Checkpoint Proteins Influence Telomeric Silencing and Length Maintenance in Budding Yeast

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ABSTRACT

A complex network of surveillance mechanisms, called checkpoints, interrupts cell cycle progression when damage to the genome is detected or when cells fail to complete DNA replication, thus ensuring genetic integrity. In budding yeast, components of the DNA damage checkpoint regulatory network include the *RAD9*, *RAD17*, *RAD24*, *MEC3*, *DDC1*, *RAD53*, and *MEC1* genes that are proposed to be involved in different aspects of DNA metabolism. We provide evidence that some DNA damage checkpoint components play a role in maintaining telomere integrity. In fact, *rad53* mutants specifically enhance repression of telomere-proximal transcription via the Sir-mediated pathway, suggesting that Rad53 might be required for proper chromatin structure at telomeres. Moreover, Rad53, Mec1, Ddc1, and Rad17 are necessary for telomere length maintenance, since mutations in all of these genes cause a decrease in telomere size. The telomeric shortening in *rad53* and *mec1* mutants is further enhanced in the absence of *SIR* genes, suggesting that Rad53/Mec1 and Sir proteins contribute to chromosome end protection by different pathways. The finding that telomere shortening, but not increased telomeric repression of gene expression in *rad53* mutants, can be suppressed by increasing dNTP synthetic capacity in these strains suggests that transcriptional silencing and telomere integrity involve separable functions of Rad53.

IN eukaryotic cells, the consequences of DNA damage are minimized by the simultaneous activation of DNA repair mechanisms and signal transduction pathways, called checkpoints, which lead to a transient delay of cell cycle progression when alterations in DNA structure are detected. Checkpoint pathways ensure the proper order and timing of cell cycle events and contribute to the maintenance of genetic integrity as they increase the repair capacity of a damaged cell and prevent replication and segregation of damaged chromosomes (reviewed in Hartwell and Weinert 1989; Paulovich *et al.* 1997). In *Saccharomyces cerevisiae*, a number of genes have been identified that control the ability of cells to arrest the cell cycle and/or to activate the transcriptional response of DNA repair genes. Upstream components acting at the early steps in the DNA damage checkpoint regulatory network include the *RAD9*, *RAD17*, *RAD24*, *MEC3*, and *DDC1* genes (reviewed in Longhese *et al.* 1998; Weinert 1998). Conversely, the DNA replication proteins Polε, Dpb11, Rfc2, and Rfc5 appear to sense DNA alterations specifically during DNA synthesis, thus linking entry into mitosis to a proper completion of S phase (Araki *et al.* 1995; Navas *et al.* 1995; Sugimoto *et al.* 1996, 1997; Noskov *et al.* 1998). Once DNA alterations are sensed the signaling process involves a protein phosphorylation cascade propagated through the two protein kinases Mec1 and Rad53 (reviewed in Longhese *et al.* 1998; Weinert 1998), indicating that these two gene products play a central role in the checkpoint signal transduction cascade. Rad53 is an essential Ser/Thr/Tyr protein kinase (Zheng *et al.* 1993), whereas Mec1 belongs to the PI-3 kinase motif family, including Tel1 (Greenwell *et al.* 1995; Morrow *et al.* 1995) and *Schizosaccharomyces pombe* Rad3 (Bentley *et al.* 1996), as well as human DNA-PK (DNA-dependent protein kinase) (Jeggo *et al.* 1995) and the ATM (ataxia-telangiectasia mutated gene) gene product (Savitsky *et al.* 1995). In response to DNA insults, Mec1 is required to phosphorylate and activate several substrates, including Rad53 (Zheng *et al.* 1993; Sanchez *et al.* 1996), Ddc1 (Paciotti *et al.* 1998), and Rad9 (Emili 1998; Sun *et al.* 1998; Vialard *et al.* 1998), suggesting a pivotal role for Mec1 in the checkpoint signal transduction pathway. Moreover, Mec1 and Rad53 induce transcription of the genes encoding ribonucleotide reductase (RNR), which catalyzes the rate-limiting step in dNTP synthesis that is necessary for both replication and repair (Zhou and Elledge 1993; Allen *et al.* 1994).

Since DNA is packaged into chromatin, chromatin is the context in which any alteration in DNA structure is recognized by the checkpoint pathways. In *S. cerevisiae*, transcriptionally silent chromatin is found at telomeres and at the *HML* and *HMR* loci, encoding the cryptic mating-type cassettes (Aparicio *et al.* 1991). Transcription of genes located at telomeres and at *HML* or *HMR* loci is subject to reversible but mitotically inheritable

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repression (Gottschling *et al.* 1990; reviewed in Loo and Rine 1995). A number of proteins are required for telomeric repression. These include repressor activator protein 1 (Rap1; Kyrion *et al.* 1993), the regulators Sir2-4 (Aparicio *et al.* 1991), and the N termini of histones H3 and H4 (Kayne *et al.* 1988; Thompson *et al.* 1994), suggesting that these factors may contribute to establish a higher-order repressed state of chromatin. Associated with telomeres are also the yeast homologs of the DNA end-binding Ku proteins, yKu70 and yKu80. yKu70 was shown to bind to Sir4 by two-hybrid assay (Tsukamoto *et al.* 1997) and, similar to *sir* mutants, deletion of *YKU70* or *YKU80* causes the loss of telomere-adjacent gene silencing (Boulton and Jackson 1998; Laroche *et al.* 1998; Nugent *et al.* 1998). While the absence of Sir proteins does not cause loss of Ku proteins from telomeres, the absence of Ku proteins results in loss of telomere-associated Sir proteins, indicating a prominent role of Ku proteins in heterochromatin assembly at telomeres. The investigation of the relationships between telomeres, chromatin, and checkpoints is crucial for the understanding of how chromatin affects DNA repair and checkpoint controls and how telomeres avoid being recognized as broken DNA ends. In addition to exerting profound influences on the transcription of adjacent genes, yeast telomeres are required for the maintenance of chromosome integrity. In fact, one of the essential functions of telomeres is to protect chromosomal termini from degradation and fusion that can lead to DNA changes and genomic instability. These protective structures also provide a mechanism to replicate chromosomal ends (reviewed in Zakian 1995). Telomere length is kept within a narrow size range in a cell population and can be viewed as the result of a balance between elongation and shortening. Telomerase, a ribonucleoprotein complex responsible for the addition of DNA sequences to telomeres of eukaryotic chromosomes, is specifically required to maintain telomere length, preventing progressive shortening of chromosomal ends (reviewed in Nugent and Lundblad 1998).

Recent lines of evidence in budding and fission yeast suggest that proteins involved in DNA damage checkpoints have a role in telomere length maintenance. First, in fission yeast, DNA replication checkpoint mutants display altered telomeres (Dahlen *et al.* 1998; Matsuura *et al.* 1999), and mutations in *rad3*$^+$, encoding the homologue of *S. cerevisiae* Mec1, and *tel1*$^+$ cause dramatically shortened telomeres (Naito *et al.* 1998), suggesting that telomere synthesis is at least partially dependent on both kinases. This appears to be a conserved mechanism, since in budding yeast, strains carrying mutations in both *MEC1* and *TEL1* undergo continual loss of telomeric repeats that is associated with a senescence phenotype (Ritchie *et al.* 1999). Moreover, in budding yeast, Mec3 shows genetic and physical interaction with Set1, a protein required for transcriptional telomeric silencing and telomere integrity (Corda *et al.* 1999). Mec3 and Set1 have antagonistic effects on both telomere length maintenance and repression of telomere-proximal transcription. Finally, Ku, which is involved in double-strand break repair and telomere maintenance, and Sir proteins residing at telomeres relocalize in response to DNA damage and this process is under the control of the DNA damage checkpoint pathways (Martin *et al.* 1999; Mills *et al.* 1999). To further investigate the role of the checkpoint proteins in modulating chromatin and telomere structure, we studied the effects of mutations in the *RAD53* and *MEC1* genes and in other checkpoint components on telomere-directed transcriptional silencing and telomere length maintenance.

