2Δ double mutant behaved like the single rad9Δ mutant [Fig. 4i–l]. Similarly, the cdc13-1 rad9Δ mad2Δ triple mutants formed colonies with efficiency similar to that of the cdc13-1 rad9Δ double mutants.

To confirm that MAD2 was responsible partially for arrest of yku70Δ mutants and to produce evidence that it contributed to the residual arrest observed in yku70Δ chk1Δ mutants at 37°C, we performed liquid culture experiments. Figure 4m shows that the decrease in growth observed in yku70Δ chk1Δ mutants after several hours at 37°C [vs. YKU70+ cells] could be overcome by a mad2Δ deletion, because yku70Δ chk1Δ mad2Δ triple mutants grew almost as well as the YKU70+ strain. Figure 4n shows that the increase of yku70Δ chk1Δ mutants at medial nuclear division, during 9–18 h of incubation at 37°C, did not occur in the yku70Δ chk1Δ mad2Δ triple mutants. The growth of yku70Δ bub2Δ mutants is most similar to yku70Δ cells, indicating that BUB2 does not play a role in the arrest of yku70Δ mutants at 37°C. Consistent with the hypothesis that yku70Δ damage induces a MAD2-dependent arrest, yku70Δ mad2Δ mutants reached an approximately fivefold higher cell density than yku70Δ cells over an 18-h period [Fig. 4m]. In addition, a maximum of 75% of yku70Δ mad2Δ mutants arrested at medial nuclear division, whereas about 95% of yku70Δ cells arrested [Fig. 4n].

We have shown that arrest of yku70Δ mutants at 37°C is due to CHK1- and MAD2-dependent pathways. Figure 4n allows us to estimate their respective contributions. A CHK1-dependent pathway is responsible for 75% of arrest [determined from the percentage of yku70Δ mad2Δ mutants arrested at the time point of maximum arrest, 12 h], whereas a MAD2-dependent pathway is responsible for 20% of the arrest [determined from the percentage of yku70Δ mutants arrested minus the percentage of yku70Δ mad2Δ cells arrested at 12 h]. In the case of yku70Δ chk1Δ cells, the percentage of cells arrested by MAD2 only noticeably increased at later time points [over 15 h], consistent with the idea that at early times arrested cells were diluted by the large mass of dividing cells. Thus, the effects of the CHK1-dependent DNA damage and MAD2-dependent spindle checkpoint pathways are additive and together they contribute to all (95%) of the arrest observed in yku70Δ mutants.
yku70Δ mutants accumulate ssDNA in subtelomeric Y′ sequences at 37°C

There is much evidence that single-stranded DNA is an important stimulus for DNA damage checkpoint pathways. For example, cdc13-1 mutants accumulate ssDNA up to 20kb from their telomeres, when cultured at restrictive temperatures (Garvik et al. 1995). If ssDNA is an important component of the signal that activates checkpoint pathways in yku70Δ mutants at 37°C, then increased levels should be observed at restrictive temperatures. It was known that yku70Δ and yku80Δ mutants contain more ssDNA in their repetitive TG telomeric sequences than do YKU′ cells [Gravel et al. 1998; Polotnianka et al. 1998; Teo and Jackson 2001]. However, yku80Δ mutants appear to contain as much single-stranded TG DNA at their permissive temperatures of 23°C and 30°C as at their restrictive temperature of 37°C [Gravel et al. 1998; Teo and Jackson 2001], suggesting that ssDNA at telomeric sequences is not necessarily an important stimulus for cell cycle arrest [Teo and Jackson 2001]. We reasoned that the ssDNA in yku70Δ mutants might extend beyond the telomeres, as it does in cdc13-1 mutants, and that there may be a better correlation between the appearance of ssDNA in subtelomeric repeats and cell cycle arrest.

