Inactivation of Ku-Mediated End Joining Suppresses *mec1Δ* Lethality by Depleting the Ribonucleotide Reductase Inhibitor Sml1 through a Pathway Controlled by Tel1 Kinase and the Mre11 Complex

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***RAD53* and *MEC1* are essential *Saccharomyces cerevisiae* genes required for the DNA replication and DNA damage checkpoint responses. Their lethality can be suppressed by increasing the intracellular pool of deoxynucleotide triphosphates. We report that deletion of *YKU70* or *YKU80* suppresses *mec1Δ*, but not *rad53Δ*, lethality. We show that suppression of *mec1Δ* lethality is not due to Ku[−]-associated telomeric defects but rather results from the inability of Ku[−] cells to efficiently repair DNA double strand breaks by nonhomologous end joining. Consistent with these results, *mec1Δ* lethality is also suppressed by *lif1Δ*, which like *yku70Δ* and *yku80Δ*, prevents nonhomologous end joining. The viability of *yku70Δ mec1Δ* and *yku80Δ mec1Δ* cells depends on the ATM-related Tel1 kinase, the Mre11-Rad50-Xrs2 complex, and the DNA damage checkpoint protein Rad9. We further report that this Mec1-independent pathway converges with the Rad53/Dun1-regulated checkpoint kinase cascade and leads to the degradation of the ribonucleotide reductase inhibitor Sml1.**

Cells maintain the integrity of their genetic information by activating complex pathways in response to DNA damage. The activation of these signal transduction pathways leads to a delay of cell cycle progression to ensure replication and/or segregation of damaged DNA molecules and to activate DNA repair pathways (61). In the budding yeast *Saccharomyces cerevisiae*, the central components of these checkpoint pathways are *MEC1* and *RAD53*, the budding yeast homologs of the ATR-Rad3-related gene and *CHK2* (*CDS1*), respectively (1, 50, 56). In addition to their involvement in checkpoint pathways, Mec1 and Rad53 are required to induce the transcription of repair genes and of the genes encoding ribonucleotide reductase (RNR) that catalyze the rate-limiting step in deoxynucleoside triphosphate (dNTP) synthesis (2).

In contrast to most other checkpoint genes, *MEC1* and *RAD53* are essential for cell viability. The lethality associated with the disruption of *MEC1* and *RAD53*, but not their checkpoint defects, can be suppressed by increasing the intracellular concentration of dNTPs (15, 22, 56).

Sml1 inhibits Rnr1 through a direct interaction (59). Mec1 and Rad53 relieve the Sml1-Rnr1 interaction in S phase, allowing synthesis of sufficient amounts of dNTPs for DNA replication (59). Consistent with this idea, *sml1* missense mutations that rescue *mec1Δ* and *rad53Δ* lethality abolish the Sml1-Rnr1 interaction (58). More recently, it has been shown that Dun1 phosphorylates and removes Sml1 during S phase (57, 60). From all these results, it has been proposed that the absence of Mec1 or Rad53 would lead to insufficient dNTP

levels and subsequent cell death. Both *MEC1* and *RAD53* also regulate the activation of late-firing origins of DNA replication (44, 46). Firing of replication origins with insufficient nucleotides would effectively cause a condition of higher dNTP deprivation (15).

The Ku heterodimer is conserved in a wide range of eukaryotes and plays multiple roles in DNA metabolism in yeast. Ku is involved in double strand break repair by nonhomologous end joining (NHEJ) (4, 6, 33, 38). Inactivation of *YKU70* or *YKU80* also results in telomere shortening, loss of telomere clustering and silencing, deregulation of the normally cell cycle-dependent telomeric G overhang, earlier activation of replication origins close to telomeres, and synthetic lethality with mutations that impair telomere replication (3, 7, 12, 18, 25, 36, 40). *yku70* and *yku80* mutants are viable at 30°C but are unable to grow at 37°C, which reflects a defect in telomere maintenance rather than a more generalized DNA repair defect (18, 30, 31, 48). *CHK1*, *MEC1*, and *RAD9* checkpoint genes contribute to the inhibition of cell division of *yku70Δ* mutants cultured at 37°C (30). Recently, it was suggested that Mec1, Rad9, and Rad53 inhibit degradation of double-stranded DNA in and near telomere repeats (23).

In this study, we initially asked whether the inactivation of *YKU* genes would affect *mec1Δ* and/or *rad53Δ* lethality. We report that *YKU70* or *YKU80* deletion suppress *mec1Δ*, but not *rad53Δ*, lethality. We showed that in the absence of Mec1, a deletion of *YKU70* or *YKU80* associated with a defective end-joining function induces a Tel1-Mre11-dependent response. Rad9, Rad53, and Dun1 are all required for the degradation of the RNR inhibitor Sml1 in *yku70Δ* and *yku80Δ*. Our results bring new insights into the way cells respond to DNA lesions in *yku70Δ* and *yku80Δ* cells and unmask for the first time a

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connection between the NHEJ pathway and the checkpoint response.

MATERIALS AND METHODS

Strains and plasmids. The genotypes of the yeast strains used in this study are listed in Table 1. The *mec1Δ::KAN*, *rad24Δ::KAN*, *rad17Δ::KAN*, and *rif2Δ::KAN* null mutations were introduced as described previously (16). The *mec1Δ::TRP1*, *lcd1Δ::TRP1*, *ddc1Δ::TRP1*, *mrc1Δ::TRP1*, *chk1Δ::TRP1*, *mad2Δ::TRP1*, *rad9Δ::TRP1*, *mre11Δ::TRP1*, *tel1Δ::TRP1*, *dun1Δ::TRP1*, *mr3Δ::TRP1*, *lif1Δ::TRP1*, and *yku80Δ::TRP1* null mutations were obtained after PCR amplification of a disruption cassette from plasmid pF6a-*TRP1*. The *exo1Δ::URA3* mutation was introduced using SphI-linearized pDL684 plasmid (from David Lydall). The *mec3Δ::TRP1* mutation was introduced as described previously (11). The *rad53-K227A* mutation was introduced using EcoRI-linearized pCH3 plasmid (39). *est2Δ::NAT*, *mec1Δ::NAT*, *rad53Δ::NAT*, *sml1Δ::NAT*, and *tel1Δ::NAT* were obtained after PCR amplification of a disruption cassette containing the nourseothricin (*nat*) resistance gene (17). To disrupt *TEL1* with *URA3* or *LEU2*, we linearized plasmid pPG47 (*URA3* or *LEU2*) with *SacI* and transformed the appropriate yeast strains (20). To construct strains carrying the *RAD52* chromosomal deletion, we transformed the appropriate strains with the BamHI-linearized plasmid pSM21 (a gift from M. Fasullo, Loyola University, Chicago, IL), which carries a *rad52Δ::TRP1* cassette.

Protein extracts and Western blot analysis. Protein extracts for Western blot analysis were prepared from trichloroacetic acid-treated cells and resolved by electrophoresis on an appropriate sodium dodecyl sulfate-polyacrylamide gel (80:1 acrylamide-bisacrylamide). Immunoblots were performed with anti-protein A (Sigma) and anti-green fluorescent protein (GFP; Roche) monoclonal antibodies for Mre11-ProA detection and for yellow fluorescent protein (YFP)-Sml1 detection, respectively.