MATERIALS AND METHODS

Strains and media: All the oligonucleotides mentioned below and used for PCR amplification are reported in Table 1. The strains used in this study are listed in Table 2. Strains UCC3537, UCC1001, UCC3511, and UCC3515 were kindly provided by D. Gottschling (Fred Hutchinson Cancer Research Center, Seattle), and strains Y300 and Y300tel1 were kindly provided by S. Elledge (Howard Hughes Medical Institute, Houston, TX). Strains DMP2696/3D, DMP2696/4A, DMP2698/1D, and DMP2694/2C were derivatives of W303 (*MAT***a** or *MAT*α *ade2-1 trp1-1 leu2-3, 112 his3-11, 15, ura3*; Paciotti *et al.* 1998). One-step replacement of *RAD53* with the *rad53K227A* allele was carried out by transforming strains UCC3537, UCC1001, W303, Y300, Y300tel1, UCC3511, and UCC3515 with the *Eco*RI-digested pCH8 plasmid (Pellicioli *et al.* 1999), kindly provided by M. Foiani (University of Milan, Italy), to generate strains YLL410, YLL392, DMP2760/1A/DMP2760/3B, YLL678, YLL677, YLL416, and YLL422, respectively. Strains YLL430, DMP2840/3D, DMP2831/1B, and YLL439, in which the *URA3* gene has been integrated into the telomeric region of chromosome VII, were generated by transforming strains W303, DMP2760/1A, DMP2696/3D, and DMP2698/1D, respectively, with *Sal*I-*Eco*RI-digested pVII-L *URA3-TEL* plasmid, kindly provided by D. Gottschling. Strain DMP3141/2B is a meiotic segregant from a cross between strains YLL488 and DMP2831/1B. Strains DMP2952/2B and DMP2952/2C were meiotic segregants from a cross between strains YLL430 and DMP2854/2B. Strains DMP2955/4A and DMP2955/4C were derived from a cross between strains YLL430 and DMP2855/7C. To construct the *SIR3* chromosomal deletion, *sir3*Δ*::HIS3* and *sir3*Δ*::KanMX4* cassettes were constructed by PCR using, respectively, pFA6a-*HIS3* and pFA6a-*kanMX4* plasmids (Wach *et al.* 1994) as templates and oligonucleotides PRP109 and PRP110 as primers. One-step replacement of 2866 bp of the *SIR3* coding region with the *Kluyveromyces lactis HIS3* cassette was carried out by transforming strains W303, YLL410, YLL392, DMP2760/1A, and DMP2696/3D with the *sir3*Δ*::HIS3* PCR product to give rise to strains YLL521, YLL465, YLL462, YLL522, and YLL525, respectively. One-step replacement of 2866 bp of the *SIR3* coding region with the *KanMX4* cassette was carried out by transforming strains UCC1001 and UCC3537 with the *sir3*Δ*::KanMX4* PCR product to give rise to strains YLL387 and YLL405, respectively. To construct the *SIR4* chromosomal deletion, a *sir4*Δ*::HIS3* cassette was constructed by PCR using plasmid pFA6a-*HIS3* as a template and oligonucleotides PRP105 and PRP106 as primers. One-step replacement of 3808 bp of the *SIR4* coding region with the *K. lactis HIS3* cassette

TABLE 1

Oligonucleotides used for PCR amplification

PRP21	5′-GCT TAG ACA TAT ATG TCA TTT AAG GCA ACT ATC ACC GAG TCG GGG CGT ACG CTG CAG GTC GAC-3′
PRP22	5′-TAT ACC CCT TGG CTT TTC TAC TTG TGT TAG ACC CAG CCC ATC TTC ATC GAT GAA TTC GAG CTC G-3′
PRP64	5′-TTC AGC TTA CTG CAC ATT TTG AGA ACA CCA TGA ATC TTT ACG AAC AGG TCC GCT GCA CGG TCC TGT TCC TGT-3′
PRP65	5′-TGG CCA GCA ACA CTC AAT ACC AGG CCA TCC TGC GGA TCT ATA CCG TCC TCG GGG ACA CCA AAT ATG-3′
PRP75	5′-GTC GAA GAA TTC CTT TGG CCA TAG TGG GG-3′
PRP105	5′-CCC ACA ATA CCA AAA AAG CGA AGA AAA CAG CCA ATG CCA AAT GAC CGT ACG CTG CAG GTC GAC-3′
PRP106	5′-TTT TCA TCC AGC GCC GAT GCT GCT TTC GAC AAA ACG ATA TCC AAT CGA TGA ATT CGA GCT CG-3′
PRP109	5′-TGT TGG TGG TCA AAT GCA GTC CAT ATT TTT GAA TTC TTC ATC CAT CGC GTA CGC TGC AGG TCG AC-3′
PRP110	5′-GGA TTA GCT AAA ATG GCT AAA ACA TTG AAA GAT TTG GAC GGT TGG CAT CGA TGA ATT CGA GCT CG-3′
PRP119	5′-AAG GAT TTC AAC TAT GCG AAT CAA CAG TGA GCT AGC GAA CAA GCG TAC GCT GCA GGT CGA C-3′
PRP120	5′-TCC TTT GTT GGA TAC TTG CAG TGA TTC TCT TCA TCC TCA CTT ATC GAT GAA TTC GAG CTC G-3′
PRP127	5′-CAA GTT TGT TCC TGT CTG AAT GAT ATG GAT AGT ACG AAT TTG AAC GTA CGC TGC AGG TCG AC-3′
PRP128	5′-TTA GAG TAT TTC CAG ATC TGA ATC TGA AAG GGA CTC ACT GAT AAT CGA TGA ATT CGA GCT CG-3′
PRP131	5′-AAT CTT CAA CAT CAG GGC TAT GTC AGG CCA GTT AGT TCA ATG GAA CGT ACG CTG CAG GTC GAC-3′
PRP132	5′-GTA TAT ATC ATT GTC CGT AAT ATC ATC GTG AAA ACC AGT GTC CTC GAT CGA TGA ATT CGA GCT CG-3′
PRP134	5′-CGC GGA TCC ATA TGG AAT CAC ACG TCA AAT ATC TTG AC-3′
PRP148	5′-CCT CTC TTC AAC TGC TCA ATA ATT TCC CGC TAT GCA AAA TTC CCC GTA CGC TGC AGG TCG AC-3′
PRP149	5′-GAA AGG AAC TTT AGA AGT CCA TTT CCT CGA CCT TAC CCT GGT TGA TCG ATG AAT TCG AGC TCG-3′
PRP158	5′-CAC ACA GCA ATC CAC GCA GGC TAC TCA AAG GTT TTT GAT TGA GAA GCG TAC GCT GCA GGT CGA C-3′
PRP159	5′-TGC AAA TTC TCG GGG CCT TTT GAG GTT TGG TCC AAT TTT GCC CTT ATC GAT GAA TTC GAG CTC G-3′

TABLE 2

Strains used in this study

Strain	Genotype	Reference/source
W303	*MAT***a** or *MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3*	Longhese *et al.* (1997)
DMP2760/1A	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4*	This study
DMP2760/3B	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4*	This study
DMP2696/3D	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1-14*	This study
DMP2696/4A	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1-14*	This study
DMP2694/2C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad17*Δ*::LEU2*	This study
DMP2698/1D	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sad1-1*	This study
YLL244	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc1*Δ*::KanMX4*	Longhese *et al.* (1997)
YLL430	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L*	This study
DMP2840/3D	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L rad53K227A::KanMX4*	This study
YLL439	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L sad1-1*	This study
DMP2955/4A	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L rad53*Δ*::HIS3 sml1*Δ*::KanMX4*	This study
DMP2955/4C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L sml1*Δ*::KanMX4*	This study
DMP2831/1B	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L mec1-14*	This study
DMP3141/2B	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L mec1-14 sml1*Δ*::KanMX4*	This study
DMP2952/2B	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L mec1*Δ*::HIS3 sml1*Δ*::KanMX4*	This study
DMP2952/2C	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 URA3-TEL-VII-L sml1*Δ*::KanMX4*	This study
UCC1001	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L*	Gottschling (1992)
YLL606	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L sml1*Δ*::HIS3*	This study
YLL392	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L rad53K227A::KanMX4*	This study
YLL607	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L rad53K227A::KanMX4 sml1*Δ*::HIS3*	This study
YLL387	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L sir3*Δ*::KanMX4*	This study
YLL462	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L rad53K227A::KanMX4 sir3*Δ*::HIS3*	This study
YLL388	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L ddc1*Δ*::KanMX4*	This study
YLL389	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L rad17*Δ*::KanMX4*	This study
YLL390	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L rad24*Δ*::KanMX4*	This study
YLL424	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L rad9*Δ*::KanMX4*	This study
UCC3537	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L ADE2-TEL-V-R*	Huang *et al.* (1997)
YLL410	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L ADE2-TEL-V-R rad53K227A::KanMX4*	This study
YLL409	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L ADE2-TEL-V-R mec3*Δ*::TRP1*	This study
YLL405	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L ADE2-TEL-V-R sir3*Δ*::KanMX4*	This study
YLL465	*MAT***a** *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 URA3-TEL-VII-L ADE2-TEL-V-R rad53K227A::KanMX4 sir3*Δ*::HIS3*	This study
YLL521	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sir3*Δ*::HIS3*	This study
YLL522	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4 sir3*Δ*::HIS3*	This study
YLL541	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sir4*Δ*::HIS3*	This study
YLL543	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4 sir4*Δ*::HIS3*	This study
YLL488	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1*Δ*::KanMX4*	This study

(*continued*)

