EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast *yku70*Δ mutants

Laura Maringele and David Lydall[1]

School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK

We have examined the role of checkpoint pathways in responding to a *yku70*Δ defect in budding yeast. We show that *CHK1*, *MEC1*, and *RAD9* checkpoint genes are required for efficient cell cycle arrest of *yku70*Δ mutants cultured at 37°C, whereas *RAD17*, *RAD24*, *MEC3*, *DDC1*, and *DUN1* play insignificant roles. We establish that cell cycle arrest of *yku70*Δ 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 *yku70*Δ mutants. In contrast, *MRE11* is not required for ssDNA generation. The behavior of *yku70*Δ *exo1*Δ double mutants strongly indicates that ssDNA is an important component of the arrest signal in *yku70*Δ 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 *yku70*Δ mutants. Therefore, subsets of both DNA-damage and spindle checkpoint pathways cooperate to regulate cell division of *yku70*Δ mutants.

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

Received January 10, 2002; revised version accepted June 6, 2002.

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 *ku80*$^{-/-}$ 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

[1]**Corresponding author.**
E-MAIL lydall@man.ac.uk; FAX 44-0-161-275-5600.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.225102.

the Yku70p/Yku80p heterodimer. Mutants with deletions of *YKU70* or *YKU80* contain short telomeres (Boulton 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 *yku70*Δ and *yku80*Δ 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 *yku70*Δ 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 *yku70*Δ 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 *yku70*Δ 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 *yku70*Δ mutants. Finally, we show that the mismatch repair-associated exonuclease *EXO1* is essential for ssDNA generation in *yku70*Δ 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* yku70Δ*-induced checkpoint*

To determine how checkpoint pathways interact with *yku70*Δ-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 nonlethal levels of DNA damage to divide and form colonies. Figure 1 shows the growth of serial dilutions of *yku70*Δ and checkpoint mutant cells at 28°C, 36°C, and 37°C. At 28°C, a permissive temperature for *yku70*Δ 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 *chk1*Δ mutation had the most profound effect and significantly increased the ability of *yku70*Δ mutants to form colonies at both 36°C and 37°C (Fig. 1g–i). *rad9*Δ and *mec1*Δ also increased *yku70*Δ colony size, but the colonies were smaller than the *yku70*Δ *chk1*Δ colonies (Fig. 1d–i). In contrast, *mec3*Δ, *ddc1*Δ, *rad17*Δ, *rad24*Δ, and *dun1*Δ mutations had minor effects on the growth of *yku70*Δ mutants (Fig. 1a–i). The growth of *rad9*Δ *rad24*Δ *yku70*Δ triple mutants at 36°C and 37°C was most similar to that of *yku70*Δ *rad9*Δ 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 *yku70*Δ mutants at 36°C and 37°C.

To determine whether checkpoint pathways are activated in *yku70*Δ mutants, we examined the growth and cell cycle distribution of *yku70*Δ and checkpoint mutants in liquid cultures (Fig. 2). In four separate experiments, the growth of *yku70*Δ 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 *yku70*Δ cells began to plateau after about 6 h in liquid culture (Fig. 2a–d; data not shown). The poor growth of *yku70*Δ 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 budded cells that accumulated in *yku70*Δ (*hdf1*Δ) 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 *yku70*Δ mutants is in contrast to the behavior of *cdc13-1* mutants, because 94% of *cdc13-1* mutants 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 *yku70*Δ *chk1*Δ, *yku70*Δ *rad9*Δ, and *yku70*Δ *mec1*Δ mutants was most like *YKU70*$^+$ *RAD*$^+$ 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 *yku70*Δ 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 *yku70*Δ *ddc1*Δ, *yku70*Δ *rad24*, and *yku70*Δ *dun1*Δ mutants were most similar to *yku70*Δ strains, suggesting that *yku70*Δ-induced checkpoint pathways are intact in *rad24*Δ, *ddc1*Δ, and *dun1*Δ mutants (Fig. 2a,b,e,f). Both *yku70*Δ *mec3*Δ and *yku70*Δ

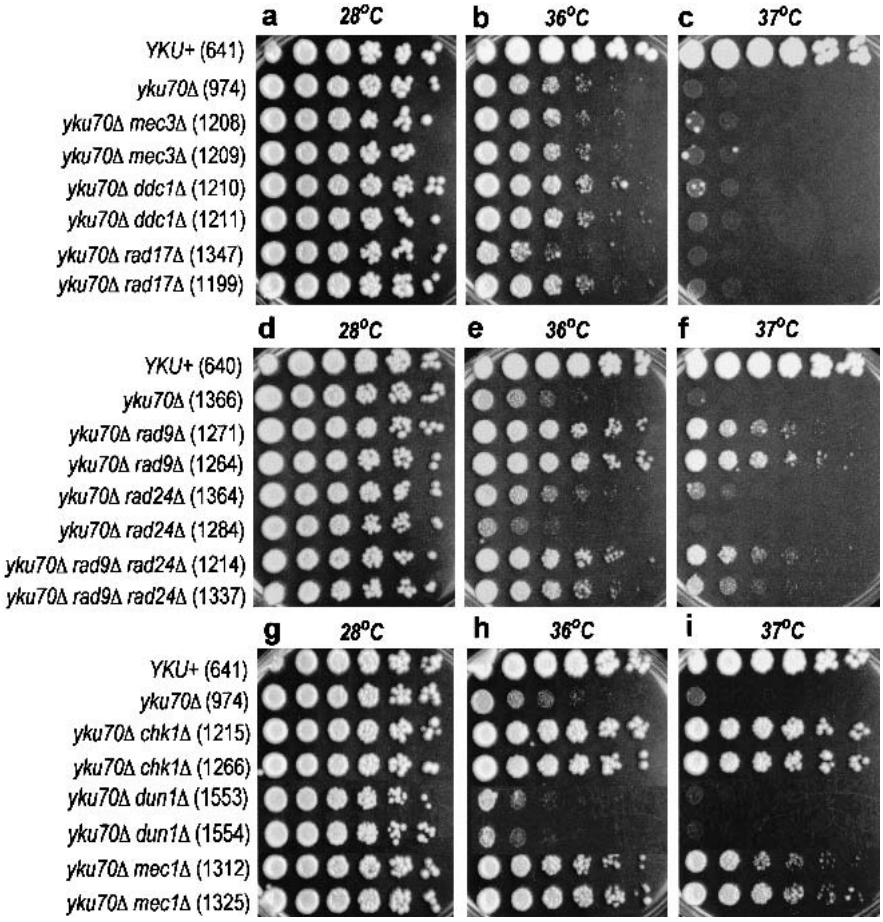

Figure 1. *RAD9*, *MEC1*, and *CHK1* inhibit growth of *yku70*Δ strains at high temperatures. Small aliquots of fivefold dilution series of several yeast strains were transferred to plates and incubated at the temperatures indicated for 2 d before being photographed. The relevant genotypes of the strains are indicated on the *left,* and the strain numbers shown in parentheses. The *mec1*Δ strains also carried an *sml1*Δ mutation. To ensure reproducibility we routinely examined two independent strains with the same genotypes. All strains grouped together were grown on the same plates, except for the *yku70*Δ *dun1*Δ strains, which were grown on different plates. The photographs of the *yku70*Δ *dun1*Δ strains were superimposed in *g,h,i*.

*rad17*Δ mutants behaved similarly to *yku70*Δ *rad24*Δ mutants (data not shown). We found that *rad53*Δ *sml1*Δ single mutants, as well as *yku70*Δ *rad53*Δ *sml1*Δ 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, *yku70*Δ *mec1*Δ *and yku70*Δ *chk1*Δ mutants begin to slow cell division and start to accumulate at medial nuclear division, after long periods (8 h) at 37°C (Fig. 2 c,d,g,h). This suggests that another checkpoint pathway, independent of *CHK1* and *MEC1*, can arrest cell division of *yku70*Δ mutants after long periods at 37°C. This arrest is due to the activation of spindle checkpoint pathways (see Fig. 4, below). Both *yku70*Δ *rad24*Δ *rad9*Δ and *yku70*Δ *rad24*Δ *mec1*Δ triple mutants showed the exponential growth phenotype of *yku70*Δ *rad9*Δ and *yku70*Δ *mec1*Δ mutants respectively, indicating that the exponential growth phenotype is epistatic to the poor growth phenotype (Fig. 2d,h; data not shown). In summary, these liquid culture experiments suggest that a checkpoint pathway that arrests *yku70*Δ mutants at medial nuclear division at 37°C is dependent on *CHK1*, *RAD9*, and *MEC1*, but independent of *RAD17*, *RAD24*, *MEC3*, *DDC1*, and *DUN1*.