RNA extraction and RT-PCR. Total RNA was extracted from yeast cells using the SV total RNA isolation system kit (Promega). RNA (1 μg) was reverse transcribed using the Titan one-tube reverse transcriptase PCR (RT-PCR) kit (Roche) using specific primers for *TEL1* (5'-CCACAGGATTGTCCCTGCC-3' and 5'-AGCTGCGACACCTTTTGTGTA-3') and *ACT1* (5'-CCAATTGCTCGA GAGATTTC-3' and 5'-CATGATACCTTGGTGTCTTG-3'). PCR cycling conditions for *TEL1* and *ACT1* were as follows: a denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s, as well as a final extension of 68°C for 7 min. PCR products (30% of reaction mixture) were then separated on 2% agarose gels, and bands were visualized with ethidium bromide. The gels were scanned with a Molecular Dynamics PhosphorImager, and the signals were quantified with Kodak software. The *TEL1* transcript levels were arbitrarily set at 1 in the wild-type cells, and *TEL1* levels in mutant cells were normalized accordingly.

Analysis of the YFP-Sml1 level. For G₁ phase, cells were grown to early log phase and arrested by the addition of α-factor (5 μg/ml) for 60 to 90 min, at which point arrest was verified by the absence of budded cells. For S phase, cells were grown to early log phase and arrested by the addition of hydroxyurea (10 mM) for 90 min. For G₂ phase, cells were grown to early log phase and arrested by the addition of nocodazole (30 μg/ml) for 90 to 120 min. Cells were analyzed by fluorescence microscopy, and the YFP-Sml1 level was detected by Western blot analysis.

Analysis of telomere length. Genomic DNA was isolated from overnight cultures of the strains indicated, cut with *XhoI*, separated on a 0.8% agarose gel, and subjected to Southern blot analysis with poly(GT) telomeric probe, which was obtained by PCR using plasmid sp100 (a gift from E. Gilson, Ecole Normale Supérieure, Lyon) as a template.

Micromanipulation. Cells were micromanipulated with the MSM system from Singer Instruments (26).

RESULTS

Strains initially defective in yKu70 rescue *mec1Δ*, but not *rad53Δ*, lethality. To test if the essential function of *MEC1* could be suppressed by inactivation of *YKU70*, we disrupted one allele of *MEC1* in either wild-type, heterozygous, or homozygous for *yku70Δ* diploid yeast strains. As expected, we were not able to obtain *mec1Δ* spores after 3 days of growth at 30°C when the spores were obtained from a *mec1Δ/MEC1 YKU70/YKU70* strain or from the heterozygous *mec1Δ/MEC1*

yku70Δ/YKU70 diploid. Surprisingly, tetrads derived from the *mec1Δ/MEC1 yku70Δ/yku70Δ* diploid gave more than two colonies (Fig. 1A). One hundred tetrads were dissected. A total of 160/200 *mec1Δ yku70Δ* spores germinated to form colonies, whereas 166/200 *yku70Δ* cells grew. Subsequently, several viable *mec1Δ yku70Δ* spores were backcrossed to unrelated wild-type strains. The resulting diploids were sporulated and dissected. We never obtained viable *mec1Δ* or *mec1Δ yku70Δ* colonies (not shown). These results were reproducibly obtained using multiple independent disruptions. We concluded that *mec1Δ yku70Δ* spores have not acquired an inheritable suppressor and that the viability of *mec1Δ yku70Δ* segregants is entirely dependent on the genotype of the parental diploid. *mec1Δ* is viable only in *mec1Δ yku70Δ* spores that were generated from diploids homozygous for *yku70Δ*. Similar results were obtained with the deletion of *LCD1/DDC2* (not shown), the ATRIP partner of Mec1 that is involved in both its essential and checkpoint functions (37, 41, 53). The germinating *yku70Δ* and *mec1Δ yku70Δ* spores of several tetrads were examined microscopically after 4, 8, 18, and 72 h (not shown). All of them start to germinate after 8 h at 30°C. After 18 h, the spores grew into microcolonies consisting of at least 20 cells, but often more. We could not detect any difference in colony size between *mec1Δ yku70Δ* and *MEC1 yku70Δ* microcolonies after 18 h, indicating that these cells are growing at the same rate. As previously shown, after 3 days, most of the tetrads arising from *mec1Δ/MEC1 yku70Δ/yku70Δ* gave four big colonies with a size of 100 to 150 cells each. If the *mec1Δ yku70Δ* cells are separated into individual cells 3 days after germination, each *mec1Δ* cell divides at the same rate as *yku70Δ*. In each case, the viable *mec1Δ yku70Δ* colonies were sensitive to hydroxyurea and UV irradiation. We checked whether the HU sensitivity is different in *mec1Δ* compared to *mec1Δ yku70Δ* cells. Since *mec1Δ* strains are unable to grow in the presence of yKu70, their ability was maintained by the presence of a 2 μm *RNR1* plasmid (15). We found that even at a low dose of HU, *mec1Δ* and *mec1Δ yku70Δ* cells presented a similar sensitivity (Fig. 1B). Taken together, these results indicate that both types of cells, *yku70Δ* and *mec1Δ yku70Δ*, are fully viable but only if the parental diploid strain was homozygous *yku70Δ/yku70Δ*.

An important role of yKu70 and yKu80 is maintenance of telomere integrity (40). Mec1 is associated with telomeres (47), and its absence causes a shortening of telomere length. We investigated whether the deletion of *MEC1* (in the presence of *SML1*) aggravated the telomeric phenotypes associated with *yku70Δ* deletion. As shown in Fig. 1C, *mec1Δ* did not confer senescence to a *yku70Δ* strain and *mec1Δ yku70Δ* cells exhibit telomeric defects similar to those observed in *yku70Δ* cells. The suppression of *mec1Δ* by *YKU70* or *YKU80* gene deletion was confirmed by our ability to disrupt *MEC1* in *yku70Δ* or *yku80Δ* haploid strains and by the discovery that *yku70Δ mec1Δ* cells were able to lose a p*MEC1* expression plasmid, whereas *mec1Δ* cells were not (Fig. 1D). Deletion of *YKU70* or *YKU80* did not suppress *rad53Δ* lethality (not shown).