Quantitative amplification of ssDNA (QAOS) (Booth et al. 2001) was used to examine the appearance of ssDNA in telomere proximal sequences of yku70Δ mutants at 37°C [Fig. 5]. This quantitative PCR-based method can be used to measure ssDNA levels in the range 0.2% to 100% at single-copy loci in the genome. We found that yku70Δ mutants cultured at the restrictive temperature of 37°C generated increasing amounts of ssDNA at telomeric loci. We measured ssDNA at a locus situated 600 bp from the telomeric end of the Y′ subtelomeric repeat. In telomeres that contain Y′ repeats, this locus is about 900–1000 bp from the very end of the chromosome [Fig. 5a]. At these positions, the amount of ssDNA in yku70Δ mutants increased from 1.6% at the beginning of the experiment to values between 5% and 8%, after 6–10 h of incubation at 37°C.
the telomere of chromosome V in budding yeast. The amount of ssDNA in the two strains. The yeast stains and the amount of ssDNA at their telomeres was measured by meric sequences. A series of yeast strains was cultured at 37°C, the Y\textsubscript{KU70}/H9004: DLY1364 and DLY1430. The error bars indicate the standard amount of ssDNA observed in two independent strains DLY1271. The results shown for (Fig. 5b). The increase in ssDNA is less rapid and less accurate ssDNA more rapidly than cdc13-1 cells, which in turn generate ssDNA more rapidly than cdc13-1 rad24Δ cells (Lydall and Weinert 1995). To investigate whether yku70Δ mutants generate ssDNA beyond the Y sequence, we examined ssDNA production at the YER188W locus, 8,500 bp from the telomere. This is the first unique gene close to the right telomere of chromosome V. Figure 5e shows that yku70Δ mutants generate considerably less ssDNA at this locus compared to cdc13-1 mutants, and compared to the amount of ssDNA they generate at their Y sequences. Interestingly, yku70Δ rad9Δ mutants generated some ssDNA at YER188W, which suggests that RAD9 may inhibit ssDNA production in yku70Δ mutants, as it does in cdc13-1 mutants (Lydall and Weinert 1995).

In summary, we find that yku70Δ mutants contain significantly more subtelomeric ssDNA at restrictive temperatures than at permissive temperatures. This suggests that the subtelomeric ssDNA is an important stimulus for activation of checkpoint control pathways in yku70Δ mutants.

EXO1 is required for ssDNA generation and arrest of yku70Δ mutants

If ssDNA contributes to the signal that arrests cell division of yku70Δ mutants, then mutations that reduce the

**Figure 5.** yku70Δ mutants accumulate ssDNA in subtelomeric sequences. A series of yeast strains was cultured at 37°C, and the amount of ssDNA at their telomeres was measured by quantitative amplification of ssDNA (QAOS). The yeast stains used were cdc13-1: DLY1230; yku70Δ chk1Δ: DLY1215; yku70Δ: DLY1412; YKU70+: DLY640; and yku70Δ rad24Δ: DLY1271. The results shown for yku70Δ rad24Δ are the average amount of ssDNA observed in two independent strains DLY1364 and DLY1430. The error bars indicate the standard error of the mean derived from three independent measurements of the amount of ssDNA in a sample, except for the yku70Δ rad24Δ, where they indicate the difference in the amount of ssDNA in the two strains. (a) A schematic model of the telomere of chromosome V in budding yeast. (b,c) Detection of ssDNA on the TG strand 600 bases from the telomeric end of the Y sequence. (d) Detection of ssDNA on the AC strand, 600 bases from the telomeric end of the Y sequence. (e) Detection of ssDNA on the TG strand at YER188W, 8,500 bases from the right end of chromosome V.
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amount of ssDNA should alleviate arrest. To test this hypothesis, we examined the effect of an exo1Δ mutation on arrest of yku70Δ mutants. EXO1 encodes an exonuclease that is normally recruited to DNA by the mismatch repair machinery (Tishkoff et al. 1997) and is involved in the resection of meiotic DSBs (Tsubouchi and Ogawa 2000), but does not appear to affect telomere length [Tsubouchi and Ogawa 2000]. The resection of meiotic DSBs by 5’ to 3’ exonucleases, to generate 3’ ssDNA tails, is in many ways similar to the processes that occur at damaged telomeres, which also produce 3’ ssDNA tails. Figure 6a shows that an exo1Δ mutation strongly increases the ability of yku70Δ mutants to form colonies at 36°C and 37°C. The effect is as strong as that seen with a chk1Δ mutation (cf. Figs. 6 and 1). To determine whether the strong growth is due to alleviation of checkpoint control, we examined the cell cycle distribution of yku70Δ exo1Δ double mutants in liquid cultures and found that yku70Δ exo1Δ mutants do not accumulate in medial nuclear division [Fig. 6b].