Microcolony assays were used to confirm that *yku70*Δ mutants are able to divide several times before ceasing growth at 37°C, and that *rad9*Δ and *rad24*Δ mutations had different effects on cell division. *MAT**a*** 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, *YKU70*$^+$ *RAD*$^+$ and *yku70*Δ *rad9*Δ cells had formed much larger and more uniformly sized colonies (>1000 cells) than *yku70*Δ and *yku70*Δ *rad24*Δ cells (2–200 cells) (Fig. 3a–d). This shows that deletion of *RAD9*, but not *RAD24*, allows *yku70*Δmutants to grow over 20 h, at 37°C, at rates indistinguishable from the rate of *YKU70*$^+$ cells. The behavior of *yku70*Δ *checkpoint*Δ double mutants cultured at 37°C is in contrast to the behavior of *cdc13-1 checkpoint*Δ double mutants cultured at a similar temperature, as *cdc13-1 rad9*Δ mutants formed smaller microcolonies at 36°C compared to the *cdc13-1 rad24*Δ mutants (Lydall and Weinert 1997).

A model that might explain the different roles of *RAD9* and *RAD24* in responding to *yku70*Δ and *cdc13-1*-induced damage is that *RAD9* is required for the primary checkpoint response in *yku70*Δ and *cdc13-1* mutants, and that *RAD24* is required for arrest only when

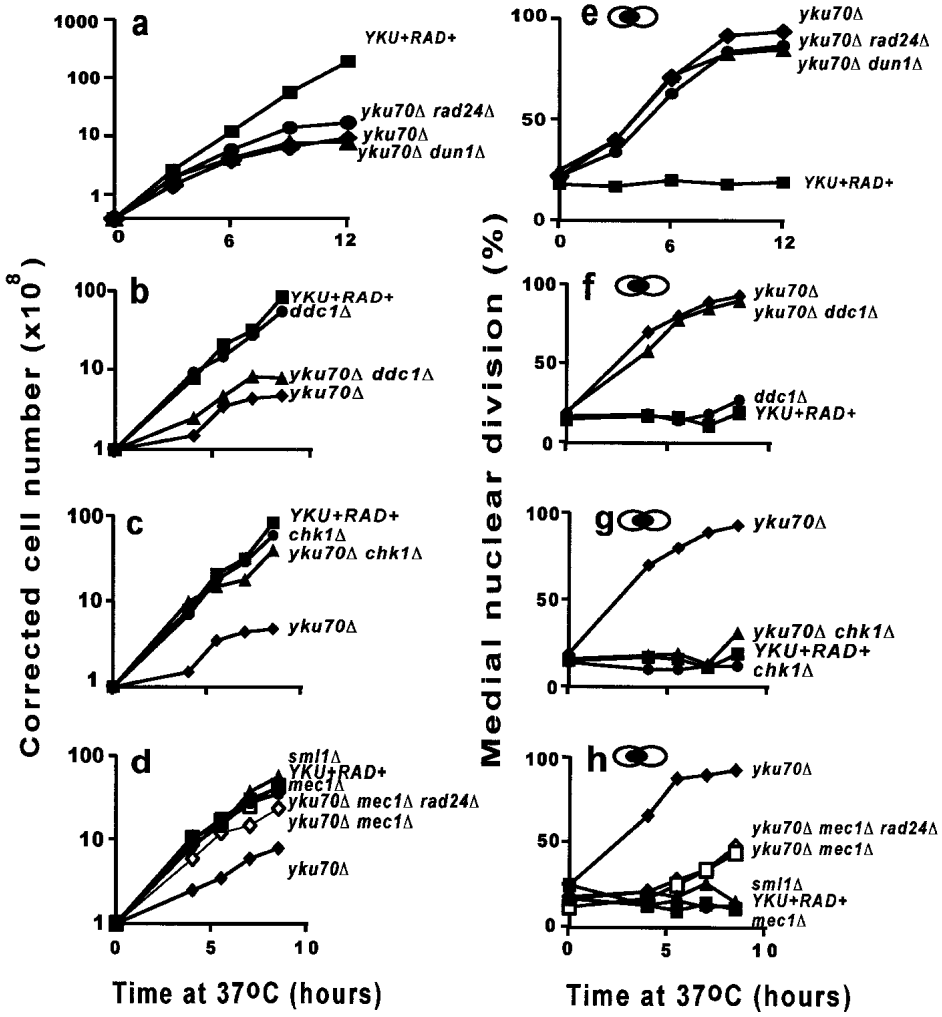

Figure 2. *RAD9*, *MEC1*, and *CHK1* cause G2/M arrest of *yku70*Δ strains at 37°C. A series of yeast strains dividing exponentially at 23°C were placed at 37°C and their growth and cell cycle distribution were monitored. At indicated time points, cells density was determined by hemocytometer (*a–d*) and cell cycle distribution was determined by staining with DAPI (*e–h*). The yeast stains used were *YKU*$^+$ *RAD*$^+$: DLY640 in *a,d,e,h,* and DLY641 in *b,c,e,f*; *yku70*Δ: DLY1412 in *a,e*, and DLY1366 in *b,c,d,f,g,h*; *yku70*Δ *rad24*Δ: DLY 1505; *yku70*Δ *dun1*Δ: DLY 1553; *ddc1*Δ: DLY883; *yku70*Δ *ddc1*Δ: DLY1220; *chk1*Δ: DLY1095; *yku70*Δ *chk1*Δ: DLY1215; *sml1*Δ: DLY1248; *mec1*Δ: DLY1249; *yku70*Δ *mec1*Δ *rad24*Δ: DLY1327; and *yku70*Δ *mec1*Δ: DLY1325.

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 *yku70*Δ 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 *yku70*Δ 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 rad9*Δ cells formed medium-sized colonies (20–200 cells), whereas *cdc13-1 rad24*Δ 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 rad9*Δ mutants form smaller colonies than *cdc13-1 rad24*Δ mutants. In contrast, *yku70 rad9*Δ mutants form larger colonies than *yku70*Δ *rad24*Δ mutants at 37°C. Therefore, we conclude that *RAD9*- and *RAD24*-dependent checkpoint pathways play different roles in responding to *yku70*Δ 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, *yku70*Δ *mec1*Δ and *yku70*Δ *chk1*Δ mutants began to

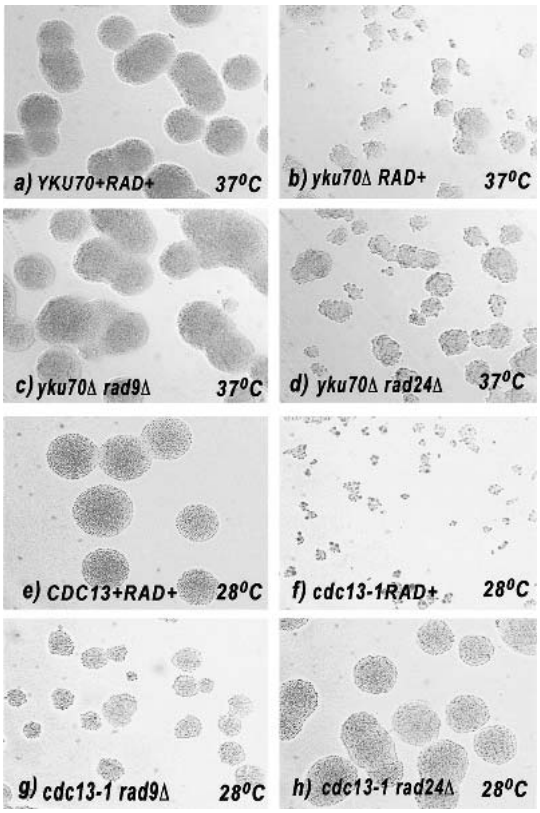

Figure 3. *RAD9* and *RAD24* have different effects on the growth of *yku70*Δ and *cdc13-1* colonies. Single G1-arrested cells were spread on YEPD (ade) plates and photographed after 20-h growth. The yeast strains were (*a*) DLY640, (*b*) DLY1412, (*c*) DLY1271, (*d*) DLY1364, (*e*) DLY640, (*f*) DLY1230, (*g*) DLY1255, and (*h*) DLY1257.

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 *yku70*Δ *chk1*Δ mutants, we examined the effect of *mad2*Δ and *bub2*Δ mutations on the growth of *yku70*Δ strains at 37°C (*MAD2* and *BUB2* belong to different arms of the spindle checkpoint pathways (Gardner and Burke 2000). Whereas a *mad2*Δ mutation increased the growth of *yku70*Δ mutants (Fig. 4a–c), a *bub2*Δ mutation had no effect on the growth of *yku70*Δ mutants (Fig. 4d–f). Interestingly, simultaneous disruption of both *RAD24* and *MAD2* increased the growth of *yku70*Δ mutants more than either single mutation (Fig. 4b,c), suggesting that perhaps *RAD24* plays a small role in the arrest of *yku70*Δ mutants, a role that can be unmasked by deletion of *MAD2*. A *yku70*Δ *chk1*Δ *mad2*Δ 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 *mad2*Δ and *bub2*Δ mutations with *cdc13-1*. Curiously, and once again, *cdc13-1* and *yku70*Δ mutants showed different, almost opposite interactions with checkpoint pathways. A *bub2*Δ deletion had a moderate effect on the growth of *cdc13-1* mutants (Fig. 4j–l), whereas a *mad2*Δ deletion had less effect (Fig. 4g–l). The effect of the *bub2*Δ mutation was not as strong as a *rad9*Δ DNA damage checkpoint mutation, and the *rad9*Δ *bub2*Δ double mutant behaved like the single *rad9*Δ mutant (Fig. 4j–l). Similarly, the *cdc13-1 rad9*Δ *mad2*Δ triple mutants formed colonies with efficiency similar to that of the *cdc13-1 rad9*Δ double mutants.