Genetic dependence of the suppression of *mec1Δ* lethality by *yku70Δ*. Because the DNA damage pathway regulates dNTP levels by increasing *RNR* gene transcription (22) and *RNR* activity by phosphorylation-mediated removal of Sml1, an inhibitor of *RNR* (57), we next addressed the importance of known genetic pathways involved in the response to DNA

TABLE 1. *S. cerevisiae* strains used in this study^a

Strain	Genotype	Source and/or reference
JKM179/120	<i>MATα/mata Δho/Δho Δhml::ADE1/HMLα Δhmr::ADE1/HMRa ade1-100/ade1-100 leu2-3,112/leu2-3,112 lys5 trp1::hisG/lys5 trp1::his ura3-52/ura3-52 ade3::GAL::HO/ADE3</i>	J. Haber
JKM120Ku	<i>MATa Δho ade1-100 leu2-3,112 lys5 trp1::hisG' ura3-52 yku70::LEU2</i>	J. Haber
JKM179Ku	<i>MATα Δho Δhml::ADE1 Δhmr::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG' ura3-52 ade3::GAL::HO yku70::LEU2</i>	J. Haber
YVG100	JKM179/120, <i>MEC1/mec1::KAN</i>	This study
JKM YKU+/-	<i>MATa/mata Δho/Δho Δhml::LEU2/Δhml::ADE1 HMRa/Δhmr::ADE1 ade1-100/ade1-100 leu2-3,112/leu2-3,112 lys5/lys5 trp1::hisG/trp1::hisG ura3-52/ura3-52 ADE3/ade3::GAL::HO YKU70/yku70Δ::URA3</i>	J. Haber
YVG101	JKM YKU+/-, <i>YKU70/yku70::LEU2 MEC1/mec1::KAN</i>	This study
JKM SIR3 -/-	<i>MATa/mata Δho/Δho HMLα/Δhml::LEU2 HMRa/Δhmr::ADE1 ade1-100/ade1-100 leu2-3,112/leu2-3,112 lys5/lys5 trp1::hisG/trp1::hisG ura3-52/ura3-52 ADE3/ade3::GAL::HO sir3Δ::URA3/sir3Δ::URA3</i>	J. Haber
YVG102	JKM SIR3 -/-, <i>MEC1/mec1::KAN</i>	This study
JKM YKU -/-	JKM179/120, <i>yku70::LEU2/yku70::LEU2</i>	J. Haber
YVG103	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN</i>	This study
YVG104	JKM179/120, <i>yku70::LEU2/yku70::LEU2 RAD53/rad53::NAT</i>	This study
YVG105	JKM179/120, <i>yku70::LEU2/yku70::LEU2 RAD53/rad53-K227A::KAN</i>	This study
YVG106	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN SIR3/sir3::TRP1</i>	This study
YVG107	JKM179/120, <i>SIR3/sir3::LEU2 MEC1/mec1::TRP1</i>	This study
YVG108	JKM179/120, <i>yku70::LEU2/yku70::LEU2 LCD1/lcd1::TRP1</i>	This study
YVG109	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::NAT</i>	This study
YVG110	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::NAT RAD17/rad17::KAN</i>	This study
YVG111	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN DDC1/ddc1::TRP1</i>	This study
YVG112	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN MEC3/mec3::TRP1</i>	This study
YVG113	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::NAT RAD24/rad24::KAN</i>	This study
YVG114	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN MRC1/mrc1::TRP1</i>	This study
YVG115	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN CHK1/chk1::TRP1</i>	This study
YVG116	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN MAD2/mad2::TRP1</i>	This study
YVG117	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN EXO1/exo1::URA3</i>	This study
YVG118	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN RAD52/rad52::TRP1</i>	This study
YVG119	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN RAD9/rad9::TRP1</i>	This study
YVG120	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN RAD9/rad9::TRP1 SML1/sml1::NAT</i>	This study
YVG121	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN MRE11/mre11::TRP1</i>	This study
YVG122	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN MRE11/mre11::TRP1 SML1/sml1::NAT</i>	This study
YVG123	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN TEL1/tel1::TRP1</i>	This study
YVG124	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN TEL1/tel1::TRP1 SML1/sml1::NAT</i>	This study
YVG125	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN tel1::URA3/tel1::TRP1</i>	This study
YVG126	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN DUN1/dun1::TRP1</i>	This study
YVG127	JKM179/120, <i>yku70::LEU2/yku70::LEU2 MEC1/mec1::KAN RNR3/rnr3::TRP1</i>	This study
YVG128	JKM179/120, <i>lif1::TRP1/lif1::TRP1 MEC1/mec1::KAN</i>	This study
YVG130	Spore <i>est2::CLONAT</i> arising from the dissection of JKM179/120, <i>EST2/est2::CLONAT</i>	This study
W303-1A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1</i>	R. Rothstein
W303-1a	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1</i>	R. Rothstein
YVG140	W303-1A <i>yku70::TRP1</i>	This study
YVG141	W303-1A <i>yku70::TRP1 mec1::KAN</i>	This study
W303-1A-YFP-SML1	<i>MATa YFP-SML1 RAD5 ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1</i>	R. Rothstein
W303-1a-YFP-SML1	<i>MATα YFP-SML1 RAD5 ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1</i>	R. Rothstein
YVG142	W303-1A-YFP-SML1 <i>yku80::TRP1</i>	This study
YVG143	W303-1A-YFP-SML1 <i>yku80::TRP1 mec1::KAN</i>	This study
YVG144	W303-1A-YFP-SML1 <i>mre11::URA3</i>	This study
YVG145	W303-1A-YFP-SML1 <i>yku80::TRP1 mre11::URA3</i>	This study
YVG146	W303-1a-YFP-SML1 <i>rad9::TRP1</i>	This study
YVG147	YVG142 × YVG146, <i>YFP-SML1/YFP-SML1 yku80::TRP1/YKU80 RAD9/rad9::TRP1</i>	This study
YVG148	Spore <i>YFP-SML1 yku80::TRP1 rad9::TRP1</i> arising from the dissection of YVG147	This study
YVG149	W303-1a-YFP-SML1 <i>dun1::TRP1</i>	This study
YVG150	YVG142 × YVG149, <i>YFP-SML1/YFP-SML1 yku80::TRP1/YKU80 DUN1/dun1::TRP1</i>	This study
YVG151	Spore <i>YFP-SML1 yku80::TRP1 dun1::TRP1</i> arising from the dissection of YVG150	This study
YVG152	W303-1A-YFP-SML1 <i>rad53-K227A::KAN</i>	This study
YVG153	W303-1A-YFP-SML1 <i>yku80::TRP1 rad53-K227A::KAN</i>	This study
YVG154	W303-1A-YFP-SML1 <i>tel1::URA3</i>	This study
YVG155	W303-1A-YFP-SML1 <i>yku80::TRP1 tel1::URA3</i>	This study
YVG156	W303-1A-YFP-SML1 <i>rif2::KAN</i>	This study
YVG157	W303-1A-YFP-SML1 <i>yku80::TRP1 rif2::KAN</i>	This study
YVG158	W303-1A-YFP-SML1 <i>tel1::URA3 rif2::KAN</i>	This study
YVG159	W303-1A-YFP-SML1 <i>yku80::TRP1 tel1::URA3 rif2::KAN</i>	This study
YVG160	W303-1A-YFP-SML1 + pRS416-MEC1	This study
YVG161	W303-1A-YFP-SML1 <i>yku80::TRP1</i> + pRS416-MEC1	This study

