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Elevated dNTP levels suppress hyper-recombination in *Saccharomyces cerevisiae* S-phase checkpoint mutants

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ABSTRACT

MEC1*, the essential yeast homolog of the human ATR/ATM genes, controls the S-phase checkpoint and prevents replication fork collapse at slow zones of DNA replication. The viability of hypomorphic *mec1-21* is reduced in the *rad52* mutant, defective in homologous recombination, suggesting that replication generates recombinogenic lesions. We previously observed a 6-, 10- and 30-fold higher rate of spontaneous sister chromatid exchange (SCE), heteroallelic recombination and translocations, respectively, in *mec1-21* mutants compared to wild-type. Here we report that the hyper-recombination phenotype correlates with lower deoxyribonucleoside triphosphate (dNTP) levels, compared to wild-type. By introducing a *dun1* mutation, thus eliminating inducible expression of ribonucleotide reductase in *mec1-21*, rates of spontaneous SCE increased 15-fold above wild-type. All the hyper-recombination phenotypes were reduced by *SML1* deletions, which increase dNTP levels. Measurements of dNTP pools indicated that, compared to wild-type, there was a significant decrease in dNTP levels in *mec1-21*, *dun1* and *mec1-21 dun1*, while the dNTP levels of *mec1-21 sml1, mec1-21 dun1 sml1* and *sml1* mutants were ~2-fold higher. Interestingly, higher dNTP levels in *mec1-21 dun1 sml1* correlate with ~2-fold higher rate of spontaneous mutagenesis, compared to *mec1-21 dun1*. We suggest that higher dNTP levels in specific checkpoint mutants suppress the formation of recombinogenic lesions.

INTRODUCTION

MEC1 is the essential yeast homolog (1) of the human ATR/ATM genes (for review, 2) and shares some functions with both ATM (mutated in ataxia telangiectasia) and ATR (ATM and *Rad3* related). *MEC1* prevents replication fork collapse (3), a function shared with ATR, but not ATM, in mammalian cells (for review, 2). Similar to ATR, *MEC1* is required for viability; *mec1* lethality can be suppressed by mutations in *SML1, CRT1* or *DIF1,* or by over-expression of *RNR1* or *RNR3*, all of which increase the activity of ribonucleotide reductase (for review, 4). *SML1* mutations elevate deoxyribonucleoside triphosphate (dNTP) levels 2-fold compared to wild-type, implying that increasing dNTP levels is sufficient to suppress *mec1* lethality (5).

MEC1's function in controlling dNTP levels is mediated by *RAD53*, which, upon activation by DNA damage and in S phase, triggers the *DUN1*-mediated induction of ribonucleotide reductase (6). While both *MEC1* and *RAD53* are essential, *DUN1* is non-essential (7). Basal dNTP levels are lower in specific *mec1* and *rad53* hypomorphic mutants, compared to wild-type (8). Observations that *dun1* exhibits a longer S phase than wild-type and is defective in the degradation of *Sml1* suggest that basal dNTP levels are also lower in *dun1* (9). Thus, yeast can maintain viability at dNTP levels that are both higher and lower than in wild-type.

Lower dNTP levels may correlate with a hyper-recombination phenotype and decreased spontaneous point mutation rates while higher dNTP levels may correlate with an increase in spontaneous or DNA damage-associated mutagenesis, compared to wild-type. For example, *dun1* mutants exhibit higher levels of spontaneous heteroallelic recombination (10) and lower levels of spontaneous mutagenesis that results in

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canavanine resistance (11). Over-expression of *RNR1*, which results in higher dNTP levels, leads to higher levels of 4-nitroquinoline-*N*-oxide (4NQO)-associated mutagenesis (12). The *rnr1-D57N* mutant, which has 2- to 3-fold higher dNTP levels, exhibits a similar increase in spontaneous mutation rates (13). One possible explanation is that replicative DNA polymerases may require higher levels of dNTPs for insertion of particular bases opposite damaged sites (12).

Since *mec1* null mutants require *sml1* for viability, *mec1* hypomorphic mutants that retain essential function are useful in determining which genetic instability phenotypes correlate to lower dNTP levels. Such mutants include *mec1-21*, which is defective in the S-phase checkpoint (6,14) and results from a G to A substitution (G882S) at position 2644 outside of the kinase domain (15). *RAD52* is required for double-strand break repair (for review, 16), and compared to *mec1-21,* viability in the *mec1-21 rad52* mutant is significantly reduced, suggesting that double-strand breaks spontaneously arise in *mec1-21* (17). Our previous studies have indicated that compared to wild-type, *mec1-21* exhibits significantly higher rates of spontaneous homologous recombination between sister chromatids, homologs and non-homologous chromosomes (18). The hyper-recombination phenotype is dependent on *RAD52* and G2 checkpoint genes (18), suggesting that spontaneous DNA breaks generated by DNA replication are repaired by homologous recombination.