To confirm that *MAD2* was responsible partially for arrest of *yku70*Δ mutants and to produce evidence that it contributed to the residual arrest observed in *yku70*Δ *chk1*Δ mutants at 37°C, we performed liquid culture experiments. Figure 4m shows that the decrease in growth observed in *yku70*Δ *chk1*Δ mutants after several hours at 37°C (vs. *YKU70*$^+$ cells) could be overcome by a *mad2*Δ deletion, because *yku70*Δ *chk1*Δ *mad2*Δ triple mutants grew almost as well as the *YKU70*$^+$ strain. Figure 4n shows that the increase of *yku70*Δ *chk1*Δ muants at medial nuclear division, during 9–18 h of incubaion at 37°C, did not occur in the *yku70*Δ *chk1*Δ *mad2*Δ triple mutants. The growth of *yku70*Δ *bub2*Δ mutants is most similar to *yku70*Δ cells, indicating that *BUB2* does not play a role in the arrest of *yku70*Δ mutants at 37°C. Consistent with the hypothesis that *yku70*Δ damage induces a *MAD2*-dependent arrest, *yku70*Δ *mad2*Δ mutants reached an approximately five-fold higher cell density than *yku70*Δ cells over an 18-h period (Fig. 4m). In addition, a maximum of 75% of *yku70*Δ *mad2*Δ mutants arrested at medial nuclear division, whereas about 95% of *yku70*Δ cells arrested (Fig. 4n).

We have shown that arrest of *yku70*Δ 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 *yku70*Δ *mad2*Δ 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 *yku70*Δ mutants arrested minus the percentage of *yku70*Δ *mad2*Δ cells arrested at 12 h). In the case of *yku70*Δ *chk1*Δ 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 *yku70*Δ mutants.

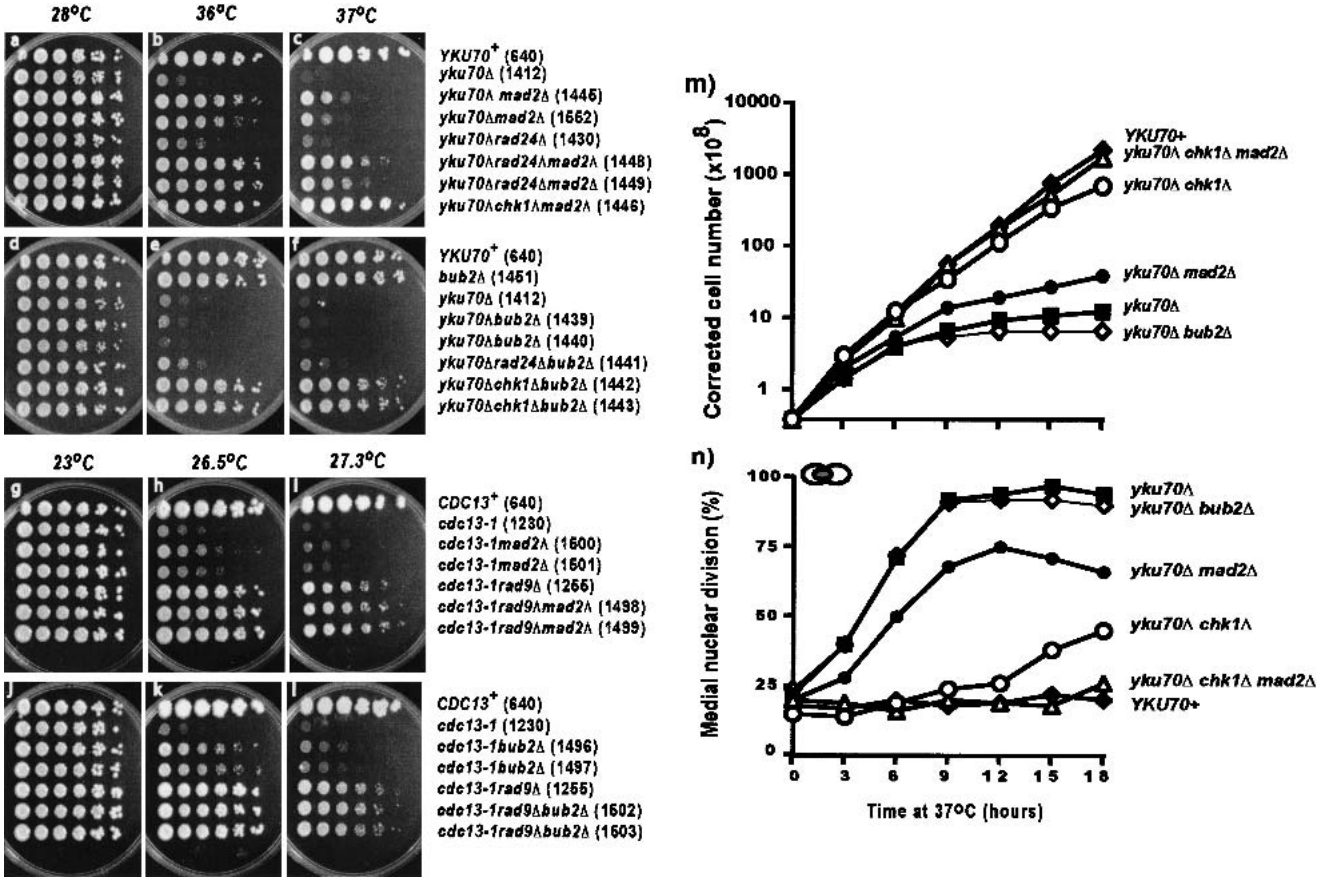

Figure 4. *MAD2* inhibits the growth of *yku70*Δ mutants, while *BUB2* inhibits growth of *cdc13-1* mutants. (*a–l*) Small aliquots of fivefold dilution series of several yeast strains were transferred to plates and incubated for 2 d (*a–f*) or 3 d (*g–l*) before being photographed. The relevant genotypes of the strains are indicated on the *left* and the strain numbers shown in parentheses. (*m*,*n*) A series of yeast strains dividing exponentially at 23°C was placed at 37°C and their growth and cell cycle distribution were monitored. At indicated time points, cell density was determined by hemocytometer and cell cycle distribution was determined by staining with DAPI. The yeast stains used were *YKU*$^+$ *RAD*$^+$: DLY640; *yku70*Δ *chk1*Δ *mad2*Δ: DLY1446; *yku70*Δ *chk1*Δ: DLY1215; *yku70*Δ *mad2*Δ: DLY1445; *yku70*Δ: DLY1412; and *yku70*Δ *bub2*Δ: DLY1440.

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 *yku70*Δ mutants at 37°C, then increased levels should be observed at restrictive temperatures. It was known that *yku70*Δ and *yku80*Δ 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, *yku80*Δ 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 *yku70*Δ 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 *yku70*Δ 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 *yku70*Δ mutants cultured at the restrictive temperature of 37°C generated increasing amounts of ssDNA at subtelomeric 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 *yku70*Δ 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

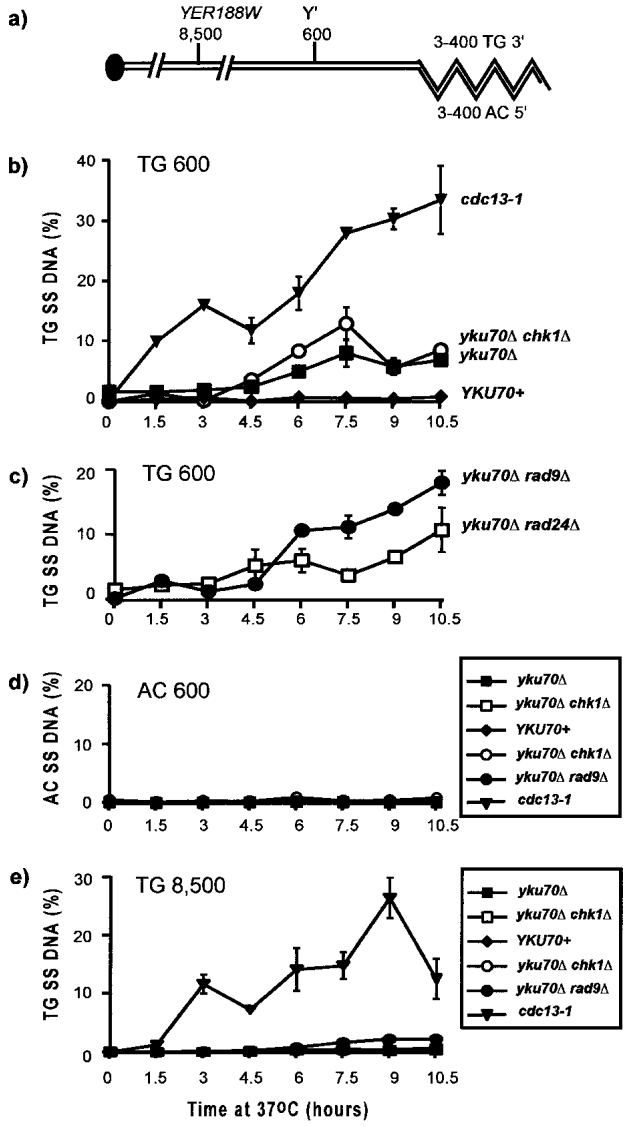

Figure 5. *yku70*Δ mutants accumulate ssDNA in subtelomeric sequences. A series of yeast strains was cultured at 37°C, and the amount of ssDNA at their telomeres was measured by quantitative amplification of ssDNA (QAOS). The yeast stains used were *cdc13-1*: DLY1230; *yku70*Δ *chk1*Δ: DLY1215; *yku70*Δ: DLY1412; *YKU70*$^+$: DLY640; and *yku70*Δ *rad9*Δ: DLY1271. The results shown for *yku70*Δ *rad24*Δ 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 *yku70*Δ *rad24*Δ, 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*, 8500 bases from the right end of chromosome V.