Continued on facing page

TABLE 1—Continued

Strain	Genotype	Source and/or reference
YVG162	W303-1A-YFP-SML1 <i>yku80::TRP1 mec1::KAN</i> + pRS416-MEC1	This study
YVG163	W303-1A-YFP-SML1 <i>mec1::KAN</i> + pRS416-MEC1	This study
LPY917	MATa <i>ade2-101 trp1Δ1 leu2Δ1 his3Δ-200 lys2-801 TELadh4::URA3</i>	L. Pillus
YVG164	LPY 917 <i>yku70::TRP1</i>	This study
YVG165	LPY 917 <i>yku80::TRP1</i>	This study
YVG166	LPY 917 <i>yku80::TRP1</i> + pRS413-YKU80	This study
YVG167	W303-1a-YFP-SML1 <i>yku80::TRP1</i>	This study
YVG168	LPY 917 <i>yku80::TRP1</i> + pRS413- <i>yku80PF437,438AA</i>	This study
YVG169	LPY 917 <i>yku70::TRP1 mec1::KAN</i>	This study
YVG170	LPY917 <i>yku80::TRP1 mec1::KAN</i>	This study
YVG171	LPY 917 <i>sm1::NAT</i>	This study
YVG172	LPY 917 <i>sm1::NAT mec1::KAN</i>	This study
W303-1A	MATa <i>ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1</i>	R. Rothstein
W3483-10A	MATa <i>MRE11-YFP</i>	R. Rothstein, 27
YVG173	W3483-10A × YVG167, <i>MRE11-YFP/MRE11 SML1/YFP-SML1 YKU80/yku80::TRP1</i>	This study
YVG174	Spore <i>MRE11-YFP yku80::TRP1</i> arising from the dissection of YVG173	This study
YVG175	Spore <i>MRE11-YFP</i> arising from the dissection of YVG173	This study
W1379-3C	MATa <i>bar1Δ::LEU2</i>	R. Rothstein, 57
W2057-11A	MATa <i>bar1Δ::LEU2 GAL-SML1</i>	R. Rothstein, 57

^a Most yeast strains used in this study have an isogenic JKM, W303, LPY917, or YPH250 background. Relevant genotypes are shown. Where strains are the products of a genetic cross, the numbers of the parent strains are also indicated.

damage in the suppression of the *mec1Δ* lethality by *yku70Δ*. We constructed diploid strains homozygous for *yku70Δ* and heterozygous for *mec1Δ* and for several DNA damage checkpoint genes. For each combination, the diploids were sporulated, 100 tetrads were dissected, and the genotypes of the viable spores were determined. The results shown in Table 2 indicate that the suppression of *mec1Δ* lethality by *yku70Δ* does not depend on the PCNA-like proteins Rad17, Ddc1, and Mec3, the RFC-like protein Rad24, the DNA replication checkpoint Mrc1, or the downstream signal transduction kinase Chk1. In contrast, deletion of the DNA damage checkpoint gene *RAD9* prevented suppression of *mec1Δ* lethality by *yku70Δ*. This result indicates that *RAD9* has an essential function in rescuing *mec1Δ* lethality in *yku70Δ* cells.

EXO1 and *MAD2* are required to activate checkpoint pathways in *yku70Δ* mutants at 37°C (30). We found that neither *exo1Δ* nor *mad2Δ* mutations affect the ability of *yku70Δ mec1Δ* mutants to form colonies (Table 2). Similar results were observed using haploid *yku80Δ* for analyzing the genetic dependence of suppression of *mec1Δ* lethality.

It has been suggested that the *RAD52* recombinational repair pathway is required to repair double strand breaks (DSBs) caused by defective DNA replication in *mec1* mutants. Indeed, several viable *mec1* mutations that display synthetic lethality with *rad52Δ* have been isolated (32). We asked if Rad52 was required in the suppression of *mec1Δ* by inactivation of *YKU70* and observed that *yku70Δ* is able to suppress the lethality of *mec1Δ* cells even in the absence of *RAD52*.

The TEL1/MRX checkpoint pathway is required for *yku70Δ* suppression of *mec1Δ* lethality. Rad9 is implicated with Tel1 and the Mre11-Rad50-Xrs2 (MRX) complex in a checkpoint pathway that recognizes unprocessed DSBs and parallels the Mec1 pathway (14, 51). We considered the Mre11 nuclease and the ATM-related Tel1 kinase, which are the first proteins detected at DSBs (27), as candidates that might function in suppressing *mec1Δ* lethality. To test this, tetrads derived from

the *tel1Δ/TEL1 mec1Δ/MEC1 yku70Δ/yku70Δ* and *mre11Δ/MRE11 mec1Δ/MEC1 yku70Δ/yku70Δ* diploid strains were analyzed. We observed the appearance of *mec1Δ yku70Δ*, *tel1Δ yku70Δ*, and *mre11Δ yku70Δ* but not of *mec1Δ tel1Δ yku70Δ* and *mec1Δ mre11Δ yku70Δ* spores. Thus, in the absence of *TEL1* or *MRE11*, *mec1Δ yku70Δ* strains are not able to grow. These results suggest that *TEL1* and *MRE11* have an essential function in rescuing *mec1Δ* lethality in *yku70Δ* cells.

One interpretation of our data is that the absence of *mec1Δ yku70Δ tel1Δ* and *mec1Δ yku70Δ mre11Δ* spores is the consequence of the synthetic lethality of the triple mutation due to telomere shortening and eventual cellular senescence. To address this possibility, we took advantage of the fact that deleting the *RIF2* gene causes telomere elongation (even in a *yku70Δ* or a *yku80Δ* background) and bypasses the requirement for Mec1 and Tel1 kinases in telomere maintenance (9, 34). We disrupted one allele of *RIF2* in the diploid yeast strain *tel1Δ/TEL1 mec1Δ/MEC1 yku70Δ/yku70Δ* and analyzed the spores derived from this diploid. We never obtained *mec1Δ tel1Δ yku70Δ* and *rif2Δ mec1Δ tel1Δ yku70Δ* spores (not shown). We were also unable to obtain *mec1Δ* transformants in *rif2Δ tel1Δ yku80Δ* cells, although telomeres of *rif2Δ tel1Δ yku80Δ* cells were significantly longer than those of *tel1Δ yku80Δ* cells and slightly greater than in *yku80Δ* cells (Fig. 2A). These results predict that a DNA damage pathway controlled by Tel1 and the Mre11 complex is activated in *yku70Δ* and *yku80Δ* cells.

Because Mre11 is phosphorylated after DNA damage in a Tel1-dependent manner (14), we investigated whether Mre11 is phosphorylated in *yku70Δ* and *yku70Δ mec1Δ* cells at 30°C by using a Mre11-ProtA fusion. Figure 2B shows that Mre11-ProtA migrates as a single band in extracts obtained from wild-type cells and also in *mre11Δ* cells complemented with pMre11-ProtA, whereas a slower-migrating form of Mre11, corresponding to the phosphorylated form of Mre11, was detected in *yku70Δ mre11Δ* and *yku70Δ mec1Δ mre11Δ* cell ex-