We demonstrate that, compared to wild-type, *mec1-21* hyper-recombination phenotype is *SML1*-dependent and correlates with lower basal levels of dNTPs. Deleting *SML1* in *mec1-21 dun1* mutant also correlates to a modestly higher rate of spontaneous mutagenesis. These studies suggest that higher dNTP levels may suppress hyper-recombination in hypomorphic *mec1* mutants. Thus, an adequate supply of dNTPs is an important *MEC1* function in maintaining genomic stability.

MATERIALS AND METHODS

Media and yeast strains

Standard media for the culture of yeast, SC (synthetic complete, dextrose), SC-HIS (SC lacking histidine), SC-TRP (SC lacking tryptophan), SC-URA (SC lacking uracil), YP (yeast extract, peptone) and YPD (YP, dextrose), are described by Burke *et al.* (19). YP(A)D contains YPD with 80 mg/l adenine. CAN plates contain SC medium and 60 mg/l of canavanine.

Relevant yeast strains are listed in Table 1. The *mec1-21* strain YA197 (Y620), and the YA184 and YA185 strains used to PCR amplify *sml1::URA3* and *mec1-Δ::TRP1*, respectively, are derived from W303; all other strains are of the S288c background. Strains used to measure SCE contain two overlapping *his3* fragments, positioned in tandem at *trp1*, and were derived from YB163 (20,21). Diploid strains were used to measure translocations that were derived from a cross of one haploid (YB109) that contains the *his3* fragments on one copy of chromosomes II and IV, and another which did not contain the *his3* fragments (YA102) (22). To measure heteroallelic recombination, we replaced the *ade2-101* alleles in YB109 and YA102 with *ade2-n* (YB318) and *ade2-a* (YB315), respectively, by two-step gene replacement using the plasmid pKH9 (23). Heteroallelic recombination was measured by selecting for Ade^+ recombinants.

We used the *mec1-21* missense mutant to measure spontaneous recombination. The original *MAT***a** *mec1-21* strain (Y620) (6) was backcrossed 10 times with strains in the S288c background [YB163, YA166 (21) and YB315] to generate meiotic segregants that either do (YB312) or do not (YB316, YB314) contain *his3* recombinational substrates to measure SCE. We introduced the *sml1::KanMX* and *sml1::URA3* allele in yeast strains by PCR-mediated gene replacement; the primers used for amplifying *sml1::KanMX* or *sml1::URA3* knockout fragments were the same: 5′-CAT ATCGTTACTGTTTTGGAACATCGC-3′ and 5′-TAAA GGGAAAGGAAAATGCACG-3′. The construction of *mec-Δ1 sml1::KanMX* (YB327) was described earlier (17).

To measure translocations and heteroallelic recombination in *mec1* strains, mutations were introduced into two haploids by either genetic crosses or by one-step gene replacement (24); one haploid contains the *his3* recombination substrates and *ade2-n* (YB318), while another (YB315) contained *ade2-a* but no recombination substrates. YB318 was crossed with YB313 to generate the *MAT***a**-*inc ade2-n mec1-21* meiotic segregant (YB319) that contains the *GAL1::his3-Δ5′* and *trp1::his3-Δ3′*. YB325 is a homozygous *mec1-21* diploid that was then used to measure translocations and heteroallelic recombination.

Additional checkpoint mutants were made by either one-step gene disruption (24) or by genetic crosses and screening the phenotype of the appropriate meiotic segregant. The primer pairs used to amplify *dun1::KanMX* fragments were, 5′AGAAGCCCCTGAA TACCATAAATA3′ and 5′CGATGTCAGAGATTTAG AGGAAAAA3′, respectively. We made the *mec1-21 dun1::KanMX sml1::URA3* haploid (YB380) by introducing *sml1::URA3* into *mec1-21 dun1::KanMX* (YB369) by one-step gene disruption (24). All gene disruptions were confirmed by PCR.

Determining rates of spontaneous recombination and mutations

The rates (events per cell division) of spontaneous SCE, heteroallelic recombination, translocations and mutations in *CAN1* were determined by the method of the median (25), as previously performed (21). Rates of spontaneous heteroallelic recombination were determined on cells inoculated on YP(A)D, on which there is no growth advantage for Ade^+ recombinants. Rates of mutations in *CAN1* were determined by selecting for resistance to canavanine. We determined the statistical significance by the Mann–Whitney U-test (26).