(Fig. 5b). The increase in ssDNA is less rapid and less extensive than that observed in *cdc13-1* mutants during the same experiment, but the amount of ssDNA is significantly higher than that observed in control *YKU70*$^+$ cells (Fig. 5b). No ssDNA was detected on the AC strand in any strains (Fig. 5d). Therefore, the ssDNA observed in *yku70*Δ mutants is on the TG (3′) strand of (sub) telomeres and is presumably caused by loss of the AC (5′) strand, as in *cdc13-1* mutants. The increase in the levels of ssDNA in Y′ sequences with time at 37°C is consistent with the hypothesis that the subtelomeric ssDNA in *yku70*Δ mutants contributes to activation of the *RAD9*, *CHK1*, and *MEC1*-dependent checkpoint pathway. There is a clear correlation between the accumulation of ssDNA at the Y′ 600 locus, after a 6-h incubation at 37°C (Fig. 5b) and the accumulation of cells at medial nuclear division in checkpoint-proficient *yku70*Δ cells (Figs. 2 and 4n).

Despite the fact that *yku70*Δ *rad9*Δ and *yku70*Δ *chk1*Δ mutants grow at 37°C, whereas *yku70*Δ and *yku70*Δ *rad24*Δ did not, all of these mutants accumulated comparable amounts of ssDNA, arguing that their different growth phenotypes are not due to differences in the amount of DNA damage among these strains. Interestingly, the strains that did not grow at 37°C, the *yku70*Δ and *yku70*Δ *rad24*Δ strains, had marginally less ssDNA than the strains that grew (*yku70*Δ *rad9*Δ and *yku70*Δ *chk1*Δ; Fig. 5b,c). This observation, together with the cell cycle distribution results (Fig. 2) argues that checkpoint genes differ in their ability to induce arrest, rather than in their ability to affect the production of ssDNA in Y′ sequences of *yku70*Δ mutants.

The effect of checkpoint genes on the production of ssDNA in *cdc13-1* mutants is easily detected at a locus 12 kbp from the telomere, *cdc13-1 rad9*Δ mutants generate ssDNA more rapidly than *cdc13-1* cells, which in turn generate ssDNA more rapidly than *cdc13-1 rad24*Δ cells (Lydall and Weinert 1995). To investigate whether *yku70*Δ mutants generate ssDNA beyond the Y′ sequences, 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 *yku70*Δ 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, *yku70*Δ *rad9*Δ mutants generated some ssDNA at *YER188W*, which suggests that *RAD9* may inhibit ssDNA production in *yku70*Δ mutants, as it does in *cdc13-1* mutants (Lydall and Weinert 1995).

In summary, we find that *yku70*Δ 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 *yku70*Δ mutants.

EXO1 *is required for ssDNA generation and arrest of* yku70Δ *mutants*

If ssDNA contributes to the signal that arrests cell division of *yku70*Δ mutants, then mutations that reduce the

amount of ssDNA should alleviate arrest. To test this hypothesis, we examined the effect of an *exo1*Δ mutation on arrest of *yku70*Δ 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 *exo1*Δ mutation strongly increases the ability of *yku70*Δ mutants to form colonies at 36°C and 37°C. The effect is as strong as that seen with a *chk1*Δ 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 *yku70*Δ *exo1*Δ double mutants in liquid cultures and found that *yku70*Δ *exo1*Δ mutants do not accumulate in medial nuclear division (Fig. 6b).

To determine whether *EXO1* was required for the production of ssDNA in *yku70*Δ mutants, we used QAOS to measure ssDNA production in Y′ sequences during growth at 37°C (Fig. 6c). It is clear that *yku70*Δ *exo1*Δ mutants contain extremely low levels of ssDNA at subtelomeric sequences at both 20°C and 37°C. The levels are indistinguishable from the levels in *YKU70*$^+$ 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 *yku70*Δ mutants and that in the absence of this *EXO1*-dependent ssDNA, *yku70*Δ mutants do not arrest cell division at 37°C.

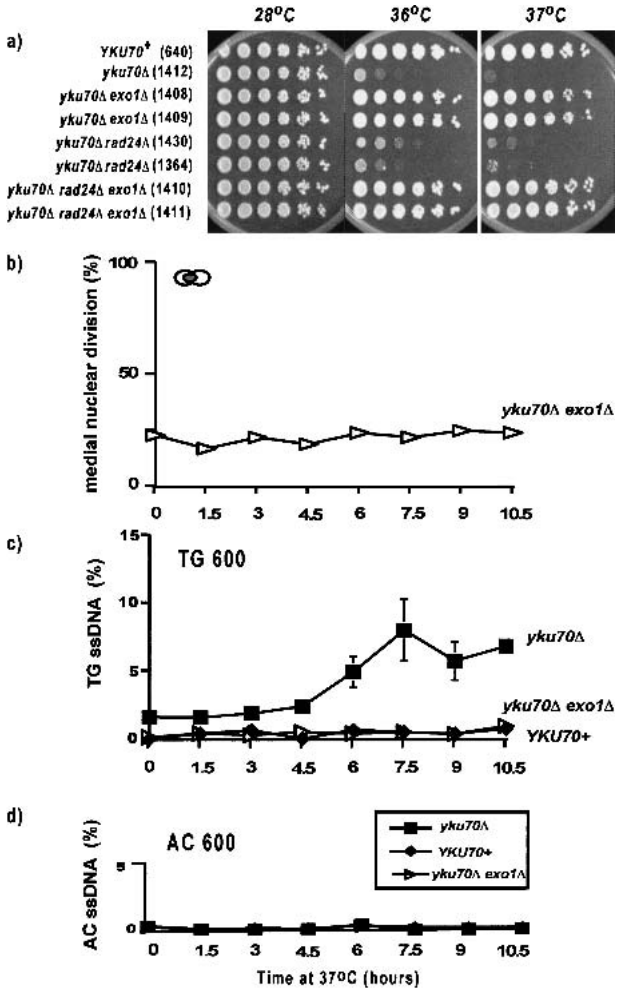

Figure 6. *EXO1* is required for arrest and ssDNA generation in *yku70*Δ mutants. (*a*) Small aliquots of fivefold dilution series of several yeast strains were transferred to plates and incubated at 28°C, 36°C, or 37°C for 2 d before being photographed. The relevant genotypes of the strains are indicated on the *left,* and the strain numbers shown in parentheses. (*b*) A *yku70*Δ *exo1*Δ (DLY1408) yeast strain dividing exponentially at 20°C was placed at 37°C, and its cell cycle distribution was monitored by staining with DAPI (see Figs. 2 and 4n for the behavior of control strains). (*c*) Detection of ssDNA on the TG strand 600 bases from the telomeric end of the Y′ sequence. The yeast strains were *yku70*Δ: DLY1412; *yku70*Δ *exo1*Δ: DLY1408; and *YKU70*$^+$: DLY640. (*d*) Detection of ssDNA on the AC strand 600 bases from the telomeric end of the Y′ sequence. The yeast strains are as in *c*.