FIG. 1. Deletion of *YKU70* suppresses *mec1Δ* lethality. (A) *mec1Δ* lethality is suppressed in cells generated from diploids homozygous for *yku70Δ*. The diploid strains *mec1Δ/MEC1 YKU70/YKU70*, *mec1Δ/MEC1 yku70Δ/YKU70*, and *mec1Δ/MEC1 yku70Δ/yku70Δ* were sporulated and dissected. Tetrads were displayed vertically on a YPD plate and incubated at 30°C for 3 days. Four representative tetrads are shown for each dissection. The arrows show *mec1Δ* spore clones. (B) HU sensitivity of *mec1Δ yku70Δ* cells. Haploid wild-type (*wt*) cells were transformed with the 2-μm *URA3*-marked plasmid expressing *RNR1* (pRS426-*RNR1*). Subsequently, *mec1Δ* or *yku70Δ* and *mec1Δ* gene deletions were created. Tenfold serial dilutions of fresh stationary-phase cultures were plated on SD-Ura and on YPD plates containing 20 mM, 5 mM, or 2 mM HU and subsequently incubated for 3 days at 30°C. (C) Effects of *mec1Δ* on viability, silencing, and telomere length of the *yku70Δ* cells. (Left) Cells of the indicated genotypes were streaked multiple times on YPD plates. Cells from the first, second, and third restreaks are shown. (Middle) Telomeric position effect was assayed by 10-fold serial dilution of the culture cells on 5-fluoroorotic acid (5-FOA) plates. (Right) Telomere lengths of *mec1Δ yku70Δ* strains. (D) *Mec1* is not required for the viability of *yku70Δ* cells. Haploid wild-type cells were transformed with the 2-μm *URA3*-marked plasmid expressing *MEC1* (pRS426-*MEC1*). Subsequently, *yku70Δ*, *mec1Δ*, or *yku70Δ* and *mec1Δ* gene deletions were created. Tenfold serial dilutions of fresh stationary-phase cultures were plated on SD-Ura and 5-FOA plates and incubated for 3 days at 30°C.

TABLE 2. Genetic dependence of *mec1Δ* lethality suppression by *yku70Δ*^a

Spore type	Result with deletion:									
	<i>rad17Δ</i>	<i>ddc1Δ</i>	<i>mec3Δ</i>	<i>rad24Δ</i>	<i>mrc1Δ</i>	<i>chk1Δ</i>	<i>rad9Δ</i>	<i>exo1Δ</i>	<i>mad2Δ</i>	<i>rad52Δ</i>
<i>yku70Δ mec1Δ</i>	+	+	+	+	+	+	–	+	+	+
<i>yku70Δ</i>	+	+	+	+	+	+	+	+	+	+

^a *mec1Δ/MEC1Δ yku70Δ/yku70Δ* strains heterozygous for the indicated deletions were sporulated and dissected. For each combination, 100 tetrads were dissected and the genotypes of the viable spores were determined. The presence (+) or absence (–) of viable spores carrying each deletion is indicated. Among the deletions, only *rad9Δ* prevents the suppression of *mec1Δ* lethality by *yku70Δ*.

tracts. These results revealed a Mec1-independent DNA damage-induced phosphorylation of Mre11 in *yku70Δ* cells.

***dun1Δ* prevents the suppression of *mec1Δ* lethality by *yku70Δ* and *yku80Δ*.** Rad53 plays an essential role in both the DNA damage and replication block checkpoints (28). Its phosphorylation correlates with activation of the checkpoint pathways (43). Overexpression of the *DUN1* gene can suppress the lethality of *mec1Δ* (35, 43); this suppression appears to reflect Dun1’s role in repressing Sml1 activity, as suppression of *mec1Δ* by *sml1Δ* did not require the activity of Dun1 (59). However, other studies have shown that a viable *mec1* mutation and *dun1Δ* are synthetically lethal (13). We tested whether *yku70Δ* or *yku80Δ* suppression of *mec1Δ* lethality is dependent on the product of *DUN1* and *RAD53* genes. Since *RAD53* is essential, we used the viable checkpoint-deficient *rad53* allele (*rad53-K227A*) that carries a substitution within the conserved kinase domain of Rad53 (39). We found that we were able to

obtain *mec1Δ* transformants in haploid *yku70Δ* and *yku80Δ* mutants but not in *yku70Δ dun1Δ*, *yku80Δ dun1Δ*, *yku70Δ rad53-K227A*, or *yku80Δ rad53-K227A* double mutants (not shown). Thus, *RAD53* and *DUN1* are required to rescue *mec1Δ* lethality in *yku70Δ* and *yku80Δ* cells. However, we observed by a gel autophosphorylation assay and by a Western blot mobility shift assay that *yku70Δ* and *mec1Δ yku70Δ* mutants do not show a high level of Rad53 phosphorylation.

Loss of essential components in nonhomologous end joining suppress *mec1Δ* lethality. yKu70 and yKu80 are DNA end-binding proteins that play various roles at different kinds of DNA ends. At telomeres, yKu70 and yKu80 are part of the structure that protects the chromosome end, whereas at broken DNA ends, they promote DNA repair as part of the NHEJ pathway. To gain insight about which aspect of yKu function, when lost, leads to the suppression of *mec1Δ* lethality, we tested a separation-of-function mutant of *YKU80*. For these

FIG. 2. Tel1 and Mre11 are required for the *mec1Δ* lethality suppression by *yku70Δ*. (A) Telomere length analysis for *rif2Δ* in wild-type (wt), *yku80Δ*, and *yku80Δ tel1Δ* cells. Lane M, ladder DNA serving as size standard. (B) Mre11 is phosphorylated in *yku70Δ* and *mec1Δ yku70Δ* cells. The indicated strains contain a plasmid allowing the expression of either wild-type Mre11-ProtA (+) or the vector (–) (14). Protein extracts of the indicated strains were analyzed by Western blotting for protein phosphorylation. Arrows indicate the position of the basal and phosphorylated (*) Mre11-ProtA bands. MW, molecular mass.

FIG. 3. Suppression of the end-joining function of yKu suppresses *mec1Δ* lethality. (A) Telomere size analysis of the telomeric defective/repair-proficient *yku80-PF437,438AA* mutant. Telomere size was examined in strains of the indicated genotypes carrying either a pRS413-YKU80 plasmid or a pRS413 plasmid expressing the mutant allele *yku80-PF437,438AA* (42). Lane M, ladder DNA serving as size standard. wt, wild type. (B) *lif1Δ* suppresses *mec1Δ* lethality. The *mec1Δ/MEC1 lif1Δ/lif1Δ* diploid strain was dissected. The presence of *mec1Δ lif1Δ* spores is indicated by arrows.

experiments, plasmids that carried the telomeric defective/repair-proficient *yku80-PF437,438AA* (*yku80-PF*) mutation (42) or the wild-type *YKU80* gene were introduced individually into a *yku80Δ* haploid strain. Cells were then examined for the suppression of *mec1Δ* lethality. We found that *yku80Δ* cells expressing the *yku80-PF* mutant protein, which are affected in telomeric silencing (42) and telomere size (Fig. 3A), were not able to suppress *mec1Δ* lethality. However, cells carrying the empty vector allowed the suppression of *mec1Δ* lethality (not shown). The inability of the *yku80-PF* mutant to suppress *mec1Δ* lethality in *yku80Δ* cells suggested that desilencing of the telomere and its size control is not sufficient to suppress *mec1Δ* lethality. Consistent with this interpretation, none of the viable segregants from 100 tetrads derived from sporulation of the diploid strain *mec1Δ/MEC1 sir3Δ/sir3Δ* was *mec1Δ sir3Δ*. Moreover, *yku70Δ mec1Δ sir3Δ* cells are viable (data not shown). We concluded that the suppression of *mec1Δ* lethality in a *ykuΔ* strain is not due to the loss of telomeric position effect.