Determining dNTP levels

The methods of measuring dNTPs in yeast are as described in Chabes *et al.*, (13). At a density between 0.5×10^7 and 1.5×10^7 cells/ml, $\sim 1 \times 10^8$ cells were

Table 1. Yeast strains

Strain	Genotype	Source (synonym)
YA102	*MAT***a**-*inc ura3-52 his3-Δ200 lys2-801 trp1-Δ1 ade2-101*	This lab
YA165	*MATα ura3-52 his3-Δ200 trp1-Δ1 leu2-Δ1*	F. Winston (FY250)
YA166	*MAT***a** *ura3-52 his3-Δ200 trp1-Δ1 leu2-Δ1*	F. Winston (FY251)
YA184	*MAT***a** *trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100 sml1::URA3 rad53::HIS3 RAD5*	R. Rothstein (W2105-17B)
YA185	*MAT***a** *trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100 mec1-Δ::TRP1 sml1::HIS3 RAD5*	R. Rothstein (U963-61A)
YA195	*MAT***a** *his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0 sml1::KanMX*	ResGene (512)
YA196	*MAT***a**/*MATα his3-Δ1/− leu2Δ0/− ura3Δ0/− met15Δ0/+ lys2Δ0/− mec1::KanMX/+*	ResGene (23275)
YA197	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1 can1-100 mec1-21*	S. Elledge (Y620)
YA224	*MAT***a** *his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0 dun1::KanMX*	ResGen (3798)
YB313	*MAT***a**-*inc ura3-52 his3-Δ200 lys2-801 trp1-Δ1 gal3⁻ mec1-21*	Derived from cross of YB311 × YA165
YB314	*MATα ura3-52 his3-Δ200 lys2-801 trp1-Δ1 gal3⁻ mec1-21*	Derived from cross of YB312 × YA166
YB315	*MAT***a** *ura3-52 his3-Δ200 ade2-a lys2-801 trp1-Δ1 gal3⁻*	Derived from YA102
YB316	*MAT***a** *ura3-52 his3-Δ200 ade2-a lys2-801 trp1-Δ1 gal3⁻ mec1-21*	Derived from cross of YB315 × YB314
YB317	*MAT***a** *ura3-52 his3-Δ200 ade2-a lys2-801 trp1-Δ1 gal3⁻sml1::KanMX*	*sml1::KanMX* disruption in YB315
Strains to monitor translocations and heteroallelic events[a]		
YB109	*MATα ura3-52 his3-Δ200 ade2-101 trp1-Δ1 gal3⁻ leu23, 112 GAL1::his3-Δ5′ trp1::his3-Δ3′::HOcs lys2⁻ (leaky)*	This laboratory
YB318	*MATα ura3-52 his3-Δ200 ade2-n trp1-Δ1 gal3⁻ leu23, 112 GAL1::his3-Δ5′ trp1::his3-Δ3′::HOcs lys2⁻ (leaky)*	Derivative of YB109
YB319	*MATα ura3-52 his3-Δ200 ade2-a trp1-Δ1 gal3⁻ leu23, 112 GAL1::his3-Δ5′ trp1::his3-Δ3′::HOcs lys2⁻ (leaky) mec1-21*	Derived from cross of YB313 × YB318
YB320	*MATα ura3-52 his3-Δ200 ade2-n trp1-Δ1 gal3⁻ leu23, 112 GAL1::his3-Δ5′ trp1::his3-Δ3′::HOcs lys2⁻ (leaky) sml1::KanMX*	*sml1::KanMX* disruption in YB318
YB323	YB317 × YB320	This laboratory
YB325	YB316 × YB319	This laboratory
YB348	YB315 × YB318	This laboratory
YB373	*MAT***a** *ura3-52 his3-Δ200 ade2-n trp1-Δ1 gal3⁻ leu23, 112 mec1-21 sml1::KanMX*	*sml1::KanMX* disruption in YB316
YB374	*MATα ura3-52 his3-Δ200 ade2-n trp1-Δ1 gal3⁻ leu23, 112 GAL1::his3-Δ5′ trp1::his3-Δ3′::HOcs lys2⁻ (leaky) mec1-21 sml1::KanMX*	*sml1::KanMX disruption* in YB319
YB375	YB373 × YB374	This laboratory
Strains to monitor SCE[a]		
YB163	*MAT***a**-*inc ura3-52 his3-Δ200 ade2-101 lys-801 trp1-Δ1 gal3⁻ trp1::[his3-Δ3′::HOcs, his3-Δ5′]*	This laboratory
YB204	*MATα leu2-Δ1*	This laboratory
YB311	*MAT***a**-*inc mec1-21*	Tenth backcross of Y620 with YB163
YB312	*MATα mec1-21*	Tenth back cross of Y620 with YB163
YB326	*MAT***a**-*inc sml1::KanMX*	*sml1::KanMX* disruption in YB163
YB327	*MAT***a**-*inc sml1::KanMX mec1-Δ::TRP1*	*mec1::TRP1* disruption in YB326
YB329	*MAT***a**-*inc sml1::KanMX mec1-21*	From cross of YB312 with YB326
YB336	*MATα sml1::KanMX mec1-21*	From cross of YB312 with YB326
YB347	*MAT***a**-*inc mec1-21 rad52:KanMX*	From cross of YB329 with YB328
YB330	*MAT***a**-*inc sml1::KanMX mec1-21 rad52:KanMX*	From cross of YB329 with YB328
YB368	*MAT***a**-*inc mec1-21 leu2-Δ1*	From cross of YB311 × YA165
YB369	*MAT***a**-*inc mec1-21 dun1::KanMX*	*dun1::KanMX* disruption in YB311
YB370	*MAT***a**-*inc dun1::KanMX*	*dun1::KanMX* disruption in YB163
YB379	*MATα mec1-21 sml1::URA3*	*sml1::URA3* disruption in YB312
YB380	*MATα mec1-21 dun1::KanMX sml1::URA3*	Derived from cross of YB379 × YB369