EXO1 *contributes to ssDNA production in* cdc13-1 *mutants*

Since *EXO1* is required to generate ssDNA at the telomeres of *yku70*Δ 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 exo1*Δ 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 exo1*Δ 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 *yku70*Δ mutants. It was shown previously that *yku80*Δ *mre11*Δ double mutants display a synthetic poor growth phenotype (Nugent et al. 1998), which is opposite to the phenotype observed in *yku70*Δ *exo1*Δ strains (Fig. 6a), suggesting that *MRE11* does not have *EXO1*-type properties when combined with a *yku70*Δ defect. To test this directly, we combined *yku70*Δ,

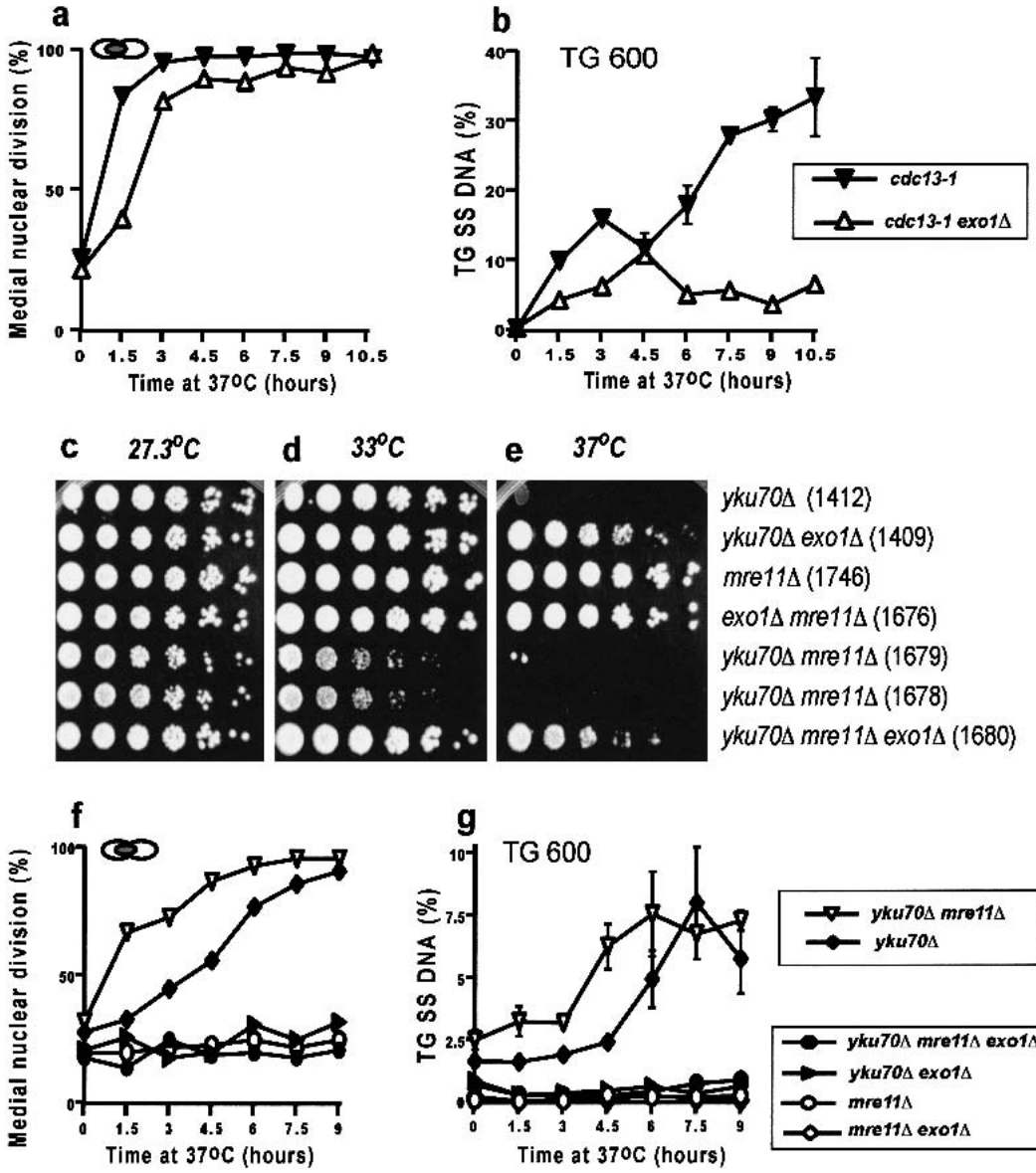

Figure 7. *EXO1* contributes to cell cycle arrest and ssDNA production in *cdc13-1* and *yku70*Δ *mre11*Δ mutants. (*a*) Yeast strains 1230 (*cdc13-1*) and 1296 (*cdc13-1 exo1*Δ) dividing exponentially at 20°C were placed at 37°C, and the fraction of cells in medial nuclear division was monitored by staining with DAPI. (*b*) Detection of ssDNA on the TG strand 600 bases from the telomeric end of the Y′ sequence. The yeast strains were as in *a*. (*c*,*d*,*e*) Small aliquots of fivefold dilution series of several yeast strains were transferred to plates and incubated for 2 d before being photographed. The relevant genotypes of the strains are indicated on the *right*, and the strain numbers are shown in parentheses. (*f*) Yeast strains dividing exponentially at 20°C were placed at 37°C, and their cell cycle distribution was monitored. The strains were *yku70*Δ: DLY1412; *yku70*Δ *mre11*Δ: DLY1679; *yku70*Δ *mre11*Δ *exo1*Δ: DLY1680; *yku70*Δ *exo1*Δ: DLY1408; Δ *mre11*Δ: DLY1746; and *mre11*Δ *exo1*Δ: DLY1676. (*g*) Detection of ssDNA on the TG strand 600 bases from the telomeric end of the Y′ sequence. The yeast strains were as in *f*.

*exo1*Δ, and *mre11*Δ mutations and examined their effects on growth, cell cycle arrest, and ssDNA production. Figure 7c–e shows that *yku70*Δ *mre11*Δ double mutants are more temperature-sensitive than *yku70*Δ mutants. Interestingly, this temperature-sensitive growth phenotype is dependent on *EXO1* (Fig. 7e). Liquid culture experiments demonstrated that *yku70*Δ *mre11*Δ double mutants arrested at medial nuclear division more rapidly than *yku70*Δ single mutants, arguing that *MRE11* functions to maintain telomere structure in *yku70*Δ mutants, rather than to degrade telomere structure, as *EXO1* does. The *mre11*Δ *yku70*Δ *exo1*Δ 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 *yku70*Δ *mre11*Δ mutants cultured at 37°C.

Accumulation of telomeric ssDNA in *yku70*Δ *mre11*Δ 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), *yku70*Δ *mre11*Δ double mutants contain more ssDNA than *yku70*Δ mutants in their Y′ sequences (Fig. 7g). Furthermore, the amount of ssDNA increases more rapidly in *yku70*Δ *mre11*Δ double mutants than in *yku70*Δ mutants, which is consistent with the more rapid arrest observed in these strains. Finally, all of the ssDNA in *yku70*Δ *mre11*Δ 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 *yku70*Δ mutant cells. We found that *yku70*Δ mutants, like *cdc13-1* mutants cultured at 37°C, contain increased levels of ssDNA in subtelomeric sequences. However, the amount of ssDNA observed in *yku70*Δ 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 *yku70*Δ 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 *yku70*Δ mutants activates a *RAD9*, *CHK1*, and *MEC1*-dependent checkpoint pathway, but is independent of *RAD17*, *RAD24*, *MEC3*, *DDC1*, and *DUN1*, whereas arrest of *cdc13-1* mutants is dependent on all eight genes. *yku70*Δ-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 *dmc1*Δ mutants, which cannot complete meiotic recombination, depends on *RAD24* but is independent of *RAD9* and *CHK1* (Bishop et al. 1992; Lydall et al. 1996; Roeder and Bailis 2000).

We propose a model to explain the functions of checkpoint proteins in responding to (sub) telomere defects in *cdc13-1* and *yku70*Δ mutants (Fig. 8). According to this model, in *cdc13-1* mutants, unprotected telomeres are perceived as DSBs with a short 3′ overhang. The Rad24p/Rfc2-5p clamp loader complex (Green et al. 2000) recognizes this structure and loads the Ddc1p, Mec3p, Rad17p sliding clamp (Venclovas and Thelen 2000). The sliding clamp tethers an unknown protein, "P" (e.g., a helicase), which processes the telomeric termini to generate the "1st processed DNA damage". The "1st processed DNA damage" is the substrate that activates a Mec1p/Rad53p/Dun1p-dependent checkpoint pathway. Rad9p might also participate in Rad53p activation (Gilbert et al. 2001). Rad53p and Dun1p are known to be responsible for 50% of the arrest observed in *cdc13-1* mutants (Gardner et al. 1999). We suggest that protein "P" is then replaced by Exo1p and by another 5′ to 3′ exonuclease ExoXp, which have affinity for the "1st processed DNA damage." The ssDNA generated by Exo1p and ExoXp activates a Rad9p/Mec1p/Chk1p- and Pds1p-dependent pathway. Chk1p/Pds1p are required for 50% of the arrest observed in *cdc13-1* mutants. Thus, together Rad53p/Dun1p and Chk1p/Pds1p pathways are responsible for 100% arrest of *cdc13-1* cells (Gardner et al. 1999; Sanchez et al. 1999). In *yku70*Δ mutants, unprotected telomeres are also perceived as DSBs with a short 3′ overhang. But in cells lacking Yku70p, Exo1p can be recruited independently of Rad24p and the Rad17p, Mec3p, Ddc1p sliding clamp. Exo1p generates ssDNA that activates the Rad9p/Mec1p/Chk1p- and Pds1p-dependent checkpoint pathway. In *yku70*Δ mutants, this checkpoint pathway is responsible for the arrest of 75% of cells, and together with a *MAD2*-dependent pathway, results in arrest of 95% of cells (see Fig. 4n).