TABLE 2

Continued

Strain	Genotype	Reference/source
YLL590	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4 sml1*Δ*::HIS3*	This study
YLL509	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53*Δ*::HIS3 sml1*Δ*::KanMX4*	This study
YLL525	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1-14 sir3*Δ*::HIS3*	This study
YLL623	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1-14 sml1*Δ*::HIS3*	This study
YLL546	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1-14 sir4*Δ*::HIS3*	This study
YLL490	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1*Δ*::HIS3 sml1*Δ*::KanMX4*	This study
Y300	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	S. Elledge
Y300tel1	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 tel1*Δ*::HIS3*	S. Elledge
YLL678	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad53K227A::KanMX4*	This study
YLL677	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 tel1*Δ*::HIS3 rad53K227A::KanMX4*	This study
DMP2950/3B	*MAT***a** *ade2-1 can1-100 his3-11.15 leu2-3,112 trp1-1 ura3 ddc1*Δ*::KanMX4 sml1*Δ*::KanMX4*	This study
DMP2947/1C	*MAT***a** *ade2-1 can1-100 his3-11.15 leu2-3,112 trp1-1 ura3 rad17*Δ*::LEU2 sml1*Δ*::KanMX4*	This study
DMP2928/9A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4 rad17*Δ*::LEU2*	This study
DMP2932/4D	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4 ddc1*Δ*::KanMX4*	This study
YLL634	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 [YCplac33 URA3]*	This study
YLL632	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 [pML225 CEN4 URA3 GAL1-MEC1]*	This study
YLL640	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1*Δ*::KanMX4 [YCplac33 URA3]*	This study
YLL638	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1*Δ*::KanMX4 [pML225 CEN4 URA3 GAL1-MEC1]*	This study
UCC3511	*MAT*α *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 hmr::URA3*	Singer *et al.* (1998)
YLL416	*MAT*α *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 hmr::URA3 rad53K227A::KanMX4*	This study
UCC3515	*MAT*α *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 hml::URA3*	Singer *et al.* (1998)
YLL422	*MAT*α *ade2-101 ura3-52 lys2-801 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 hml::URA3 rad53K227A::KanMX4*	This study
YLL719	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 [pBAD54 2*μ *TRP1 GAP promoter]*	This study
YLL720	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 [pBAD70 2*μ *TRP1 GAP-RNR1]*	This study
YLL721	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sir3*Δ*::HIS3 [pBAD54 2*μ *TRP1 GAP promoter]*	This study
YLL722	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sir3*Δ*::HIS3 [pBAD70 2*μ *TRP1 GAP-RNR1]*	This study
YLL723	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sir4*Δ*::HIS3 [pBAD54 2*μ *TRP1 GAP promoter]*	This study
YLL724	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sir4*Δ*::HIS3 [pBAD70 2*μ *TRP1 GAP-RNR1]*	This study
YLL725	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp-1-1 ura3 ddc1*Δ*::KanMX4 [pBAD54 2*μ *TRP1 GAP promoter]*	This study
YLL726	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc1*Δ*::KanMX4 [pBAD70 2*μ *TRP1 GAP-RNR1]*	This study
YLL727	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4 [pBAD54 2*μ *TRP1 GAP promoter]*	This study
YLL728	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4 [pBAD70 2*μ *TRP1 GAP-RNR1]*	This study
YLL729	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad17*Δ*::LEU2 [pBAD54 2*μ *TRP1 GAP promoter]*	This study
YLL730	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad17*Δ*::LEU2 [pBAD70 2*μ *TRP1 GAP-RNR1]*	This study
YLL731	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1-14 [pBAD54 2*μ *TRP1 GAP promoter]*	This study
YLL732	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1-14 [pBAD70 2*μ *TRP1 GAP-RNR1]*	This study

was carried out by transforming strains W303, DMP2760/1A, and DMP2696/3D with the *sir4*Δ*::HIS3* PCR product to give rise to strains YLL541, YLL543, and YLL546, respectively. To generate the *SML1* chromosomal deletion, a *sml1*Δ*::HIS3* cassette was constructed by PCR using pFA6a-*HIS3* as a template and oligonucleotides PRP148 and PRP149 as primers. One-step replacement of 269 bp of the *SML1* coding region with the *K. lactis HIS3* cassette was carried out by transforming strains UCC1001, YLL392, DMP2760/1A, and DMP2696/3D with the *sml1*Δ*::HIS3* PCR product to give rise to strains YLL606, YLL607, YLL590, and YLL623, respectively. Strains YLL488 and DMP2947/1C were constructed by transforming, respectively, strains W303 and DMP2694/2C with the *sml1*Δ*::kanMX4* PCR product, obtained by PCR using pFA6a-*kanMX4* as a template, and oligonucleotides PRP148 and PRP149 as primers. Strain DMP2950/3B was a meiotic segregant from a cross between strains YLL244 (Longhese *et al.* 1997) and DMP2818/1B. Strains DMP2928/9A and DMP2932/4D were meiotic segregants from a cross between strains DMP2760/1A and DMP2694/2C or YLL244, respectively. To construct the *RAD53* chromosomal deletion, a *rad53*Δ*::HIS3* cassette was constructed by PCR using plasmid pFA6a-*HIS3* as a template and oligonucleotides PRP158 and PRP159 as primers. One-step replacement of 2346 bp of the *RAD53* coding region with the *K. lactis HIS3* cassette was carried out by transforming strain YLL488 with the *rad53*Δ*::HIS3* PCR product to give rise to strain YLL509. To construct the *MEC1* chromosomal deletion, a *mec1*Δ*::HIS3* cassette was constructed by PCR using plasmid pUC19His3$^+$ as a template and oligonucleotides PRP64 and PRP65 as primers. One-step replacement of 6505 bp of the *MEC1* coding region with the *HIS3* cassette was carried out by transforming strain YLL488 with the *mec1*Δ*::HIS3* PCR product to give rise to strain YLL490. Strain DMP2854/2B was derived from a cross between strains W303 (*MAT*α) and YLL490. Strain DMP2818/1B was a meiotic segregant from a cross between strains YLL488 and W303 (*MAT*α). Strain DMP2855/7C was a meiotic segregant from a cross between strains W303 (*MAT*α) and YLL509. To construct the *DDC1* chromosomal deletion, a *ddc1*Δ*::kanMX4* cassette was constructed by PCR using plasmid pFA6a-*kanMX4* as a template and oligonucleotides PRP21 and PRP22 as primers. One-step replacement of 1752 bp of the *DDC1* coding region with the *KanMX4* cassette was carried out by transforming strain UCC1001 with the *ddc1*Δ*::kanMX4* PCR product to give rise to strain YLL388. To construct the *RAD17* chromosomal deletion, a *rad17*Δ*::kanMX4* cassette was constructed by PCR using plasmid pFA6a-*kanMX4* as a template and oligonucleotides PRP119 and PRP120 as primers. One-step replacement of 1057 bp of the *RAD17* coding region with the *KanMX4* cassette was carried out by transforming strain UCC1001 with the *rad17*Δ*::kanMX4* PCR product to give rise to strain YLL389. To construct the *RAD24* chromosomal deletion, a *rad24*Δ*::kanMX4* cassette was constructed by PCR using plasmid pFA6a-*kanMX4* as a template and oligonucleotides PRP127 and PRP128 as primers. One-step replacement of 1917 bp of the *RAD24* coding region with the *KanMX4* cassette was carried out by transforming strain UCC1001 with the *rad24*Δ*::kanMX4* PCR product to give rise to strain YLL390. To construct the *RAD9* chromosomal deletion, a *rad9*Δ*::kanMX4* cassette was constructed by PCR using plasmid pFA6a-*kanMX4* as a template and oligonucleotides PRP131 and PRP132 as primers. One-step replacement of 3880 bp of the *RAD9* coding region with the *KanMX4* cassette was carried out by transforming strain UCC1001 with the *rad9*Δ*::kanMX4* PCR product to give rise to strain YLL424. Strain YLL409 was derived from strain UCC3537 by transformation with *Nde*I-digested pML54 plasmid DNA (Longhese *et al.* 1996) to obtain one-step replacement of the 877-bp *Pvu*II-*Sac*I fragment of the chromosomal *MEC3* coding region with an *Eco*RI-*Bgl*II fragment containing the *TRP1* gene. The accuracy of all gene replacements was verified by Southern blot analysis or PCR.

Strains W303, YLL521, YLL541, YLL244, DMP2760/1A, DMP2694/2C, and DMP2696/4A were transformed with a *TRP1* empty vector (pBAD54; Desany *et al.* 1998) giving rise to strains YLL719, YLL721, YLL723, YLL725, YLL727, YLL729, and YLL731, respectively. Strains W303, YLL521, YLL541, YLL244, DMP2760/1A, DMP2694/2C, and DMP2696/4A were transformed with a *TRP1* plasmid carrying the GAP-controlled *RNR1* gene (pBAD70; Desany *et al.* 1998), giving rise to strains YLL720, YLL722, YLL724, YLL726, YLL728, YLL730, and YLL732, respectively.

Cells were grown at 25° in YEP medium (1% yeast extract, 2% bactopeptone) containing 2% glucose (YEPD), 2% raffinose (YP-raf), or 2% raffinose and 2% galactose (YP-gal). Transformants carrying the *KanMX4* cassette were selected on YEPD plates containing 400 μg/ml G418 (U.S. Biological).

Plasmids: To construct plasmid pML225 (*URA3 CEN4 GAL1-MEC1*), where the 7437-bp fragment spanning from the *MEC1* ATG to the *Sac*I site and containing the whole *MEC1* coding region is fused to the *GAL1* promoter, the 1302-bp *Xba*I-*Bam*HI *MEC1* fragment from plasmid pML79 (Longhese *et al.* 1997) was cloned into *Xba*I-*Bam*HI sites of plasmid SP1 (S. Piatti, University of Milan, Italy), giving rise to plasmid pML195; plasmid SP1 carried the *Eco*RI-*Bam*HI fragment containing the *GAL1-10* promoter cloned in the *Eco*RI-*Bam*HI sites within the YCplac33 polylinker region (Gietz and Sugino 1988). A *MEC1* fragment spanning from position +1 to position +99 from the translation initiation codon was then amplified by PCR using plasmid pML79 as a template and oligonucleotides PRP134 and PRP75 as primers and then cloned into the *Bam*HI site of plasmid pML195, followed by insertion of the 6049-bp *Aat*II-*Sac*I fragment of *MEC1* coding region. Plasmids pBAD54 and pBAD70 containing, respectively, the GAP promoter and the *RNR1* open reading frame under the control of the GAP promoter were kindly provided by S. Elledge.