To determine whether EXO1 was required for the production of ssDNA in yku70Δ mutants, we used QAOS to measure ssDNA production in Y' sequences during growth at 37°C [Fig. 6c]. It is clear that yku70Δ exo1Δ mutants contain extremely low levels of ssDNA at subtelomeric sequences at both 20°C and 37°C. The levels are indistinguishable from those in YKU70Δ cells. No ssDNA was detected on the AC strand at telomeres [Fig. 6d]. Thus, it appears that EXO1 plays an important role in the accumulation of ssDNA in yku70Δ mutants and that in the absence of this EXO1-dependent ssDNA, yku70Δ mutants do not arrest cell division at 37°C.

EXO1 contributes to ssDNA production in cdc13-1 mutants

Since EXO1 is required to generate ssDNA at the telomeres of yku70Δ mutants, we asked whether EXO1 is also required to generate ssDNA and induce cell cycle arrest in cdc13-1 mutants. Figure 7a shows that cdc13-1 exo1Δ double mutants arrest at medial nuclear division, as do cdc13-1 mutants, when cultured at 37°C, but with slower kinetics. Therefore, EXO1 contributes to, but is not completely required for, the arrest of cdc13-1 mutants grown at 37°C. When we examined the effect of EXO1 on the appearance of ssDNA at the Y' 600 locus in cdc13-1 mutants, we found that ssDNA did appear in cdc13-1 exo1Δ mutants, reaching a level of about 6% after 1.5 h at 37°C and largely staying at this level for the rest of the experiment. This level of ssDNA was considerably less than the 30% level of ssDNA observed in cdc13-1 EXO1 strains [Fig. 7b]. We conclude that EXO1 contributes to ssDNA generation in cdc13-1 mutants, but that another exonuclease [ExoX] must also contribute to the production of ssDNA in cdc13-1 mutants.

MRE11 protects telomeres in yku70Δ mutants

EXO1 functions redundantly with MRE11 to process DSBs to create 3’ ssDNA tails [Tsubouchi and Ogawa 2000] and in other aspects of DNA damage metabolism (Moreau et al. 2001; Lewis et al. 2002). Therefore, it was possible that MRE11 also played a role in generating ssDNA in yku70Δ mutants. It was shown previously that yku80Δ mre11Δ double mutants display a synthetic poor growth phenotype [Nugent et al. 1998], which is opposite to the phenotype observed in yku70Δ exo1Δ strains [Fig. 6a], suggesting that MRE11 does not have EXO1-type properties when combined with a yku70Δ defect. To test this directly, we combined yku70Δ,
exo1Δ, and mre11Δ mutations and examined their effects on growth, cell cycle arrest, and ssDNA production. Figure 7c–e shows that yku70Δ mre11Δ double mutants are more temperature-sensitive than yku70Δ mutants. Interestingly, this temperature-sensitive growth phenotype is dependent on EXO1 (Fig. 7e). Liquid culture experiments demonstrated that yku70Δ mre11Δ double mutants arrested at medial nuclear division more rapidly than yku70Δ single mutants, arguing that MRE11 functions to maintain telomere structure in yku70Δ mutants, rather than to degrade telomere structure, as EXO1 does. The mre11Δ yku70Δ exo1Δ triple mutant did not arrest cell division at 37°C over a 9-h time course, suggesting that EXO1-dependent ssDNA is required for the cell cycle arrest of yku70Δ mre11Δ mutants cultured at 37°C.

Accumulation of telomeric ssDNA in yku70Δ mre11Δ mutants provides an explanation for their rapid arrest at
medial nuclear division at 37°C. Even at 20°C (at the beginning of the experiment), yku70Δ mre11Δ double mutants contain more ssDNA than yku70Δ mutants in their Y' sequences [Fig. 7g]. Furthermore, the amount of ssDNA increases more rapidly in yku70Δ mre11Δ double mutants than in yku70Δ mutants, which is consistent with the more rapid arrest observed in these strains. Finally, all of the ssDNA in yku70Δ mre11Δ double mutants appears to be dependent on EXO1.