Rad24p, the sliding clamp, and protein P might play a minor role in responding to *yku70*Δ-induced DNA damage, but the "1st processed DNA damage" does not form to a sufficient extent to activate Rad53p and Dun1p. Teo and Jackson (2001) showed that Rad53p kinase is activated at 37°C in *yku80*Δ mutants, but at very low levels. Pellicioli et al. (1999) showed that cells released from hydroxyurea arrest contain residual Rad53 kinase activity, even when the cell cycle has restarted. Therefore, a threshold of Rad53p kinase activity may be necessary to cause cell cycle arrest, and this threshold may not be reached in *yku70*Δ mutants.

This model raises many questions, most importantly: Why do *DDC1*, *MEC3*, *RAD17*, *RAD24*, and *DUN1* play insignificant roles in the arrest of *yku70*Δ mutants? We suggest the following, not mutually exclusive, hypotheses: (1) Rad24p cannot bind to telomeres in *yku70*Δ mutants; (2) The "1st processed DNA damage" is not generated in *yku70*Δ mutants; (3) "ExoXp" is not recruited to telomeres in *yku70*Δ mutants; and (4) Exo1p competes with "ExoXp" for substrates and is preferentially recruited to telomeres in *yku70*Δ mutants.

Our experiments reveal for the first time the role of the spindle checkpoint in arresting *yku70*Δ mutants at 37°C. *MAD2* contributes to the arrest of *yku70*Δ mutants, whereas *BUB2* has no significant effect. Other studies have also shown interactions between mitotic spindle checkpoint pathways and cells that contain DNA damage. For example, all of the thymic lymphomas that developed in *Brca2* knockout mice, defective in a protein thought to be involved in DNA repair (Venkitaraman 2002), had generated mutations in spindle checkpoint pathways Bub1 and Mad3 (Lee et al. 1999). 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). Finally, recent experiments demonstrated that Rad53p is required to modify Bfa1p, the partner of Bub2p in response to *cdc13-1*-induced DNA damage (Hu et al. 2001).

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-

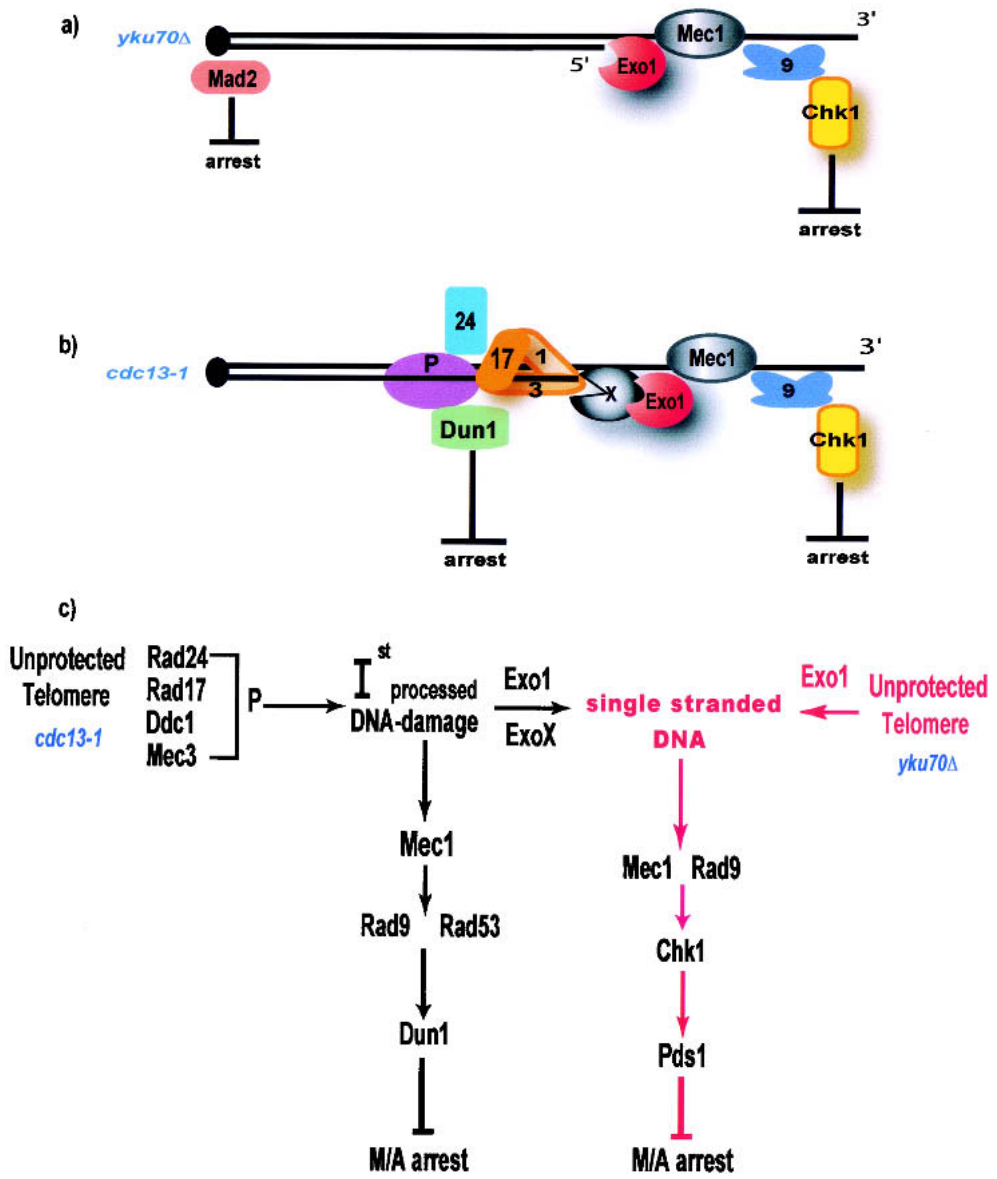

Figure 8. A model for the roles of checkpoint proteins in responding to *cdc13-1* and *yku70*Δ-induced DNA damage. (*a*) A representation of the proteins that are responsible for the arrest of *yku70*Δ mutants. (*b*) A representation of proteins that are responsible for the arrest of *cdc13-1* mutants. (*c*) A schematic model of the DNA damage checkpoint pathways responsible for metaphase/anaphase arrest of *cdc13-1* and *yku70*Δ mutants.

age checkpoint (Fig. 4n). In a culture of *yku70*Δ *chk1*Δ 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 *yku70*Δ *mad2*Δ 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 *yku70*Δ *mad2*Δ 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*