Southern analysis of telomere length: Yeast DNA was prepared according to standard methods (Guthrie and Fink 1991) and digested with an appropriate enzyme (*Xho*I or *Eco*RV). The resulting DNA fragments were separated by gel electrophoresis in 0.8% agarose gel and transferred to a GeneScreen nylon membrane (New England Nuclear, Boston) followed by hybridization with a poly(GT) probe or with a 1166-bp *Hin*dIII *URA3* fragment purified from plasmid YEp24. Standard hybridization conditions were used.

RESULTS

Mutations in *RAD53* gene increase transcriptional silencing at telomeres: We first assayed the effect of a *rad53* mutation on the expression of the *ADE2* reporter gene integrated into a telomeric region (Gottschling *et al.* 1990). Yeast cells that do not express *ADE2* form red-pigmented colonies on medium containing low levels of adenine, whereas white colonies result from cells expressing the *ADE2* gene. When the *ADE2* gene was placed at the telomere such that *ADE2* transcription was directed toward the telomere, cells developed red-sectored colonies due to heritable Sir-dependent transcriptional silencing (Gottschling *et al.* 1990; Aparicio *et al.* 1991). We introduced the kinase-defective *rad53K227A* allele (Zheng *et al.* 1993) in strain UCC-3537, carrying the only copy of the *ADE2* gene inserted

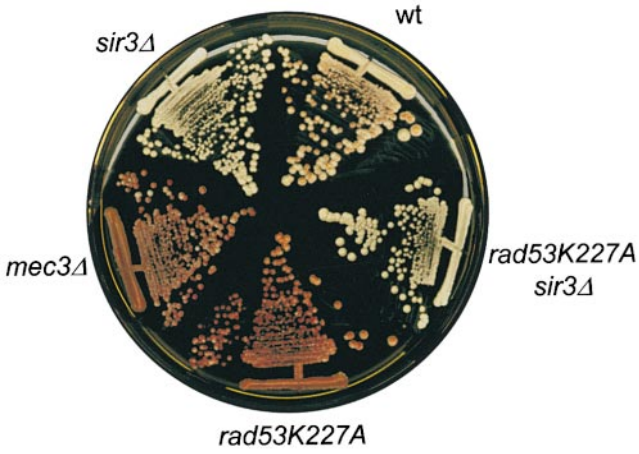

Figure 1.—Telomeric position effect on *ADE2* expression in *rad53* mutants. Isogenic wild type (UCC3537), *sir3*Δ (YLL405), *rad53K227A* (YLL410), *mec3*Δ (YLL409), and *rad53K227A sir3*Δ (YLL465) cells, carrying the *ADE2* gene at telomere V-R, were streaked onto YEPD plates. After incubation at 25° for 3 days, the plates were placed at 4° for 1 wk before being photographed. Comparable results were obtained in three independent experiments.

into the telomeric region of chromosome V-R (Huang *et al.* 1997) and scored for the colony-developed color. As shown in Figure 1, most wild-type colonies were completely white or red-sectored, suggesting that the *ADE2* gene was expressed in most cells. Conversely, a large proportion of the *rad53* mutant colonies were either completely red or contained few white sectors, indicating that the telomere-associated *ADE2* gene was transcriptionally silent (Figure 1). According to previous observations, *mec3*Δ also increased transcriptional silencing at telomeres compared to wild type (Figure 1; Corda *et al.* 1999).

The telomeric position effect (TPE) is dependent on the known Rap1-interacting proteins Sir3 and Sir4, and null mutations of *SIR3* or *SIR4* abolish silencing at telomeres (Ivy *et al.* 1986; Aparicio *et al.* 1991). To determine whether the increase in silencing caused by the *rad53* mutation was dependent on the function of the Sir proteins and not on a general bypass of the silencing machinery, we examined the silencing defects in *sir3*Δ *rad53K227A* double mutants by using the above *ADE2* system. As shown in Figure 1, both *sir3*Δ *rad53K227A* double and *sir3*Δ single mutants developed white colonies and were indistinguishable from each other, indicating that the enhanced silencing in the *rad53K227A* mutants is still dependent on the normal machinery that controls repression of gene expression at telomeres.

To further assess the enhanced transcriptional silencing at telomeres in *rad53* mutants, we assayed the telomere position effect in strains in which the only copy of the *URA3* gene (*URA3-TEL*) had been integrated into a telomeric region (Gottschling *et al.* 1990; Aparicio *et al.* 1991). Repression of *URA3-TEL* expression was then measured by the ability of cells to grow on media lacking uracil and to form colonies on 5-fluoroorotic acid (5-FOA), which kills cells expressing the *URA3* gene. We introduced the *rad53K227A* mutation in strain UCC1001, carrying *URA3* gene inserted next to the left telomere of chromosome VII (Gottschling 1992). As expected, the *sir3*Δ mutant was unable to form colonies on 5-FOA, indicating derepression of the *URA3-TEL* gene (Figure 2A). Conversely, we reproducibly observed a consistent increase in *URA3-TEL* silencing in the *rad53K227A* mutant compared to wild type, as indicated by both the increase in the frequency of 5-FOA-resistant clones and the decrease in the number of cells growing on media lacking uracil (Figure 2A). The enhanced repression of *URA3-TEL* in *rad53* mutants requires the Sir-mediated pathway. In fact, the increased repression observed in *rad53K227A* mutation was abolished by deletion of *SIR3* gene, since the ability to grow on 5-FOA of *rad53K227A sir3*Δ double mutants was similar to that observed for an otherwise isogenic *sir3*Δ single mutant strain (Figure 2A).

To generalize our results and to assess whether the transcriptional silencing defects were a peculiarity of the *rad53K227A* allele or a common feature of *rad53* mutants, we analyzed the transcriptional silencing defects of different mutations in the *RAD53* gene in the W303 background, which is different from the UCC1001 genetic background, by integrating the *URA3* gene into the telomeric region of chromosome VII (Gottschling *et al.* 1990) in a W303 derivative strain. As shown in Figure 2B, when such *rad53K227A*, *sad1-1* (a *RAD53* mutation described by Allen *et al.* 1994), and *rad53*Δ mutants were analyzed for the ability to express the *URA3-TEL* gene, we detected an enhanced telomere-directed transcriptional silencing in all of them. Therefore, different mutations in the *RAD53* gene, including its deletion, improve telomeric silencing, suggesting that Rad53 might modulate the accessibility to repressive chromatin in the telomeric silent domains.

The involvement of Rad53 in silencing prompted us to analyze the effect of mutations in *MEC1* on transcriptional silencing. Very recently it was reported that the *mec1-21* allele specifically decreases telomeric silencing (Craven and Petes 2000). As shown in Figure 3A, we did not detect any transcriptional silencing defect in strains carrying either the deletion of the *MEC1* gene or the *mec1-14* allele (Paciotti *et al.* 1998). The apparent slight growth defect of *mec1-14* cells on media lacking uracil was likely due to the slow-growth phenotype associated with this allele, which was suppressed by deletion of *SML1* gene. Since *mec3*Δ cells enhance transcriptional silencing at telomeres (Figure 1; Corda *et al.* 1999), we also analyzed the silencing defects in strains lacking *DDC1*, *RAD24*, *RAD17*, and *RAD9*. As shown in Figure 3B, *ddc1*Δ, *rad17*Δ, *rad24*Δ, and *rad9*Δ checkpoint mutants did not show any silencing defects, thus im-

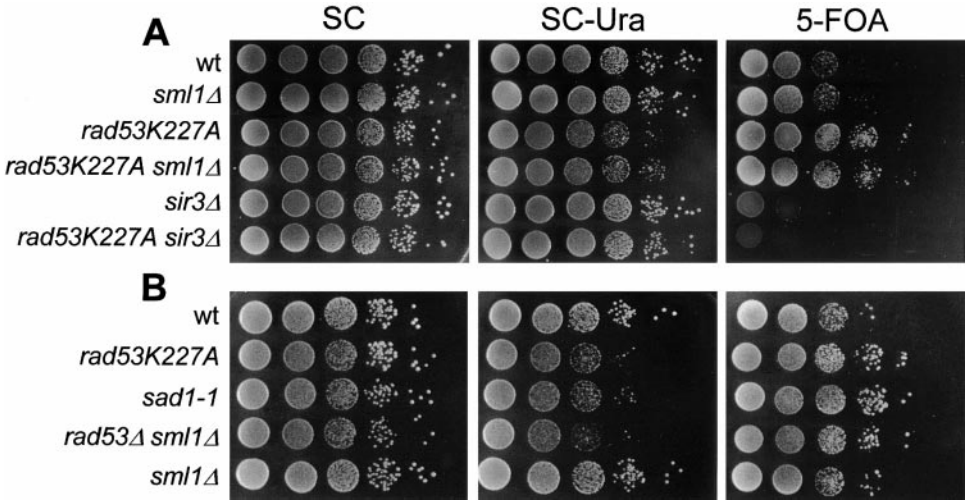

Figure 2.—*rad53* mutations increase silencing at telomeres. Isogenic strains carrying the *URA3* gene at telomere VII-L were grown overnight in rich (YEPD) medium, and then 10-fold serial dilution was spotted onto complete synthetic medium (SC) and onto the same medium lacking uracil (SC-Ura) or supplied with 5-FOA to assay for expression of the *URA3-TEL* gene. Isogenic strains were as follows: (A) wild type (UCC1001), *sml1*Δ (YLL606), *rad53 K227A* (YLL392), *rad53K227A sml1*Δ (YLL607), *sir3*Δ (YLL387), and *rad53K227A sir3*Δ (YLL462). (B) Wild type (YLL430), *rad53-K227A* (DMP2840/3D), *sad1-1* (YLL439), *rad53*Δ *sml1*Δ (DMP-2955/4A), and *sml1*Δ (DMP2955/4C). Comparable results were obtained in three independent experiments.

plying a functional difference between these genes and *MEC3*.