Discussion

In this study, we examined the interactions of checkpoint pathways with the damaged telomeres that are present in yku70Δ mutant cells. We found that yku70Δ mutants, like cdc13-1 mutants cultured at 37°C, contain increased levels of ssDNA in subtelomeric sequences. However, the amount of ssDNA observed in yku70Δ mutants is considerably less than in cdc13-1 mutants. We demonstrated that EXO1 but not MRE11 is required for the production of this ssDNA and for cell cycle arrest. The correlation between the amount of ssDNA and cell cycle arrest in yku70Δ mutant cells is in many ways analogous to the situation observed with DSBs, when strains with more ssDNA arrest cell division for longer [Lee et al. 1998].

Interestingly, the damage induced in yku70Δ mutants activates a RAD9, CHK1, and MEC1-dependent checkpoint pathway, but is independent of RAD17, RAD24, MEC3, DCC1, and DUN1, whereas arrest of cdc13-1 mutants is dependent on all eight genes. yku70Δ-induced damage is the first type of DNA damage demonstrated to have these properties. A complementary pathway appears to exist in meiosis, because prophase arrest of dmc1Δ mutants is dependent on all eight genes. A complementary pathway appears to exist in meiosis, because prophase arrest of dmc1Δ mutants is dependent on all eight genes. However, the amount of ssDNA observed in yku70Δ mutants is considerably less than in cdc13-1 mutants.

We propose a model to explain the functions of checkpoint proteins in responding to (sub) telomere defects in yku70Δ mutants [Bishop et al. 1992; Lydall et al. 1996; Roeder and Bailis 2000].

In our present experiments, the fraction or cells arrested by the DNA damage and spindle checkpoint genes to block cell division [Garner et al. 2001]. Drosophila double parked mutants, defective in a homolog of CDT1, a gene whose product is required for DNA replication in fission yeast and Xenopus, depend on both DNA damage and spindle checkpoint genes to block cell division [Garner et al. 2001]. bub2 mutations, but not mad2 mutations, allow cdc13-1 mutants to rebud and reduplicate their DNA, without completing anaphase [Wang et al. 2000]. In our present experiments, the fraction or cells arrested by the MAD2-spindle checkpoint pathway was about fourfold lower than that arrested by the DNA dam-
In a culture of \textit{yku70}/H9004 cells, missing DNA damage checkpoint control, the fraction of cells arrested at the spindle checkpoint increased to about 20% after 18 h. This shows that the spindle checkpoint can substitute for the DNA damage checkpoint and stop cells with damaged telomeres from dividing. In a culture of \textit{yku70}/H9004 \textit{mad2}/H9004 cells, missing a spindle checkpoint pathway, the fraction of cells arrested at the DNA damage checkpoint reached a maximum of 75% (after 12 h; Fig. 4n). Why did the remaining 25% of cells fail to arrest? Presumably these cells were not arrested by the DNA damage checkpoint because they did not contain DNA damage. Instead, they appear to have generated another defect that triggers arrest by the MAD2-dependent spindle checkpoint pathway. It is interesting that during the time course of our experiment the fraction of dividing cells did not significantly increase, suggesting that the cells that are not arrested at medial nuclear division carry lesions [perhaps chromosome losses!] that limit cell division. Consistent with this interpretation, only 18% of \textit{yku70}/H9004 \textit{mad2}/H9004 cells were able to form colonies after 18 h at 37°C (data not shown).

Why should cells with damaged telomeres activate spindle checkpoint pathways? One explanation is that cells with damaged telomeres generate telomere fusions and dicentric chromosomes at high rates. Indeed, it is known that mammalian cells lacking Ku suffer from...
Table 1. Yeast strains