DLY	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	*MATalpha ada2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2 RAD5*	R. Rothstein
883	*MATa ddc1::KanMX4 rad5-535*	M.P. Longhese
974	*MATa yku70::HIS3 RAD5 rDNA::ADE2*	L. Guarente
1028	*MATalpha yku70::LEU2 rad5-535*	S. Jackson
1095	*MATa chk1::HIS3 RAD5*	640 transformation
1199	*MATa yku70::HIS3 rad17::LEU2 rad5-535*	974 × 607
1208	*MATalpha yku70::LEU2 mec3::TRP1 rad5-535*	886 × 1028
1209	*MATa yku70::LEU2 mec3::TRP1 rad5-535*	886 × 1028
1210	*MATa ddc1::KanMX4 yku70::LEU2 rad5-535*	886 × 1028
1211	*MAT alpha ddc1::KanMX4 yku70::LEU2 rad5-535*	886 × 1028
1214	*MATalpha yku70::LEU2 rad9::HIS3 rad24::TRP1 rad5-535*	1028 × 261
1215	*MATa yku70::LEU2 chk1::HIS3 RAD5*	1028 × 1096
1230	*Mata cdc13-1int RAD5*	1108 transformation
1248	*MATa rad5-535 sml1:KANMX4*	M.P. Longhese
1249	*MATa rad5-535 sml1del::KANMX4 mec1::HIS3*	M.P. Longhese
1255	*MATa cdc13-1int rad9::HIS3 RAD5*	662 × 1218
1257	*MATa cdc13-1int rad24::TRP1 RAD5*	662 × 1218
1264	*MATalpha yku70::LEU2 rad9::HIS3 RAD5*	1218 × 1214
1266	*MAT a yku70::LEU2 chk1::HIS3 RAD5*	1028 × 1096
1271	*MATa yku70::LEU2 rad9::HIS3 RAD5*	1264 × 1285
1284	*MATa rad24::TRP1 yku70::HIS3 RAD5*	974 × 1258
1296	*MATa exo1::LEU2 cdc13-1int RAD5*	1272 × 1230
1312	*MATalpha yku70::LEU2 mec1::HIS3 rad5-535 sml1::KANMX4*	1028 × 1249
1325	*MATa yku70::LEU2 mec1::HIS3 rad5-535 sml1::KANMX4*	1028 × 1249
1327	*MATa yku70::LEU2 mec1::HIS3 rad24::TRP1 sml1del::KANMX4*	1312 × 1285
1337	*MATA yku70::LEU2 rad9::HIS3 rad24::TRP1 rad5-535*	1028 × 261
1347	*MATalpha yku70::HIS3 rad17::LEU2 RAD5*	1308 × 1284
1364	*MATa rad24::TRP1yku70::HIS3 RAD5*	1308 × 1284
1366	*MATalpha yku70::HIS3 RAD5*	1308 × 1284
1408	*MATa yku70::HIS3 exo1::LEU2 RAD5*	1273 × 1364
1409	*MATalpha yku70::HIS3 exo1::LEU2 RAD5*	1273 × 1364
1410	*MATalpha yku70::HIS3 exo1::LEU2 rad24::TRP1 RAD5*	1273 × 1364
1411	*MATa yku70::HIS3 exo1::LEU2 rad24::TRP1 RAD5*	1273 × 1364
1412	*MATa yku70::HIS3 RAD5*	1273 × 1364
1430	*MATa yku70::HIS3 rad24::TRP1 RAD5*	1399 × 1364
1439	*MATalpha bub2::URA3 yku70::LEU2 rad5-535*	1429 × 1371
1440	*MATalpha bub2::URA3 yku70::LEU2 RAD5*	1429 × 1371
1441	*MATalpha bub2::URA3 yku70::LEU2 rad24::TRP1 RAD5*	1429 × 1371
1442	*MATa bub2::URA3 yku70::LEU2 chk1::HIS3 rad5-535*	1429 × 1371
1443	*MATa bub2::URA3 yku70::LEU2 chk1::HIS3 rad24::TRP1 RAD5*	1429 × 1371
1445	*MATa mad2::URA3 yku70::LEU2 rad5-535*	1429 × 1372
1446	*MATa mad2::URA3 yku70::LEU2 chk1::HIS3 RAD5*	1429 × 1372
1448	*MATa mad2::URA3 yku70::LEU2 rad24::TRP1 RAD5*	1429 × 1372
1449	*MATalpha mad2::URA3 yku70::LEU2 rad24::TRP1RAD5*	1429 × 1372
1451	*MATalpha bub2::URA3 RAD5*	1429 × 1371
1496	*MATalpha cdc13-1int bub2::URA3 RAD5*	1451 × 1255
1497	*MATalpha cdc13-1int bub2::URA3 RAD5*	1451 × 1255
1498	*MATa cdc13-1int mad2::URA3 rad9::HIS3 RAD5*	1452 × 1255
1499	*MATalpha cdc13-1int mad2::URA3 rad9::HIS3 RAD5*	1452 × 1255
1500	*MATalpha cdc13-1int mad2::URA3 RAD5*	1452 × 1255
1501	*MATa cdc13-1int mad2::URA3 RAD5*	1452 × 1255
1502	*MATa cdc13-1int bub2::URA3 RAD5 rad9::HIS3*	1451 × 1255
1503	*MATalpha cdc13-1int bub2::URA3 RAD5 rad9::HIS3*	1451 × 1255
1505	*MATalpha yku70::LEU2 rad24::TRP1 RAD5*	1429 × 1371
1552	*MATa mad2::URA3 yku70::LEU2*	1400 × 1449
1553	*MATa dun1::HIS3 yku70::LEU2*	1400 × 1449
1554	*MATalpha dun1::HIS3 yku70::LEU2*	1400 × 1449
1676	*MATalpha mre11::hisG::URA3 exo1::LEU2 RAD5*	1330 × 1409
1678	*MATalpha yku70::HIS3 mre11::hisG::URA3 RAD5*	1330 × 1409
1679	*MATalpha yku70::HIS3 mre11::hisG::URA3 RAD5*	1330 × 1409
1680	*MATa yku70::HIS3 mre11::his G::URA3 exo1::LEU2 RAD5*	1330 × 1409
1746	*MATalpha 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 (Wellinger 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 *yku70*Δ mutants, and so it is conceivable that *EXO1* also plays a role in physiological metabolism of telomeres. *MRE11*, in contrast to *EXO1*, stablizes telomeres of *yku70*Δ mutants. If Exo1p does play a role in the physiology of telomeres, then other exonucleases must function redundantly with Exo1p because the telomeres of *exo1*Δ 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 *rad5-535* strains (Fan et al. 1996), but we observed no effect of the *rad5-535* 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 *yku70*Δ 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 pYS51 (Sanchez et al. 1999). The *mec1*Δ and *sml1*Δ deletion strains were obtained from M.P. Longhese (Paciotti et al. 2000). An *exo1::LEU2* disruption was constructed using pHT246 and an mre::hisg::URA3 deletion with pHT16 (Tsubouchi and Ogawa 2000). *mad2*Δ and *bub2*Δ deletion strains were obtained from L Dirick. *Dun1*Δ strains were obtained from T. Weinert. Other deletions have been described elsewhere (Lydall and Weinert 1997). *cdc13-1*int 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 *yku70*Δ*mre11*Δ (*exo1*Δ) mutants enter crisis after several generations, and therefore we assume the strains analysed in Figure 7 have escaped or avoided crisis.

Serial dilution and growth on plates

Colony-purified yeast strains were inoculated into 1mL 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 (1×10^8 cells in 50 mL, or 0.4×10^8 cells in 20 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 multicounter, 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 *yku70*Δ 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 *PDA*1 "loading control." *PDA1* is 30 kbp from the telomere and does not become single-stranded in *yku70*Δ 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.

Acknowledgments

We thank L. Dirick, S. Elledge, H. Ogawa, S. Jackson, L. Guarente, M.P.Longhese, V. Lundblad, S. Piatti, R. Rothstein, Y. Sanchez, L. Symington, H. Tsubouchi, and T. Weinert for plasmids, protocols, or yeast strains. We thank all members of our

lab, Keith Caldecott, and Elspeth Stewart for comments on the manuscript. L.M. was partially supported by the Vorarlberger Landesregierung. D.L. is a Wellcome Senior Research Fellow in Basic Biomedical Science.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Adams, A., Gottshcling, D.E., Kaiser, C.A., and Stearns, T. 1997. *Methods in yeast genetics*. pp. 131–132. Cold Spring Harbor Laboratory Press, New York.

Bailey, S.M., Meyne, J., Chen, D.J., Kurimasa, A., Li, G.C., Lehnert, B.E., and Goodwin, E.H. 1999. DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. *Proc. Natl. Acad. Sci.* **96:** 14899–14904.

Barnes, G. and Rio, D. 1997. DNA double-strand-break sensitivity, DNA replication, and cell cycle arrest phenotypes of Ku-deficient Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci.* **94:** 867–872.

Bellacosa, A. 2001. Functional interactions and signaling properties of mammalian DNA mismatch repair proteins. *Cell Death Differ.* **8:** 1076–1092.

Bishop, D.K., Park, D., Xu, L., and Kleckner, N. 1992. DMC1: A meiosis-specific yeast homolog of E. coli recA required for recombination, synaptonemal complex formation, and cell cycle progression. *Cell* **69:** 439–456.

Booth, C., Griffith, E., Brady, G., and Lydall, D. 2001. Quantitative amplification of single-stranded DNA (QAOS) demonstrates that cdc13-1 mutants generate ssDNA in a telomere to centromere direction. *Nucleic Acids Res.* **29:** 4414–4422.

Boulton, S.J. and Jackson, S.P. 1996. Identification of a Saccharomyces cerevisiae Ku80 homologue: Roles in DNA double strand break rejoining and in telomeric maintenance. *Nucleic Acids Res.* **24:** 4639–4648.

———. 1998. Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing. *EMBO J.* **17:** 1819–1828.

Caspari, T. and Carr, A.M. 2002. Checkpoints: How to flag up double-strand breaks. *Curr. Biol.* **12:** R105–R107.

d'Adda di Fagagna, F., Hande, M.P., Tong, W., Roth, D., Lansdorp, P.M., Wang, Z., and Jackson, S.P. 2001. Effects of DNA nonhomologous end-joining factors on telomere length and chromosomal stability in mammalian cells. *Curr. Biol.* **11:** 1192–1196.

Diede, S.J. and Gottschling, D.E. 2001. Exonuclease activity is required for sequence addition and Cdc13p loading at a de novo telomere. *Curr. Biol.* **11:** 1336–1340.

Fan, H.Y., Cheng, K.K., and Klein, H.L. 1996. Mutations in the RNA polymerase II transcription machinery suppress the hyperrecombination mutant hpr1 delta of Saccharomyces cerevisiae. *Genetics* **142:** 749–759.

Feldmann, H. and Winnacker, E.L. 1993. A putative homologue of the human autoantigen Ku from Saccharomyces cerevisiae. *J. Biol. Chem.* **268:** 12895–12900.

Fellerhoff, B., Eckardt-Schupp, F., and Friedl, A.A. 2000. Subtelomeric repeat amplification is associated with growth at elevated temperature in yku70 mutants of Saccharomyces cerevisiae. *Genetics* **154:** 1039–1051.

Gardner, R.D. and Burke, D.J. 2000. The spindle checkpoint: Two transitions, two pathways. *Trends Cell Biol.* **10:** 154–158.

Gardner, R., Putnam, C.W., and Weinert, T. 1999. RAD53, DUN1 and PDS1 define two parallel G(2)/M checkpoint pathways in budding yeast. *EMBO J.* **18:** 3173–3185.

Garner, M., van Kreeveld, S., and Su, T.T. 2001. mei-41 and bub1 block mitosis at two distinct steps in response to incomplete DNA replication in Drosophila embryos. *Curr. Biol.* **11:** 1595–1599.