***rad53* mutations do not affect transcriptional silencing at mating-type loci:** Since several regulatory proteins function in silencing at both telomeres and *HM* loci, we tested whether *rad53* mutations affected silencing also at the mating-type loci by introducing the *rad53K227A* allele in strains UCC3511 and UCC3515 containing the *URA3* gene inserted at the *HMR* and *HML* loci, respectively (Singer *et al.* 1998). Silencing at both *HMR* and *HML* loci was not enhanced in *rad53* mutants, since the *rad53K227A* mutation did not affect the ability of cells to grow on media lacking uracil or in the presence of 5-FOA compared to the isogenic wild-type strain (Figure 4). These data suggest that *rad53* mutants have primarily a telomere-specific silencing defect.

Rad53 and Mec1 are involved in telomere length maintenance: Many mutations that influence telomeric silencing also influence telomere length. For example, *sir3* and *sir4* mutants, where telomeric silencing is disrupted, have telomeric repeats that are 50–100 bp shorter than wild type (Palladino *et al.* 1993). Since *RAD53* gene modulates TPE, we asked whether it also has a role in telomere length regulation. To this purpose, we first examined the effect of the *rad53K227A* mutation on the length of the left telomere of chromosome VII in UCC1001 strain, carrying the *URA3-TEL* gene as described above. As shown in Figure 5, Southern blot analysis with a *URA3* probe revealed that telomeres were shorter in *rad53K227A* mutant than in isogenic wild-type cells, and this telomere shortening was comparable to that observed for *sir3*Δ mutants. The *rad53 K227A sir3*Δ double mutants showed further decrease

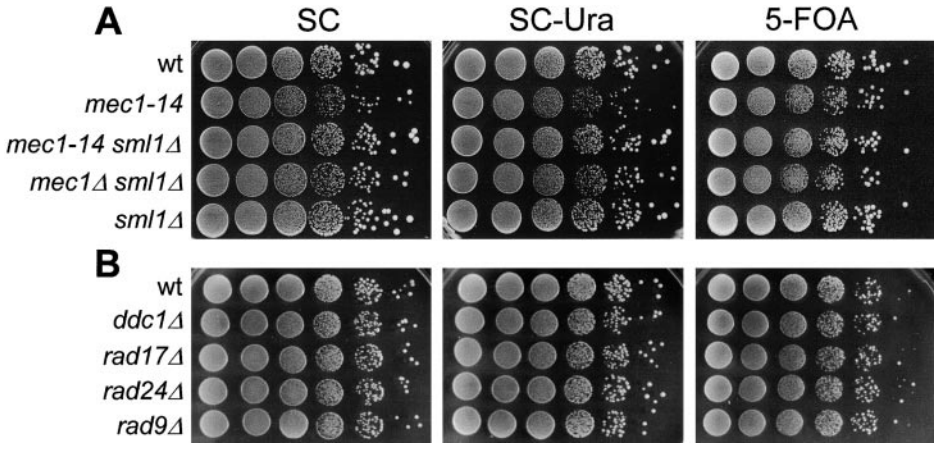

Figure 3.—Silencing assay in *mec1*, *ddc1*Δ, *rad17*Δ, *rad24*Δ, and *rad9*Δ mutants. Isogenic strains carrying the *URA3* gene at telomere VII-L were grown overnight in rich (YEPD) medium, and then 10-fold serial dilution was spotted onto complete synthetic medium (SC) and onto the same medium lacking uracil (SC-Ura) or supplied with 5-FOA to assay for expression of the *URA3-TEL* gene. Isogenic strains were as follows: (A) wild type (YLL430), *mec1-14* (DMP2831/1B), *mec1-14 sml1*Δ (DMP3141/2B), *mec1*Δ *sml1*Δ (DMP2952/2B), and *sml1*Δ (DMP2952/2C). (B) Wild type (UCC1001), *ddc1*Δ (YLL388), *rad17*Δ (YLL389), *rad24*Δ (YLL390), and *rad9*Δ (YLL424).

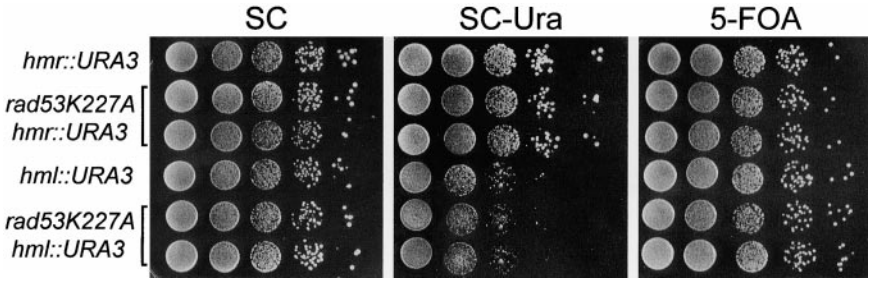

Figure 4.—Silencing at mating-type loci. Isogenic strains carrying the *URA3* gene inserted into the *HML* or *HMR* locus were grown overnight in rich (YEPD) medium, and then 10-fold serial dilution was spotted onto complete synthetic medium (SC) and onto the same medium lacking uracil (SC-Ura) or supplied with 5-FOA to assay for expression of the *URA3* gene. Isogenic strains were as follows: *hmr::URA3* (UCC 3511), *hmr::URA3 rad53K227A* (YLL416), *hml::URA3* (UCC3515), and *hml::URA3 rad53K227A* (YLL422). The more efficient silencing of the *URA3* gene at *HML* than at *HMR* has been previously reported (Singer *et al.* 1998) and depends on the difference in the way the *URA3* gene was inserted within the two *HM* loci.

in telomere length compared to both *rad53K227A* and *sir3*Δ single mutants (Figure 5), suggesting that Rad53 and Sir3 proteins contribute to chromosome end protection by different pathways.

To confirm the requirement for Rad53 function in telomere length maintenance and to analyze the effect of mutations in the *MEC1* gene on the same process, we examined the effects of *rad53* and *mec1* mutations in W303 genetic background by measuring the length of the telomeric $(C_{1-3}A)_n$ repeat. To this end, we introduced the mutations under analysis in the W303 strain and analyzed the genomic DNA recovered from each strain by Southern hybridization analysis with a poly (GT) probe. As shown in Figure 6A, the *rad53K227A* mutant showed telomere shortening, thus confirming the above indication that Rad53 contributes to telomere integrity. Moreover, not only *rad53K227A sir3*Δ, but also *rad53K227A sir4*Δ double mutants showed a more pronounced telomere shortening compared to each single mutant. According to what was previously observed in the *mec1-21* mutant (Ritchie *et al.* 1999), a comparable telomere length defect was also detectable in the *mec1-14* mutant. In fact, as shown in Figure 6B, *mec1-14* mutant cells displayed telomere shortening that was further enhanced when the *mec1-14* allele was combined with the deletion of the *SIR3* or *SIR4* genes. Therefore, both Rad53 and Mec1 proteins are required for controlling telomere length by a mechanism that appears to be different from that involving the Sir3 and Sir4 proteins. We also analyzed telomere length in a *rad53K227A mec1-14* double mutant and we found that it was undistinguishable from that observed in the most defective single mutant (*rad53K227A*; data not shown), suggesting that *rad53* and *mec1* mutations impair telomere length control through the same mechanism.

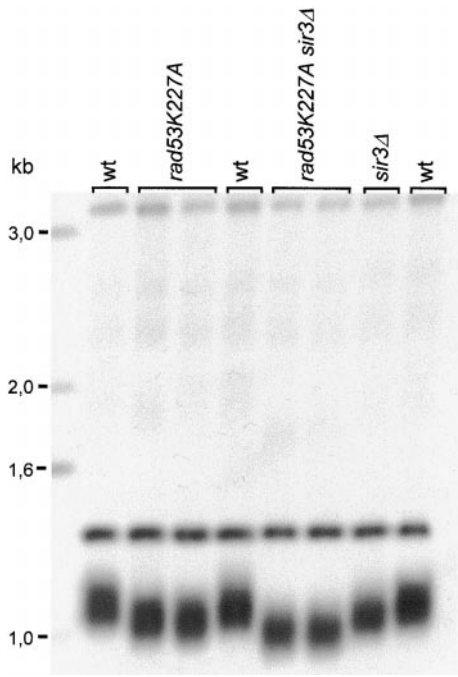

Figure 5.—*rad53* deficient strains display defects in telomere VII-L length. Genomic DNA from wild type (UCC1001), *rad53K227A* (YLL392; two independent transformants), *rad53K227A sir3*Δ (YLL462; two independent transformants), and *sir3*Δ (YLL387) isogenic strains, all carrying a subtelomeric *URA3* gene on chromosome VII-L, was prepared after 45 generations of growth, digested with *Eco*RV, separated on a 0.8% agarose gel, and hybridized to an *URA3* probe.