Given that yKu70/yKu80 and Lif1 proteins are involved in a common NHEJ pathway, it was conceivable that *lif1Δ* would be able to suppress *mec1Δ* lethality. To test this possibility, we attempted to generate *mec1Δ* in *lif1Δ* spores, starting from *mec1Δ/MEC1 lif1Δ/lif1Δ*. As shown in Fig. 3B, we were able to obtain *mec1Δ lif1Δ* viable spores. In agreement with this result, we were also able to obtain *mec1Δ* transformants in *lif1Δ* haploid cells, and *lif1Δ mec1Δ* haploid cells are able to lose a *pMEC1* expression plasmid (not shown). These results indicate

that NHEJ defects in general rescue the lethal phenotype of *Mec1*-deficient yeast. We concluded that the suppression of *mec1Δ* lethality in *yku70Δ* and *yku80Δ* cells is probably associated with the loss of repair function.

TEL1 mRNA expression is slightly affected by YKU80 deletion. Because *TEL1* overexpression can suppress both cell lethality and hypersensitivity to DNA-damaging agents of the *mec1Δ* mutant, indicating that excess Tel1 can bypass the requirement for *Mec1* (10, 43), it remains possible that *YKU80* deletion increases *TEL1* expression. Total RNA were prepared from wild-type, *yku80Δ*, or *tel1Δ* cells, and *TEL1* mRNA levels were examined by RT-PCR (Fig. 4). Our data indicate that *TEL1* is not overexpressed in *yku80Δ* mutants. However, a reproducible 1.5 enrichment of *TEL1* mRNA was detected in *yku80Δ* cells.

Sml1 is depleted in G₁ and G₂ in *yku80Δ*. Sml1 is phosphorylated and then degraded during S phase and after DNA damage to provide sufficient dNTPs to complete DNA replication (57, 60). We used a chromosomally integrated YFP-Sml1 fusion protein (kindly provided by R. Rothstein; unpublished results) to monitor the amount of Sml1 in *yku80Δ* mutants (Fig. 5). Cells were arrested either in G₁, S, or G₂. By analyzing the population of cells in the cultures, we confirmed the efficiency of the G₁, S, and G₂ arrests (not shown). Both epifluorescence and Western blot analysis were performed to determine the amount of YFP-Sml1. In agreement with previous results (60), YFP-Sml1 levels are reduced in S phase, whereas it is detected in G₁ and G₂, both by epifluorescence (Fig. 5A)

FIG. 4. TEL1 mRNA level is slightly increased in *yku80Δ* cells. Total RNA was extracted from wild-type (wt), *yku80Δ*, and *tel1Δ* cells and processed for RT-PCR with the appropriate primers to measure *TEL1* and *ACT1* mRNA levels. The signals specific for the TEL1 transcripts were normalized to that of the actin signal. The TEL1 transcript level was arbitrarily set at 1 in the wild-type cells, and RNA levels in mutant cells were determined accordingly. Bar graphs represent an analysis of the results from three experiments.

and Western blotting (Fig. 5B). The amount of YFP-Sml1 was clearly reduced, about 60%, in the *yku80Δ* mutant in G₁ and in G₂. YFP-Sml1 remained undetectable in S phase. When we reintroduced a wild-type *YKU80* in the *yku80Δ* mutant, we restored the level of YFP-Sml1. Next, we tested whether the decrease of Sml1 levels in G₁, S, and G₂ phases in *yku80Δ* depended on Mec1. Since *mec1Δ* strains are unable to grow in the presence of Sml1, their ability was maintained by the presence of a 2 μm *RNR1* plasmid (15). Sml1 levels are highly reduced at G₁ and G₂ phases in *yku80Δ mec1Δ* strains compared with *mec1Δ* cells (Fig. 5C). We deduced from these results that the protein amount of Sml1 is influenced by yKu80 protein and that degradation of Sml1 can occur independently of Mec1. The lower level of Sml1 is a plausible explanation for the suppression of *mec1Δ* lethality by *yku80Δ*.

To correlate YFP-Sml1 levels with suppression of *mec1Δ* lethality, we analyzed YFP-Sml1 levels in G₁ and G₂ phases, in *mec3Δ*, *rad24Δ*, *rad9Δ*, *mre11Δ*, *tel1Δ*, *rad53-K227A*, and *dun1Δ* mutants in the presence or absence of *YKU80* (data not shown). Western blot analyses indicate that Sml1 depletion, at least in *yku80Δ* mutant cells, depends on Rad9, on the kinase activity of Rad53, and also on Dun1 but not on Mec3 and Rad24. Deleting *MRE11* and, to a lesser extent, *TEL1* produces by itself a depletion of the Sml1 protein amount, a situation that occurs in the *yku80* mutant; however, these mutations are likely to cause Sml1 degradation via the canonical Mec1-dependent pathway. On the other hand, we found Sml1 depletion in *lif1Δ* cells and a wild-type level of Sml1 in the telomeric defective/repair-proficient *yku80-PF437,438AA* mutant which does not suppress *mec1Δ* lethality (not shown). To confirm the importance of Sml1 depletion in the suppression of *mec1Δ* lethality by *YKU80* deletion, we deleted *YKU80* and *MEC1* genes in a *GAL-SML1* haploid strain (57). We found that *GAL-SML1 yku80Δ mec1Δ* strains grow normally in non-inducible medium but not after galactose induction (not shown). Finally, we believed that it was important to check the viability of *mec1Δ yku70Δ* combined with *rad9Δ*, *tel1Δ*, or *mre11Δ* deletion, in the absence of *SML1*. We were able to obtain *sml1Δ rad9Δ mec1Δ yku70Δ* viable spores that grew as

well as *rad9Δ yku70Δ* spores (Fig. 6A). Genetic analysis also showed that a *sml1Δ* mutation rescues the lethality of *mre11Δ mec1Δ yku70Δ* and *tel1Δ mec1Δ yku70Δ* cells (Fig. 6B and C). However, these mutants both present a significant growth defect. On this base, we conclude that Rad9, Tel1, and Mre11 are required for Sml1 depletion and, consequently, for suppression of *mec1Δ* lethality in *yku70Δ* and *yku80Δ* cells. This suggests that *YKU70* and *YKU80* deletions contribute to Sml1 degradation via both Tel1/MRX- and Mec1-dependent pathways.

Spontaneous Mre11 foci are observed in *yku80Δ* cells. In response to DSBs, a number of DNA checkpoint and repair proteins in *S. cerevisiae* relocalize from a diffuse nuclear distribution to distinct subnuclear foci acting as centers of recombinational DNA repair. To investigate the presence of DNA damage in *yku80Δ* cells, we used a chromosomally integrated Mre11-YFP fusion protein (27). The Mre11-Rad50-Xrs2 complex proteins are the first proteins detected at DSBs. As reported previously (27), we found that spontaneous Mre11 foci form in a low percentage of wild-type cells (4.5%) even in the absence of exogenous DNA damage. In contrast, approximately 8-fold more Mre11 foci (35% of the cells) are detected in *yku80Δ* cells (Fig. 7). When a plasmid that encoded the telomeric defective/repair-proficient *yku80-PF437,438AA* (*yku80-PF*) mutant (42) was introduced into the *yku80Δ* haploid strain, we observed a significant reduction of Mre11 foci compared to *yku80Δ* cells (Fig. 7). These in vivo observations are consistent with the presence of DNA damage associated with the loss of repair function in *yku80Δ* cells, providing an explanation for the Sml1 depletion.