Garvik, B., Carson, M., and Hartwell, L. 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. *Mol. Cell. Biol.* **15:** 6128–6138.

Gilbert, C.S., Green, C.M., and Lowndes, N.F. 2001. Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. *Mol. Cell* **8:** 129–136.

Gravel, S., Larrivee, M., Labrecque, P., and Wellinger, R.J. 1998. Yeast Ku as a regulator of chromosomal DNA end structure. *Science* **280:** 741–744.

Green, C.M., Erdjument-Bromage, H., Tempst, P., and Lowndes, N.F. 2000. A novel Rad24 checkpoint protein complex closely related to replication factor C. *Curr. Biol.* **10:** 39–42.

Hartwell, L.H. 1974. Saccharomyces cerevisiae cell cycle. *Bacteriol. Rev.* **38:** 164–198.

Hsu, H.L., Gilley, D., Galande, S.A., Hande, M.P., Allen, B., Kim, S.H., Li, G.C., Campisi, J., Kohwi-Shigematsu, T., and Chen, D.J. 2000. Ku acts in a unique way at the mammalian telomere to prevent end joining. *Genes & Dev.* **14:** 2807–2812.

Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J., and Elledge, S.J. 2001. Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. *Cell* **107:** 655–665.

Hwang, L.H., Lau, L.F., Smith, D.L., Mistrot, C.A., Hardwick, K.G., Hwang, E.S., Amon, A., and Murray, A.W. 1998. Budding yeast Cdc20: A target of the spindle checkpoint. *Science* **279:** 1041–1044.

Laroche, T., Martin, S.G., Gotta, M., Gorham, H.C., Pryde, F.E., Louis, E.J., and Gasser, S.M. 1998. Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres. *Curr. Biol.* **8:** 653–656.

Lee, H., Trainer, A.H., Friedman, L.S., Thistlethwaite, F.C., Evans, M.J., Ponder, B.A., and Venkitaraman, A.R. 1999. Mitotic checkpoint inactivation fosters transformation in cells lacking the breast cancer susceptibility gene, Brca2. *Mol. Cell* **4:** 1–10.

Lee, S.E., Moore, J.K., Holmes, A., Umezu, K., Kolodner, R.D., and Haber, J.E. 1998. Saccharomyces Ku70, Mre11/Rad50, and RPA proteins regulate adaptation to G2/M arrest after DNA damage. *Cell* **94:** 399–409.

Lewis, L.K., Karthikeyan, G., Westmoreland, J.W., and Resnick, M.A. 2002. Differential suppression of DNA repair Deficiencies of yeast rad50, mre11 and xrs2 mutants by EXO1 and TLC1 (the RNA component of telomerase). *Genetics* **160:** 49–62.

Lowndes, N.F. and Murguia, J.R. 2000. Sensing and responding to DNA damage. *Curr. Opin. Genet. Dev.* **10:** 17–25.

Lydall, D. and Weinert, T. 1995. Yeast checkpoint genes in DNA damage processing: Implications for repair and arrest. *Science* **270:** 1488–1491.

———. 1997. G2/M checkpoint genes of Saccharomyces cerevisiae: Further evidence for roles in DNA replication and/or repair. *Mol. Gen. Genet.* **256:** 638–651.

Lydall, D., Nikolsky, Y., Bishop, D.K., and Weinert, T. 1996. A meiotic recombination checkpoint controlled by mitotic checkpoint genes. *Nature* **383:** 840–843.

Martin, S.G., Laroche, T., Suka, N., Grunstein, M., and Gasser, S.M. 1999. Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. *Cell* **97:** 621–633.

Mishra, K. and Shore, D. 1999. Yeast Ku protein plays a direct role in telomeric silencing and counteracts inhibition by rif proteins. *Curr. Biol.* **9:** 1123–1126.

Moreau, S., Morgan, E.A., and Symington, L.S. 2001. Overlapping functions of the Saccharomyces cerevisiae Mre11, Exo1 and Rad27 nucleases in DNA metabolism. *Genetics* **159:** 1423–1433.

Neff, M.W. and Burke, D.J. 1992. A delay in the Saccharomyces cerevisiae cell cycle that is induced by a dicentric chromosome and dependent upon mitotic checkpoints. *Mol. Cell Biol.* **12:** 3857–3864.

Nugent, C.I., Bosco, G., Ross, L.O., Evans, S.K., Salinger, A.P., Moore, J.K., Haber, J.E., and Lundblad, V. 1998. Telomere maintenance is dependent on activities required for end repair of double-strand breaks. *Curr. Biol.* **8:** 657–660.

Paciotti, V., Clerici, M., Lucchini, G., and Longhese, M.P. 2000. The checkpoint protein Ddc2, functionally related to S. pombe Rad26, interacts with Mec1 and is regulated by Mec1-dependent phosphorylation in budding yeast. *Genes & Dev.* **14:** 2046–2059.

Pellicioli, A., Lucca, C., Liberi, G., Marini, F., Lopes, M., Plevani, P., Romano, A., DiFiore, P.P., and Foiani, M. 1999. Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. *EMBO J.* **18:** 6561–6572.

Peterson, S.E., Stellwagen, A.E., Diede, S.J., Singer, M.S., Haimberger, Z.W., Johnson, C.O., Tzoneva, M., and Gottschling, D.E. 2001. The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku. *Nat. Genet.* **27:** 64–67.

Polotnianka, R.M., Li, J., and Lustig, A.J. 1998. The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities. *Curr. Biol.* **8:** 831–834.

Porter, S.E., Greenwell, P.W., Ritchie, K.B., and Petes, T.D. 1996. The DNA-binding protein Hdf1p (a putative Ku homologue) is required for maintaining normal telomere length in Saccharomyces cerevisiae. *Nucleic Acids Res.* **24:** 582–585.

Pryde, F.E. and Louis, E.J. 1999. Limitations of silencing at native yeast telomeres. *EMBO J.* **18:** 2538–2550.

Rizki, A. and Lundblad, V. 2001. Defects in mismatch repair promote telomerase-independent proliferation. *Nature* **411:** 713–716.

Roeder, G.S. and Bailis, J.M. 2000. The pachytene checkpoint. *Trends Genet.* **16:** 395–403.

Samper, E., Goytisolo, F.A., Slijepcevic, P., van Buul, P.P., and Blasco, M.A. 2000. Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. *EMBO Rep.* **1:** 244–252.

Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S.J. 1999. Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. *Science* **286:** 1166–1171.

Sandell, L.L. and Zakian, V.A. 1993. Loss of a yeast telomere: Arrest, recovery, and chromosome loss. *Cell* **75:** 729–739.

Smith, G.C. and Jackson, S.P. 1999. The DNA-dependent protein kinase. *Genes & Dev.* **13:** 916–934.

Teo, S.H. and Jackson, S.P. 2001. Telomerase subunit overexpression suppresses telomere-specific checkpoint activation in the yeast yku80 mutant. *EMBO Rep* **2:** 197–202.

Tishkoff, D.X., Boerger, A.L., Bertrand, P., Filosi, N., Gaida, G.M., Kane, M.F., and Kolodner, R.D. 1997. Identification and characterization of Saccharomyces cerevisiae EXO1, a gene encoding an exonuclease that interacts with MSH2. *Proc. Natl. Acad. Sci.* **94:** 7487–7492.

Tsubouchi, H. and Ogawa, H. 2000. Exo1 roles for repair of DNA double-strand breaks and meiotic crossing over in Saccharomyces cerevisiae. *Mol. Biol. Cell* **11:** 2221–2233.

Tsukamoto, Y., Taggart, A.K., and Zakian, V.A. 2001. The role of the Mre11-Rad50-Xrs2 complex in telomerase-mediated lengthening of Saccharomyces cerevisiae telomeres. *Curr. Biol.* **11:** 1328–1335.

Venclovas, C. and Thelen, M.P. 2000. Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes. *Nucleic Acids Res.* **28:** 2481–2493.

Venkitaraman, A.R. 2002. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108:** 171–182.

Wang, Y., Hu, F., and Elledge, S.J. 2000. The Bfa1/Bub2 GAP complex comprises a universal checkpoint required to prevent mitotic exit. *Curr. Biol.* **10:** 1379–1382.

Weinert, T.A. and Hartwell, L.H. 1993. Cell cycle arrest of cdc mutants and specificity of the RAD9 checkpoint. *Genetics* **134:** 63–80.

Weinert, T.A., Kiser, G.L., and Hartwell, L.H. 1994. Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes & Dev.* **8:** 652–665.

Wellinger, R.J., Ethier, K., Labrecque, P., and Zakian, V.A. 1996. Evidence for a new step in telomere maintenance. *Cell* **85:** 423–433.

Yan, T., Schupp, J.E., Hwang, H.S., Wagner, M.W., Berry, S.E., Strickfaden, S., Veigl, M.L., Sedwick, W.D., Boothman, D.A., and Kinsella, T.J. 2001. Loss of DNA mismatch repair imparts defective cdc2 signaling and G(2) arrest responses without altering survival after ionizing radiation. *Cancer Res.* **61:** 8290–8297.