It has been previously observed that strains carrying mutations in both *MEC1* and *TEL1* genes exhibit a senescence phenotype and a more pronounced shortening of telomeres compared to each single mutant (Ritchie *et al.* 1999), suggesting that Tel1 and Mec1 contribute to telomere length control by different pathways. Since we showed that Rad53, together with Mec1, is necessary to maintain telomere integrity, we analyzed the telomere length defect in the *rad53 tel1* double mutants. To this purpose, we introduced the kinase-defective *rad53K227A* allele in a strain carrying the deletion of *TEL1* gene. As shown in Figure 7, *tel1*Δ *rad53K227A* double mutants exhibited a telomere shortening indistinguishable from that observed in a *tel1*Δ single mutant, suggesting that the *rad53K227A* mutation was not able to further decrease telomere length in the absence of Tel1. Moreover, in contrast to what was observed in *tel1 mec1* double mutants (Ritchie *et al.* 1999; our unpublished observation), deletion of the *TEL1* gene did not affect the growth rate of *rad53K227A* mutants (data not shown).

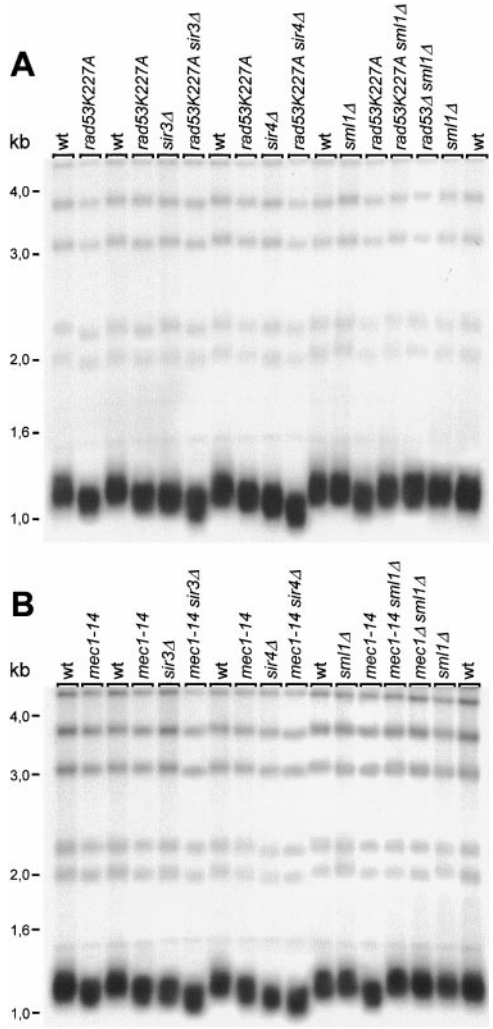

Figure 6.—Telomere length defects in *rad53* and *mec1* deficient strains. Genomic DNA from each cell culture was prepared after 45 generations of growth, digested with *Xho*I, separated on a 0.8% agarose gel, and hybridized to a poly(GT) telomere-specific probe. Isogenic strains were as follows: (A) wild type (W303), *rad53K227A* (DMP2760/1A; DMP2760/3B), *sir3*Δ (YLL521), *rad53K227A sir3*Δ (YLL522), *sir4*Δ (YLL541), *rad53K227A sir4*Δ (YLL543), *sml1*Δ (YLL488), *rad53K227A sml1*Δ (YLL590), and *rad53*Δ *sml1*Δ (YLL509). (B) Wild type (W303), *mec1-14* (DMP2696/3D; DMP2696/4A), *sir3*Δ (YLL521), *mec1-14 sir3*Δ (YLL525), *mec1-14 sml1*Δ (YLL623), *sir4*Δ (YLL541), *mec1-14 sir4*Δ (YLL546), *sml1*Δ (YLL488), and *mec1*Δ *sml1*Δ (YLL490).

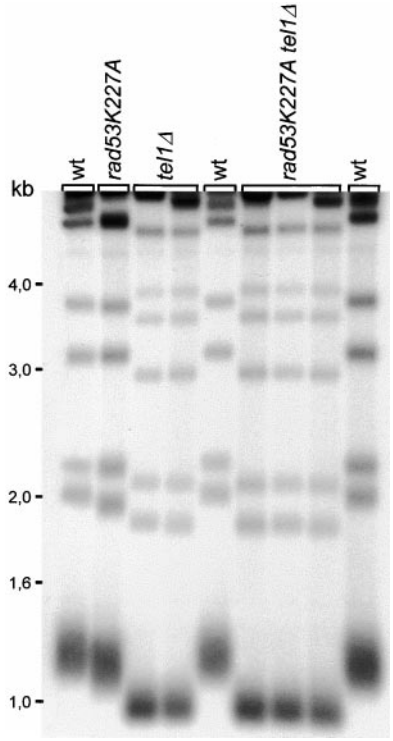

Figure 7.—Genetic interaction between *TEL1* and *RAD53*. Genomic DNA from each cell culture was prepared after 45 generations of growth, digested with *Xho*I and hybridized to a poly(GT) telomere-specific probe. Isogenic strains were as follows: wild type (Y300), *rad53K227A* (YLL678), *tel1*Δ (Y300tel1; two independent transformants), and *rad53K227A tel1*Δ (YLL677; three independent transformants).

Telomere length and transcriptional silencing controls are separable functions of Rad53: Telomere shortening usually has been found associated with reduced TPE, whereas abnormally long telomeres can hyperrepress telomere-adjacent genes (Kyrion *et al.* 1992). Since the *rad53* mutant displayed increased telomeric silencing concomitantly with decreased telomere length, the enhanced TPE observed in this mutant could be unrelated to the telomere length defect. In addition to its involvement in the DNA damage checkpoint pathway, Rad53, together with Mec1, is essential for cell viability. Their essential function can be bypassed by increasing expression of genes encoding ribonucleotide reductase (Desany *et al.* 1998) or by deleting the *SML1* gene (Zhao *et al.* 1998), which negatively affects dNTP pools likely through post-translational regulation of ribonucleotide reductase activity. On the basis of the above observations, the telomere shortening observed in *rad53* mutants might be caused by defective telomere synthesis in the presence of a reduced dNTP pool. If this were the case, we would expect to suppress the telomere length defects in *rad53K227A* and *mec1-14* mutants by deleting the *SML1* gene. As shown in Figure 6, A and B, telomere length in *sml1*Δ *rad53K227A* and *sml1*Δ *mec1-14* double mutants was comparable to that observed in a wild-type strain and was indistinguishable from that observed in a *sml1*Δ single mutant. Therefore, telomere shortening in *rad53K227A* and *mec1-14* single mutants might result from defective DNA replication caused by nucleotide depletion. This hypothesis is further supported by the finding that strains carrying deletions of the *RAD53* or *MEC1* genes, whose viability depends on the lack of Sml1, did not show any telomere shortening (Figure 6, A and B). Deletion of *SML1* did not affect transcriptional silencing in *rad53* mutants. In fact, as shown in Figure 2, A and B, both *rad53*Δ *sml1*Δ

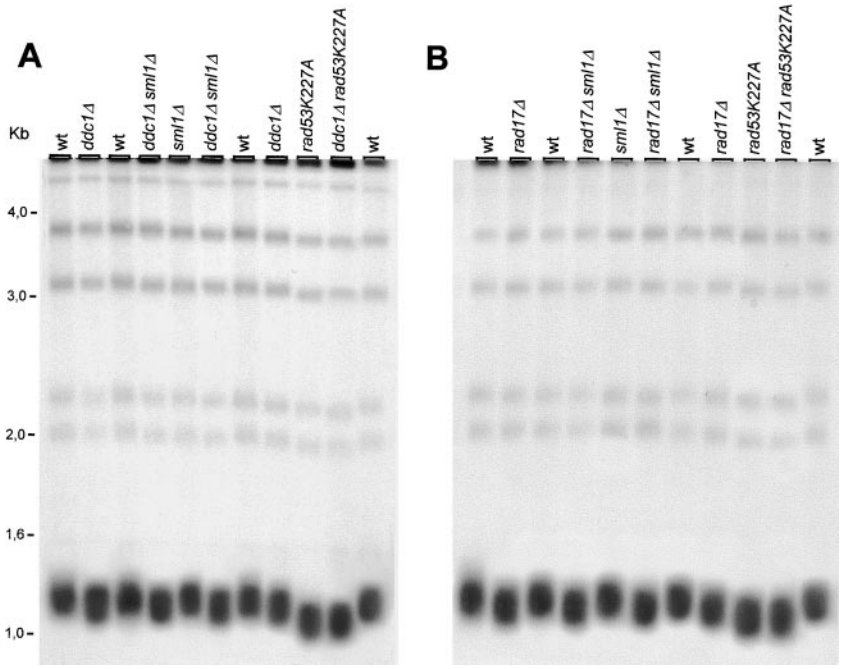

Figure 8.—Telomere length in *ddc1*Δ and *rad17*Δ mutants. Genomic DNA from each cell culture was prepared after 45 generations of growth, digested with *Xho*I, and hybridized to a poly(GT) telomere-specific probe. Isogenic strains were as follows: (A) wild type (W303), *ddc1*Δ (YLL244), *ddc1*Δ *sml1*Δ (DMP2950/3B), *sml1*Δ (YLL488), *rad53K227A* (DMP2760/1A), and *rad53K227A ddc1*Δ (DMP2932/4D). (B) Wild type (W303), *rad17*Δ (YLL244), *rad17*Δ *sml1*Δ (DMP2950/3B), *sml1*Δ (YLL488), *rad53K227A* (DMP2760/1A), and *rad53K227A rad17*Δ (DMP2928/9A).

and the *rad53K227A sml1*Δ double mutants showed an increase in the frequency of 5-FOA-resistant cells and a decrease in the number of cells growing on media lacking uracil, compared to the wild type, which were similar to those observed in *rad53K227A* single mutants. This finding suggests that the role of Rad53 in transcriptional silencing can be separated from that in telomere size control, implying distinct functions of Rad53 in modulating these processes.