| Strain Description | Genotype | Origin |
|--------------------|----------|--------|
| 640                | Mata ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2 RAD5 | R. Rothstein |
| 641                | MATαpha ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2 RAD5 | R. Rothstein |
| 883                | MATα ddc1::KanMX4 rad5-535 | M.P. Longhese |
| 974                | MATα yku70::HIS3 RAD5 rDNA::ADE2 | L. Guarente |
| 1028               | MATαpha yku70::LEU2 rad5-535 | S. Jackson |
| 1095               | MATα chk1::HIS3 RAD5 | 640 transformation |
| 1199               | MATα yku70::HIS3 rad17::LEU2 rad5-535 | 974 × 607 |
| 1208               | MATαpha yku70::LEU2 mec5::TRP1 rad5-535 | 886 × 1028 |
| 1209               | MATα yku70::LEU2 mec5::TRP1 rad5-535 | 886 × 1028 |
| 1210               | MATα ddc1::KanMX4 yku70::LEU2 rad5-535 | 886 × 1028 |
| 1211               | MAT alpha ddc1::KanMX4 yku70::LEU2 rad5-535 | 886 × 1028 |
| 1214               | MATαpha yku70::LEU2 rad9::HIS3 rad24::TRP1 rad5-535 | 1028 × 261 |
| 1215               | MATα yku70::LEU2 chk1::HIS3 RAD5 | 1028 × 1096 |
| 1230               | Mata cdc13-1int RAD5 | 1108 transformation |
| 1248               | MATα rad5-535 sm1::KANMX4 | M.P. Longhese |
| 1249               | MATα rad5-535 sm1::KANMX4 mec1::HIS3 | M.P. Longhese |
| 1255               | MATα cdc13-1int rad9::HIS3 RAD5 | 662 × 1218 |
| 1257               | MATα cdc13-1int rad24::TRP1 RAD5 | 662 × 1218 |
| 1264               | MATαpha yku70::LEU2 rad9::HIS3 RAD5 | 1218 × 1214 |
| 1266               | MATα yku70::LEU2 chk1::HIS3 RAD5 | 1028 × 1096 |
| 1271               | MATα yku70::LEU2 rad9::HIS3 RAD5 | 1264 × 1285 |
| 1284               | MATα rad24::TRP1 yku70::HIS3 RAD5 | 974 × 1258 |
| 1296               | Mata exo1::LEU2 cdc13-1int RAD5 | 1272 × 1230 |
| 1312               | MATαpha yku70::LEU2 mec1::HIS3 rad5-535 sm1::KANMX4 | 1028 × 1249 |
| 1325               | MATα yku70::LEU2 mec1::HIS3 rad5-535 sm1::KANMX4 | 1028 × 1249 |
| 1327               | MATα yku70::LEU2 mec1::HIS3 rad24::TRP1 sm1::KANMX4 | 1312 × 1285 |
| 1337               | MATα yku70::LEU2 rad9::HIS3 rad24::TRP1 rad5-535 | 1028 × 261 |
| 1347               | MATαpha yku70::HIS3 rad17::LEU2 RAD5 | 1308 × 1284 |
| 1348               | MATα rad24::TRP1 yku70::HIS3 RAD5 | 1308 × 1284 |
| 1366               | MATαpha yku70::HIS3 RAD5 | 1308 × 1284 |
| 1384               | MATα yku70::HIS3 exo1::LEU2 RAD5 | 1273 × 1364 |
| 1390               | MATαpha yku70::HIS3 exo1::LEU2 RAD5 | 1273 × 1364 |
| 1400               | MATαpha yku70::HIS3 exo1::LEU2 rad24::TRP1 RAD5 | 1273 × 1364 |
| 1411               | MATα yku70::HIS3 exo1::LEU2 rad24::TRP1 RAD5 | 1273 × 1364 |
| 1412               | MATα yku70::HIS3 RAD5 | 1273 × 1364 |
| 1430               | MATα yku70::HIS3 rad24::TRP1 RAD5 | 1399 × 1364 |
| 1439               | MATαpha bab2::URA3 yku70::LEU2 rad5-535 | 1429 × 1371 |
| 1440               | MATαpha bab2::URA3 yku70::LEU2 RAD5 | 1429 × 1371 |
| 1441               | MATαpha bab2::URA3 yku70::LEU2 rad24::TRP1 RAD5 | 1429 × 1371 |
| 1442               | MATα bab2::URA3 yku70::LEU2 chk1::HIS3 rad5-535 | 1429 × 1371 |
| 1443               | MATα bab2::URA3 yku70::LEU2 chk1::HIS3 rad24::TRP1 RAD5 | 1429 × 1371 |
| 1445               | MATα mad2::URA3 yku70::LEU2 rad5-535 | 1429 × 1372 |
| 1446               | MATα mad2::URA3 yku70::LEU2 chk1::HIS3 RAD5 | 1429 × 1372 |
| 1448               | MATα mad2::URA3 yku70::LEU2 rad24::TRP1 RAD5 | 1429 × 1372 |
| 1449               | MATαpha mad2::URA3 yku70::LEU2 rad24::TRP1RAD5 | 1429 × 1372 |
| 1451               | MATαpha bab2::URA3 RAD5 | 1429 × 1371 |
| 1496               | MATαpha cdc13-1int bab2::URA3 RAD5 | 1451 × 1255 |
| 1497               | MATαpha cdc13-1int bab2::URA3 RAD5 | 1451 × 1255 |
| 1498               | MATαpha cdc13-1int mad2::URA3 rad9::HIS3 RAD5 | 1452 × 1255 |
| 1499               | MATαpha cdc13-1int mad2::URA3 rad9::HIS3 RAD5 | 1452 × 1255 |
| 1500               | MATαpha cdc13-1int mad2::URA3 RAD5 | 1452 × 1255 |
| 1501               | MATαpha cdc13-1int mad2::URA3 RAD5 | 1452 × 1255 |
| 1502               | MATαpha cdc13-1int bab2::URA3 RAD5 rad9::HIS3 | 1451 × 1255 |
| 1503               | MATαpha cdc13-1int bab2::URA3 RAD5 rad9::HIS3 | 1451 × 1255 |
| 1505               | MATαpha yku70::LEU2 rad24::TRP1 RAD5 | 1429 × 1371 |
| 1552               | MATα mad2::URA3 yku70::LEU2 | 1400 × 1449 |
| 1553               | MATα dun1::HIS3 yku70::LEU2 | 1400 × 1449 |
| 1554               | MATα dun1::HIS3 yku70::LEU2 | 1400 × 1449 |
| 1676               | MATαpha mre11::hisG::URA3 exo1::LEU2 RAD5 | 1330 × 1409 |
| 1678               | MATαpha yku70::HIS3 mre11::hisG::URA3 RAD5 | 1330 × 1409 |
| 1679               | MATαpha yku70::HIS3 mre11::hisG::URA3 RAD5 | 1330 × 1409 |
| 1680               | MATα yku70::HIS3 mre11::hisG::URA3 exo1::LEU2 RAD5 | 1330 × 1409 |
| 1746               | MATαpha mre11::hisG::URA3 RAD5 | 1330 × 1409 |