Finally, because DSB induction appears to be the key event in suppressing *mec1Δ* lethality in *yku80Δ* cells, we analyzed whether a *pMEC1* expression plasmid can be cured from *mec1Δ* cells (in the presence of *SML1*) in a low concentration of methyl methanesulfonate (MMS) (0.01%, 0.005%, 0.001%, and 0.0005%). Our experiences indicate that MMS did not allow *mec1Δ* cells to lose the *pMEC1* expression plasmid (not shown), suggesting that DSBs generated by the absence of yKu80 are not similar and/or proceeded differently than those induced by MMS.

FIG. 5. Regulation of Sml1 protein amount in *yku80Δ* cells. Sml1 levels are reduced in *yku80Δ* cells. A chromosomally encoded YFP-Sml1 was introduced in the indicated strains. Exponentially growing cells of the indicated strains were blocked in G₁ with alpha factor (left), in S phase with hydroxyurea (center), or in G₂ with nocodazole (right), and the Sml1 level was analyzed either by epifluorescence microscopy (A) or by Western blot analysis (B) with anti-GFP antibodies (after confirmation of the efficiency of the G₁, S, and G₂ arrests by microscopic analyses). The arrowheads indicate the positions of YFP-Sml1. In each case, a band cross-reacting with anti-GFP antibodies is used as a loading control and the amount of YFP-Sml1 was normalized to that of the wild-type YFP-Sml1 level. Each value corresponds to the average of the results from three independent experiments. (C) Haploid cells of the indicated genotypes were transformed with a 2 μ m plasmid expressing *RNR1* (pRS426-*RNR1*), and the Sml1 protein amount was analyzed by Western blot analysis. Here again, in each case, a band cross-reacting with anti-GFP antibodies is used as a loading control and the amount of YFP-Sml1 was normalized to the wild-type YFP-Sml1 level. MW, size standard.

DISCUSSION

Suppression of *mec1Δ* lethality and implications for checkpoint proteins. *RAD53* and *MEC1* are essential *S. cerevisiae* genes required for the DNA damage response. Their lethality, but not their role in checkpoint responses, can be suppressed by increasing the intracellular pool of deoxynucleotides. This lethality can be explained both by observations that Mec1 and Rad53 are required to maintain sufficient dNTP levels (22) and by the findings that Mec1 and Rad53 stabilize stalled replication forks (whose occurrence would increase when dNTP levels are low) (29, 49). For example, certain alleles of *mec1* (*mec1-srf*) accumulate short DNA replication intermediates that are suppressed by the inactivation of Sml1, which raises dNTP levels (32, 59). Moreover, alteration/reduction of Mec1 function leads to fork stalling, followed by chromosome breakage (8).

We found that *mec1Δ* is viable in spores that were generated from diploids homozygous, but not heterozygous, for *yku70Δ*. One explanation for these results is that *yku70Δ mec1Δ* spores derived from heterozygous *yku70Δ/YKU70* diploids were still phenotypically *YKU70*⁺ and failed to reach the point where they are phenotypically *yku70Δ*. These observations are strongly supported by the finding that one can also obtain *mec1Δ* deletions by direct transformation of *yku70Δ* and *yku80Δ* cells. In contrast, deletion of *YKU70* or *YKU80* does not suppress *rad53Δ* lethality. This result is analogous to the previous observation that the *sml1Δ* mutation, which elevates dNTP levels, strongly suppresses *mec1Δ* lethality but only partially restores the growth of *rad53Δ* (58). It suggests that Rad53 has other, yet undefined functions that go beyond the regulation of dNTP levels (21, 45, 51).

FIG. 6. *SML1* deletion suppresses *mec1Δ* lethality in different genetic contexts. *SML1* deletion rescues *mec1Δ rad9Δ yku70Δ* (A), *mec1Δ mre11Δ yku70Δ* (B), and *mec1Δ tel1Δ yku70Δ* (C) lethality. (A, B, C) Tetrads from diploids homozygous for *yku70Δ* and heterozygous for *mec1Δ* (left) or *mec1Δ sml1Δ* (right) and either *rad9Δ* (A), *mre11Δ* (B), or *tel1Δ* (C) were dissected and analyzed for the presence of auxotrophic markers. Four tetrads are shown for each and are displayed vertically. Open squares indicate the *yku70Δ* single mutant. Diamonds indicate *sml1Δ yku70Δ* mutants. Circles indicate *mec1Δ yku70Δ* mutants. Hexagons indicate *sml1Δ mec1Δ yku70Δ* mutants. Squares indicate *rad9Δ yku70Δ* (A), *mre11Δ yku70Δ* (B), or *tel1Δ yku70Δ* (C) mutants. Crosses indicate *rad9Δ sml1Δ yku70Δ* (A), *mre11Δ sml1Δ yku70Δ* (B), or *tel1Δ sml1Δ yku70Δ* (C) mutants. Triangles indicate *mec1Δ rad9Δ yku70Δ* (A), *mec1Δ mre11Δ yku70Δ* (B), or *mec1Δ tel1Δ yku70Δ* (C) mutants. Hearts indicate *mec1Δ rad9Δ sml1Δ yku70Δ* (A), *mec1Δ mre11Δ sml1Δ yku70Δ* (B), or *mec1Δ tel1Δ sml1Δ yku70Δ* (C) mutants.

We have found that *yku70* and *yku80* mutant suppression of *mec1Δ* lethality is dependent on the genes *RAD9*, *TEL1*, *MRE11*, *RAD53*, and *DUN1*. These genes can be imagined to be part of the Tel1-MRX signal transduction pathway (14, 51, 52) that has been characterized as responding to nonresected DSB ends.

The discovery that elevation of dNTP levels by overexpressing *RNR* or *TEL1* genes would suppress *mec1Δ* made it clear that Mec1 is not truly essential (15, 22, 59). We established here that *yku70Δ* and *yku80Δ* cells only slightly elevate *RNR3* (not shown) and *TEL1* gene expression. It seems that *ykuΔ* can alleviate dNTP limitations by nontranscriptional means.

Relationship between Sml1 levels and suppression of *mec1Δ* lethality in *yku80Δ* cells. *SML1* deletion suppresses *mec1Δ* and *rad53Δ* lethality. Sml1 is normally removed during S phase and after DNA damage to provide sufficient dNTPs (57, 59). Deletion of *yku80Δ* results in a decrease in Sml1 levels in both G₁-

and G₂/M-arrested cells. The finding that the level of Sml1 protein is lower in a *yku80Δ* mutant than in wild-type cells can explain the suppression of *mec1Δ* lethality and suggests that unrepaired DNA damage or stress signals remain in *yku80Δ* and *yku80Δ mec1Δ* cells. The analysis of Mre11 foci in *yku80Δ* cells demonstrates the presence of DSBs. We found that Sml1 depletion in the *yku80Δ* mutants is dependent on Rad9, Rad53, and Dun1, but not on other checkpoint proteins, confirming the implication of these proteins in the suppression of *mec1Δ* lethality.