Effects of other DNA damage checkpoint mutants on telomere length: We also asked whether other DNA damage checkpoint mutants displayed defects in telomere length regulation. To this purpose, we examined the telomere length in *ddc1*Δ, *rad17*Δ, *rad24*Δ, and *rad9*Δ strains. Southern blot analysis using the telomere probe showed that *ddc1*Δ and *rad17*Δ mutations caused shortening of telomeres, although we reproducibly found that the extent of shortening was less than that caused by *rad53* and *mec1* mutations (Figure 8, A and B). The combination of *ddc1*Δ with *rad17*Δ did not enhance the telomere shortening (data not shown), suggesting that Ddc1 and Rad17 proteins belong to the same epistatic group not only with respect to their involvement in DNA damage checkpoint response but also with respect to their role in chromosome end protection. Conversely, we did not detect any reproducible telomere shortening in *rad9*Δ and *rad24*Δ strains (data not shown). In contrast to what was observed for the *rad53* and *mec1* mutants, the telomere length defects in *ddc1*Δ and *rad17*Δ mutants were not suppressed by deleting the *SML1* gene, since *rad17*Δ *sml1*Δ and *ddc1*Δ *sml1*Δ double mutants still showed telomere shortening (Figure 8, A and B). These data suggest that the role of Ddc1 and Rad17 in telomere length control is different from that of Rad53 and Mec1. However, the telomere shortening was not more severe by combining *rad17*Δ and *ddc1*Δ mutations with the *rad53K227A* allele (Figure 8, A and B), suggesting that Rad17/Ddc1 and Rad53/Mec1 belong to the same epistatic group with respect to telomere length control.

***RNR1* overexpression suppresses the telomere shortening in *rad53* and *mec1* mutants:** To provide further evidence that telomere shortening in *mec1* and *rad53* mutants is caused by a defective DNA replication caused by nucleotide depletion, we tested whether the telomere length defects might be suppressed by providing additional nucleotides through upregulation of ribonucleotide reductase activity. To this purpose, we analyzed whether overexpression of *RNR1* gene, encoding the large subunit of ribonucleotide reductase (RNR), the rate-limiting enzyme of deoxyribonucleotide synthesis, could suppress the telomere length shortening in *rad53* and *mec1* mutants. As shown in Figure 9, *rad53K227A* and *mec1-14* mutants overexpressing *RNR1* gene did not show any telomere shortening, while *ddc1*Δ and *rad17*Δ mutants still showed telomere length defects. According to the finding that Rad53/Mec1 and Sir proteins contribute to telomere end protection by different pathways, overexpression of *RNR1* did not suppress the telomere shortening in *sir3*Δ and *sir4*Δ mutants.

Overexpression of the *MEC1* gene affects telomere length independently of *SML1*: Since mutations in genes affecting telomere length often display the same phenotype observed when the corresponding genes are overexpressed (Aparicio *et al.* 1991; Singer *et al.* 1998), we examined telomere length in strains carrying the *MEC1* and *RAD53* genes expressed from the *GAL1* promoter. As shown in Figure 10, we observed telomere shortening

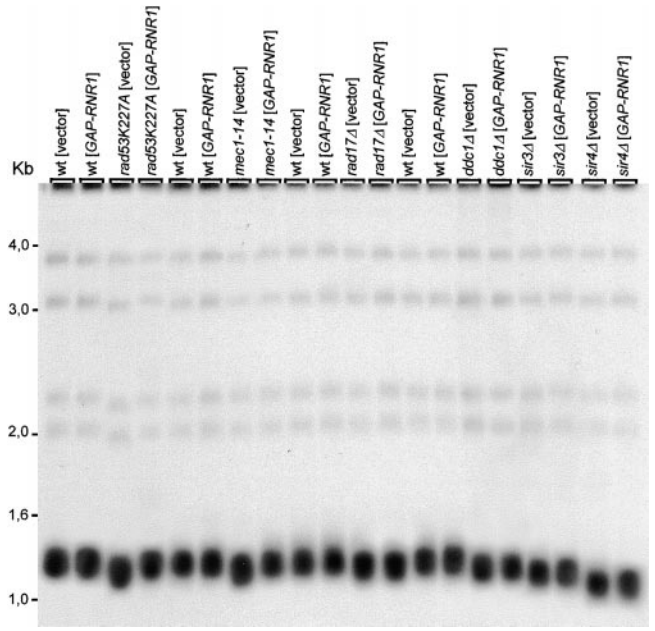

Figure 9.—Effect of *RNR1* overexpression on telomere length control. Strains were transformed with a *TRP1* plasmid carrying *GAP*-controlled *RNR1* (pBAD70) or empty vector (pBAD54; Desany *et al.* 1998). Cell cultures were grown in SC-Trp before preparing DNA. DNA was digested with *Xho*I and hybridized to a poly(GT) telomere-specific probe. Isogenic strains were as follows: wild type (YLL719), wild type [*pGAP-RNR1*] (YLL720), *rad53K227A* (YLL727), *rad53K227A* [*pGAP-RNR1*] (YLL728), *mec1-14* (YLL731), *mec1-14* [*pGAP-RNR1*] (YLL732), *rad17*Δ (YLL729), *rad17*Δ [*pGAP-RNR1*] (YLL730), *ddc1*Δ (YLL725), *ddc1*Δ [*pGAP-RNR1*] (YLL726), *sir3*Δ (YLL721), *sir3*Δ [*pGAP-RNR1*] (YLL722), *sir4*Δ (YLL723), and *sir4*Δ [*pGAP-RNR1*] (YLL724).

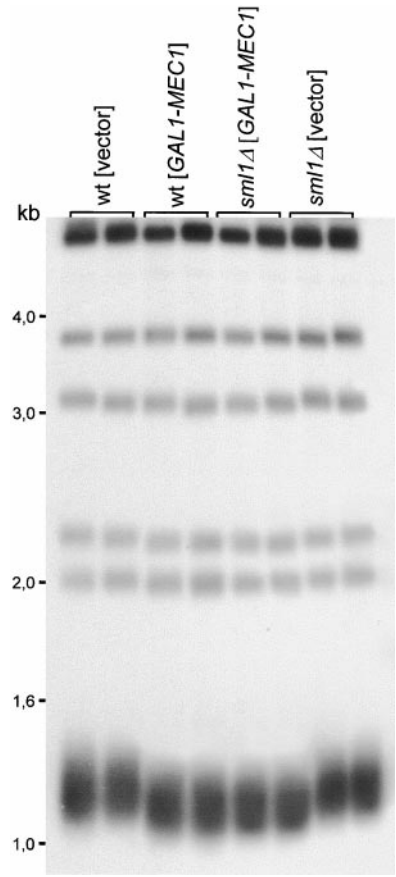

Figure 10.—Overexpression of the *MEC1* gene causes telomere shortening. Cell cultures logarithmically growing in YP-raf were transferred to YP-gal (1×10^4 cells/ml) and allowed to reach the final concentration of 2×10^7 cells/ml before preparing genomic DNA. DNA was digested with *Xho*I and hybridized to a poly(GT) telomere-specific probe. Isogenic strains were as follows: wild type (YLL634; two independent transformants), *GAL-MEC1* (YLL632; two independent transformants), *sml1*Δ (YLL640; two independent transformants), and *sml1*Δ *GAL-MEC1* (YLL638; two independent transformants).

in strains overexpressing the *MEC1* gene compared to wild-type cells. Since the lack of Sml1 suppressed the telomere shortening in *mec1* mutants, we then analyzed the effect of *MEC1* overexpression in an *sml1*Δ background. Surprisingly, *MEC1* overexpression also reduced telomere length in *sml1*Δ strains, indicating that telomere shortening caused by Mec1 overproduction occurs independently of the presence of Sml1. Conversely, overexpression of the *RAD53* gene did not affect telomere integrity in either wild-type or *sml1*Δ strains (data not shown).

DISCUSSION

DNA strand breaks existing within telomeres at the end of linear eukaryotic chromosomes are not normally recognized as DNA damage. One way in which new masking may be achieved is through telomeric DNA being sequestered into a unique type of chromatin organization. In fact, yeast telomeres contain tandem arrays of the repeated sequence $C_{1-3}A$, to which a number of telomere-associated proteins critical for their replication and maintenance are bound.