The strains are in the W303 background and relevant genotypes are shown. Where strains are the products of a genetic cross, the numbers of parent strains are also indicated.
high levels of telomere fusions [Bailey et al. 1999; Hsu et al. 2000; Samper et al. 2000; d’Adda di Fagagna et al. 2001]. In yeast, it has been shown that dicentric chromosomes, a product of telomere fusion, are activators of both spindle and DNA damage checkpoint pathways [Neff and Burke 1992].

5’ to 3’ exonucleases are thought to play a physiological role in the replication and stability of telomeres, but the nature of the exonuclease(s) responsible for telomere replication remains unclear [Wellerling et al. 1996; Diede and Gottschling 2001; Tsukamoto et al. 2001]. We have shown that EXO1 but not MRE11 is required to generate ssDNA at the telomeres of yku70Δ mutants, and so it is conceivable that EXO1 also plays a role in the physiological metabolism of telomeres. MRE11, in contrast to EXO1, stabilizes telomeres of yku70Δ mutants. If Exo1p does play a role in the physiology of telomeres, then other exonucleases must function redundantly with Exo1p because the telomeres of exo1Δ mutants appear normal [Tsubouchi and Ogawa 2000].

Defects in mismatch repair are associated with checkpoint defects in mammalian cells [Bellacosa 2001; Yan et al. 2001] and enhanced cellular proliferation of yeast cells that lack telomerase [Rizki and Lundblad 2001]. Our demonstration that the mismatch repair-associated exonuclease, Exo1p, affects the metabolism of damaged telomeres, and checkpoint responses, suggests a mechanism by which mismatch repair affects checkpoint control and tolerance of damaged telomeres.