Based on recent observations from Lisby et al. (27) which indicate that Mre11 and Tel1 are the first proteins detected at DSBs, we propose a model to explain the functions of checkpoint proteins in responding to *ykuΔ*-induced DNA damage. According to this model, Mre11 is phosphorylated in a Tel1-dependent manner, leading to Sml1 phosphorylation and deg-

FIG. 7. Mre11 foci are detected in *yku80Δ* cells. Mre11 foci were analyzed in asynchronously growing cells in the absence (–) or presence (+) of MMS (0.05%). Cells were analyzed by YFP fluorescence and phase microscopy. The numbers indicate the percentages of cells that contained Mre11 foci. At least 500 cells were analyzed for each strain. Upper panels, YFP fluorescence; lower panels, phase contrast. wt, wild type.

radiation by the subsequent activation of the Rad9, Rad53, and Dun1 pathway. We suggest that Mec1 itself might also participate in Sml1 degradation in *yku80Δ* cells because a slight but reproducible Sml1 depletion is observed in *yku80Δ MEC1* cells compared to *yku80Δ mec1Δ* cells. In cells lacking both yKu and Mec1, the Rad9-, Rad53-, and Dun1-dependent checkpoint pathway is activated by Tel1 and the MRX complex independently of Rad24, Ddc1, Mec3, and Rad17, leading to Sml1 depletion. Thus, in the *yku80Δ* mutant, the Tel1/MRX and Mec1 pathways are responsible for the regulation of the Sml1 level. Consequently, in *yku80Δ* cells, the absence of Mec1 coupled with the absence of a member of the parallel pathway (Tel1/MRX) or of any one of the downstream checkpoints (Rad9, Rad53, or Dun1) is lethal for the cell, as it was in *mec1Δ YKU* cells. Because Rad53 phosphorylation remains undetectable or very weak in *yku70Δ* and *yku70Δ mec1Δ* mutants, we suggest that Rad53 is activated in a different way, as was previously observed by Clerici et al. (10).

Implication of the NHEJ repair pathway in the suppression of *mec1Δ* lethality in *yku70Δ* cells. One of the main problems that needs to be addressed is what type of DNA damage could be responsible for the depletion of Sml1 in *ykuΔ* and *ykuΔ mec1Δ* strains? It is well known that cells devoid of *YKU70* or *YKU80* exhibit a telomere length decline and present an excess of single-stranded G-rich DNA at their telomere. Such single-stranded overhangs are an important determinant for recognition as DNA damage (18, 19). The role of checkpoint genes in responding to telomeric defects in *yku70Δ* and *yku80Δ* cells have been tested at 37°C. Previous studies have demonstrated that the cell cycle arrest exhibited by *yku70Δ* cells at 37°C is dependent on the DNA damage checkpoint genes *MEC1*, *CHK1*, and *RAD9* and on the spindle checkpoint gene *MAD2* (30). Other studies have shown that, in *yku80Δ* cells, *RAD24*, *RAD9*, and *RAD53* are associated with the checkpoint response (48). Our work suggests that, at 30°C, in *yku70Δ* and *yku80Δ* cells, the telomeric defects are not the major event responsible for the suppression of *mec1Δ* lethality. Consistent with such a suggestion, we show (i) that the suppression of *mec1Δ* lethality displayed by *yku70Δ* cells still occurred in an *exo1Δ* background that partially rescued the telomere defects of *yku70Δ* cells (5), (ii) that the lethality of *yku80Δ tel1Δ mec1Δ*

and *yku80Δ mre11Δ mec1Δ* is not rescued by telomere lengthening induced by the deletion of the gene encoding the Rif2 protein, and (iii) that telomeric defective/repair-proficient *yku80* mutants lost the capacity to suppress *mec1Δ* lethality. These results point strongly to the involvement of an NHEJ deficiency in the suppression of *mec1Δ* lethality. This is consistent with the observation that *lif1Δ* mutants, which are defective in NHEJ but are not affected at their telomeres, allow the suppression of *mec1Δ* lethality. For all these reasons, we favor the hypothesis that the absence of viability observed for *yku70Δ tel1Δ mec1Δ*, *yku80Δ tel1Δ mec1Δ*, *yku70Δ mre11Δ mec1Δ*, and *yku80Δ mre11Δ mec1Δ* cells is due to the deficiency of the Tel1/MRX checkpoint function of Tel1 and/or Mre11 rather than to the accumulation of telomeric defects. This is confirmed by the observation that *sml1Δ* partially rescues the lethality of *yku80Δ tel1Δ mec1Δ* and *yku80Δ mre11Δ mec1Δ* mutants despite their telomeric defects.

Mec1 is required for processing of potentially lethal lesions arising spontaneously during normal cellular life. The inability of *mec1Δ* cells to up-regulate dNTP synthesis is proposed to contribute to the *mec1Δ* lethality by inducing “replication stress,” that is, stalled forks that undergo irreversible collapse and/or are processed by recombination proteins into DSBs. In this study, we demonstrate that spontaneous DSBs arising in *yku80Δ* cells and, more generally, in NHEJ-deficient cells, activate the MRX-Tel1 pathway, leading to Sml1 depletion and subsequent elevation of the dNTP pool. We do not think that yKu proteins actively and directly regulate the Sml1 level in wild-type cells. The NHEJ-dependent depletion of Sml1 is a novel discovery. Since we also observed an Sml1 depletion in *lif1Δ* cells, we speculate that the failure of the NHEJ pathway is crucial for the depletion of Sml1, explaining why NHEJ mutants allow the suppression of *mec1Δ* lethality. This study unmasks for the first time a connection between the NHEJ pathway and the checkpoint response. The pathways regulating the checkpoint are conserved in organisms from yeasts to humans. The mechanisms that control the DNA damage checkpoints involve the activation of ATM (Tel1) and ATR (Mec1) kinases in mammalian cells. It was reported that *Ku*^{–/–} cells have much stronger checkpoint responses than *Ku*^{+/+} cells (54, 55), suggesting that Ku proteins affect the checkpoint

response in mammalian cells (54, 55). In agreement with this work, we also observed that the deletion of *YKU* increases the viability of some checkpoint mutants (Y. Corda and V. Géli, unpublished data). Because *sml1Δ* cells exhibit an increased viability after DNA damage, these observations may suggest that the *ykuΔ*-dependent increase of viability observed for checkpoint mutants after DNA damage is the consequence of the Sml1 depletion observed in *ykuΔ* cells. Interestingly, although no homology of Sml1 has yet been reported in mammalian cells, it has been shown that yeast Sml1 binds to the large subunit of mouse and human RNR and that the same Sml1 residues essential for the yeast RNR interaction and inhibition are also required for binding the human protein (58). These results suggest a conserved mechanism between yeasts and humans. If this is true, based on our data for yeast cells, interfering with *KU* gene expression could affect dNTP levels in human cells. Several lines of evidence suggest that the alteration of the dNTP pool is associated with spontaneous mutations and chromosome instability. Moreover, increased dNTP levels are often associated with resistance of tumor cells to drugs, and there is some evidence that down-regulation of the Ku system promotes progression of cancer from a mildly to highly aggressive malignant clinical behavior (24). In light of this, Ku and the various components of the NHEJ system may be appealing targets for cancer therapy.

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