In this study we analyzed the role of the checkpoint genes in maintaining telomere integrity. Different lines of evidence implicate DNA damage checkpoint components in chromosome end protection. Mutations in *RAD53* and *MEC1* genes affect the length of the telomere repeat sequences. One question is how a defective Rad53 or Mec1 alters telomere length control. *MEC1* and *RAD53* genes are essential for cell viability, and cell lethality in *mec1*Δ and *rad53*Δ cells is rescued by upregulation of ribonucleotide reductase activity or by deletion of the *SML1* gene that causes an increase in dNTP synthetic capacity, which is necessary for efficient DNA replication and repair. This suggests that the lethality of *mec1* and *rad53* null mutants is due to accumulation of dNTP precursors insufficient to support DNA replication. Telomere length is maintained by a balance between opposing processes such as telomere polymerization and shortening, the latter of which might involve lagging-strand DNA synthesis and an exonuclease activ-

ity (Wellinger *et al.* 1996). Since the deletion of the *SML1* gene and upregulation of the ribonucleotide reductase activity suppress the telomere length defects observed in *rad53* and *mec1* mutants, telomere shortening observed in these mutants might be caused by a defective DNA replication at telomeric ends. In this view, the loss of Rad53 and Mec1 might decrease dNTP pools, which might result in a defective DNA replication at telomeres. Rad53 and Mec1 seem to be required to support telomere replication by the same mechanism since the telomere length defect of the *rad53K227A mec1-14* double mutant is indistinguishable from the most defective single mutant (*rad53K227A*). In addition, we show that Ddc1 and Rad17 checkpoint proteins are required to maintain wild-type telomere structure. In fact, the lack of either Ddc1 or Rad17, inferred from genetic studies to operate in one pathway and to interact biochemically (Paciotti *et al.* 1998; Kondo *et al.* 1999), causes telomere shortening. The requirement for Rad17 in telomere metabolism appears to be conserved during evolution, since deletion of the *S. pombe rad1*$^+$ gene, encoding the homologue of *S. cerevisiae* Rad17, also causes telomere shortening (Dahlen *et al.* 1998; Matsuura *et al.* 1999). In contrast to what was observed in *rad53* and *mec1* mutants, the lack of Sml1 does not suppress the telomere length defects of *rad17*Δ and *ddc1*Δ mutants, suggesting that the absence of Rad17 and Ddc1 affects the telomere ends by a mechanism different from that impaired by the *rad53* and *mec1* mutations. However, combination of the *rad53* allele with *rad17*Δ or *ddc1*Δ does not further decrease telomere length, suggesting that Rad17/Ddc1 and Rad53/Mec1 function in the same pathway controlling telomere length, although their roles in maintaining telomere integrity might be different.

The evolutionary conservation of the checkpoint pathways raises the possibility that the checkpoint components play similar roles in other organisms. Major similarities between the checkpoint pathways include structural similarities between *S. cerevisiae* Mec1 and *S. pombe* Rad3 and between *S. cerevisiae* Rad53 and *S. pombe* Cds1. Like the *S. cerevisiae RAD17*, the telomere shortening of *S. pombe rad1* mutants is not further affected by combination of *rad1* with *rad3* or with other mutants affecting the DNA damage checkpoint pathway. In contrast to what was observed for *rad53* mutants, *S. pombe cds1*Δ cells apparently are not affected in telomere structure (Matsuura *et al.* 1999). This difference might not be due necessarily to a different role of the two proteins in modulating telomere length, but might be explained by the way dNTP synthesis occurs in the two yeasts. In fact, unlike the *S. cerevisiae MEC1* and *RAD53*, the *S. pombe rad3*$^+$ and *cds1*$^+$ genes are not essential for cell viability, suggesting that the nucleotide levels in *rad3*Δ and *cds1*Δ mutants are not rate limiting for some vital process like DNA replication. However, *S. pombe rad3*Δ still shows telomere shortening, which is further increased by deleting the Tel1$^+$ gene (Naito *et al.* 1998; Matsuura *et al.* 1999), suggesting a specific role for Rad3 in maintaining telomere integrity. Similarly, in *S. cerevisiae, tel1 mec1* double mutants show a more pronounced reduction in telomere length compared to each single mutant (Ritchie *et al.* 1999), while inactivation of Rad53 does not enhance the telomere shortening in *tel1*Δ strains. This suggests that *S. pombe* Rad3, and possibly its *S. cerevisiae* counterpart Mec1, might have additional functions in controlling telomere integrity compared to the Cds1 and Rad53 kinases. Interestingly, overexpression of *MEC1* leads to telomere shortening in both wild-type and *sml1*Δ background, indicating that *MEC1* overexpression affects telomere integrity by a mechanism different from that impaired by its loss of function. Since reduction in telomere length can be achieved by reducing the rate of telomere elongation or by increasing the rate of telomere degradation, an excess of Mec1 might affect the balance between the rate of telomere elongation and shortening, altering the activity of components required to maintain telomere integrity or their accessibility to the telomeric sequences. Mec1 shows homology with Tel1, whose overexpression suppresses the DNA damage sensitivity of *mec1* mutants, suggesting that the two proteins may have partially overlapping functions. In this view, overproduction of Mec1 might affect telomere length by competing with Tel1 and/or altering its activity in phosphorylating target proteins involved in maintaining telomere structure.

rad53 mutants specifically increase repression of the telomere-adjacent gene expression, suggesting that Rad53 might be involved in the relief of silencing at telomeres. The finding that telomere shortening, but not increased telomeric repression in *rad53* mutants, is suppressed by deleting the *SML1* gene suggests that the role of Rad53 in controlling telomere length might be separable from its role in modulating chromatin structure. If a defective DNA synthetic capacity likely accounts for the telomere shortening in *rad53* mutants, several possible explanations might be proposed for the increased transcriptional repression of telomere-proximal genes observed in these mutants. For example, it has been suggested that the establishment of silencing can be enhanced by slowing down cell cycle progression (Laman *et al.* 1995). Since the *rad53*Δ *sml1*Δ mutant exhibits a slow-growth phenotype, it is possible that the enhanced transcriptional silencing might be caused by a defective cell cycle progression. However, many DNA replication mutants are unable to restore repression of a silencing-deficient *HMR-E* silencer, suggesting that the slowing of cell cycle progression alone is not sufficient to reestablish silencing (Ehrenhofer-Murray *et al.* 1999). In fact, only mutations in a restricted set of replication proteins that have been implicated also in the S phase checkpoint response, such as proliferating cell nuclear antigen (PCNA), RF-C, DNA polymerase ε,

CDC45, *CDC7*, restore silencing at *HM* defective loci (Axelrod and Rine 1991; Ehrenhofer-Murray *et al.* 1999). Therefore, the enhanced reestablishment of silencing appears to be related to specific defects in DNA metabolism.

In addition to *rad53* mutants, *mec3* null mutants also show altered silencing. In fact, although to a lesser extent than *rad53* mutants, *mec3*Δ cells enhance transcriptional silencing at telomeres (Corda *et al.* 1999; Figure 1). Conversely, the *mec1-14*, *rad17*Δ, *ddc1*Δ, *rad24*Δ, and *rad9*Δ checkpoint mutants do not show any increase in telomeric repression. This would suggest that, among the analyzed checkpoint proteins, only Rad53 and Mec3 are involved in modulating the accessibility to telomeric sequences. However, recent observations indicate that the *mec1-21* allele causes a decrease in telomeric silencing, suggesting that Mec1 has a role in telomeric position effect that is detectable only in the presence of specific mutations (Craven and Petes 2000).

The finding that Mec3 shows genetic and physical interaction with Set1, a protein required for transcriptional telomeric silencing (Corda *et al.* 1999), might account for the involvement of Mec3 in modulating chromatin accessibility. Whether Rad53 directly regulates chromatin structure remains an open question. The finding that deletion of the *SML1* gene does not suppress the increased telomere silencing of *rad53* mutants suggests that Rad53 has additional functions compared to Mec1 and to the other DNA damage checkpoint components. This hypothesis is also supported by the observation that *SML1* deletion restores viability in *mec1*Δ and *rad53*Δ strains differently. In fact, while *mec1*Δ *sml1*Δ double mutants grow as well as the wild-type cells, *rad53*Δ *sml1*Δ double mutants still show severe growth defects (Zhao *et al.* 1998; our unpublished observations), implying additional functions for Rad53 in cell metabolism. Interestingly, the enhanced repression observed in *rad53* mutants is specific for the telomeric sequences. On the basis of these observations, it is tempting to speculate that Rad53 could be specifically recruited to telomeric transcriptionally inactive chromatin, where it might modulate the accessibility of chromatin by influencing the activities of proteins specifically required to establish silencing at telomeres, possibly through phosphorylation events. Since some checkpoint functions may be activated during normal DNA replication (Longhese *et al.* 1997; Paciotti *et al.* 1998), it is reasonable that the checkpoint pathway genes affect telomeres even in an unperturbed cell cycle. In this scenario, in *rad53* mutant Ku and/or Sir proteins might be more tightly bound to telomeres, resulting in more efficient telomeric silencing. The previous finding that Ku and Sir proteins are released from telomere in response to DNA double-strand breaks in a checkpoint-dependent manner further supports this hypothesis (Martin *et al.* 1999; Mills *et al.* 1999).

Altogether, our observations that gene products implicated in DNA damage checkpoint response in *S. cerevisiae* are also required for the integrity of yeast telomere ends and chromatin structure provides further evidence that checkpoint mechanisms, telomeres, and chromatin are connected by complex relationships, whose elucidation will be a challenging subject for future work.

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