Materials and methods

Yeast strains

All strains used in this study are isogenic and in the W303 background; in most cases we used RAD5 rather than rad5-355 strains [Fan et al. 1996], but we observed no effect of the rad5-355 mutation in any experiments. To construct strains, standard genetic procedures of transformation and tetrad analysis were followed [Adams et al. 1997]. Since W303 strains contain an ade2-1 mutation YEPD (yeast extract, peptone, and dextrose), the medium was routinely supplemented with adenine at 50 mg/L. The yku70Δ deletion strains were obtained from L. Guarente (Massachusetts Institute of Technology, Cambridge, MA) and S. Jackson [University of Arizona, Tuscon, AZ]. A chk1- HIS3 deletion was created using pYSS1 [Sanchez et al. 1999]. The mec1Δ and smd1Δ deletion strains were obtained from M.P. Longhese [Paciotto et al. 2000]. An exo1::LEU2 disruption was constructed using pHT246 and an mrc::hisg::URA3 deletion with pHT16 [Tsubouchi and Ogawa 2000]. mad2Δ and bub2Δ deletion strains were obtained from L. Dirick. Dun1Δ strains were obtained from T. Weinert. Other deletions have been described elsewhere [Lydall and Weinert 1997]. cdc13-1int strains contain a cdc13-1 integrated allele rather than one that was introduced by backcrossing from the A364a genetic background.

We have observed that other yku70Δmre11Δ (exo1Δ) mutants enter crisis after several generations, and therefore we assume the strains analysed in Figure 7 have escaped or avoided crisis.

EXO1-dependent ssDNA and yku70Δ checkpoints

Serial dilution and growth on plates

Colonies were inoculated into 1 mL YEPD [ade], and grown overnight with aeration. In the morning, cultures were diluted 1:10, grown for about 4 h, sonicated, counted by hemocytometer, and diluted to 1.5 × 10^7 cells/mL. Fivefold dilution series were set up in 96-well plates, and small aliquots of the dilution series were transferred to YEPD [ade] plates using metal prongs. Plates were incubated for 2 d before being photographed.

Liquid culture, medial nuclear division, and viability assays

Single purified colonies were inoculated directly into 50 mL of YEPD [ade] and cultured overnight, with aeration, at 23°C. In the morning, cell densities were determined by hemocytometer, and cultures were diluted to 2 × 10^6 cells/mL. The cultures were placed at the restrictive temperature of 37°C, and samples were taken at the times indicated. Cultures were maintained at a concentration that allowed exponential growth, diluting when necessary with prewarmed (37°C) medium. Cell densities were determined by hemocytometer, and the corrected cell number was calculated as a product of cell density and cumulative dilution factor. To score checkpoint arrest, samples were taken at the indicated time points and fixed in 70% EtOH, then washed twice with water. To visualize the DNA, cells were resuspended in 0.2μg/mL 4′,6-diamidino-2-phenylindole (DAPI), sonicated, and examined by fluorescent microscopy. At least 200 cells were counted using the microcounter, and classified as described previously [Gardner et al. 1999] as: (1) unbudded, single DAPI-stained body; (2) small budded, single DAPI-stained body, the bud <50% of the diameter of the mother cell; (3) medial nuclear division, single DAPI-stained body, bud >50% diameter of mother cell; and (4) late nuclear division, two buds, and two DAPI-stained bodies, and (5) none of these types.

Microcolony assays

Colony-purified yeast strains were inoculated into 1 mL YEPD [ade], grown overnight with aeration, at the appropriate temperature [20°C for cdc13-1 strains and 23°C for yku70Δ cells], until they reached a concentration of about 8 × 10^6 cells/mL. Cells were arrested in G1 with alpha-factor for about 2.5 h, and arrest was monitored microscopically. Arrested cells were washed twice with YEPD [ade], sonicated briefly, and spread on plates. The plates were incubated at the indicated temperature. After an appropriate length of time the colonies were photographed.

Single-stranded DNA measurements

Single-stranded DNA was measured as described [Booth et al. 2001] except that we calculated ssDNA levels by comparison with a PDA1 “loading control.” PDA1 is 30 kbp from the telomere and does not become single-stranded in yku70Δ mutants. The PDA1 and YER188W primers are as described [Booth et al. 2001]. The sequences of the primers used to detect ssDNA in the Y′ sequence are available on request.

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EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70\(^{Δ}\) mutants

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