[a]All strains listed below have the same genotype as YB163 unless indicated. Mating type is added for clarity. YB333 and YB334 may contain either *ura3-52* or *ura3Δ0* and *lys2Δ0* or *lys2-801*.

harvested by filtration through 25 mm White AAWP nitrocellulose filters (0.8 μm, Millipore AB, Solna, Sweden). The filters were immersed in 500 μl of ice-cold extraction solution (12% w/v trichloroacetic acid, 15 mM $MgCl_2$) in Eppendorf tubes. The following steps were carried out at 4°C. The tubes were vortexed for 30 s, incubated for 15 min and vortexed again for 30 s. The filters were removed and the supernatants were collected after centrifugation at 20 000*g* for 1 min and added to 800 μl of ice-cold Freon-trioctylamine mixture [10 ml of Freon (1,1,2-trichlorotrifluoroethane, Aldrich, Sigma-Aldrich Sweden AB, Stockholm, Sweden, 99%) and 2.8 ml of trioctylamine (Fluka, Sigma-Aldrich Sweden AB, Stockholm, Sweden, >99%)]. The samples were vortexed and centrifuged for 1 min at 20 000*g*. The aqueous phase was collected and added to 800 μl of ice-cold Freon-trioctylamine mixture. The mixture was vortexed and centrifuged as described earlier. A 475 μl aliquot of the aqueous phase was pH-adjusted with 25 μl of 1M NH_4HCO_3, pH 8.9, the deoxyribonucleotides were separated from the ribonucleotides by boronate affinity chromatography (Affi-Gel 601, Bio-Rad) and quantified by HPLC. Another 47.5 μl aliquot of the aqueous phase was mixed with 152.5 μl of water and used for the HPLC quantification of NTP pools. Separation of nucleotides was done on a Partisphere 5 SAX column (PolyLC Inc., Columbia, MD, USA) using a UV-2075 Plus detector (Jasco, Mölndal, Sweden). Nucleotides were isocratically

eluted with 2.5% acetonitrile, 0.36 M ammonium phosphate, pH 3.4 buffer.

Western blots

To detect Rad53 phosphorylation after HU and MMS exposure, protein samples were obtained from log phase cells ($A_{600} = 0.5$–1) exposed to 200 mM HU or 0.01% MMS for 2 h. Proteins (10 μg) were separated on 10% acrylamide/0.266% *bis*-acrylamide gels, transferred to nitrocellulose membrane and exposed to a Rad53 antibody (yC-19, Santa Cruz). The secondary antibody was peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory, Inc.). The signals were developed using ECL western lightning kit (NEL102, PerkinElmer Life Science, Inc.).

RESULTS

Viability of particular *mec1* hypomorphs is reduced in the *rad52* mutant, deficient in homologous recombination (27,17). While ~60% of either *mec1-21* or *rad52* log phase cells form colonies, only ~20% of *mec1-21 rad52* cells form colonies (17). Deleting *SML1* in *mec1-21 rad52* increased the plating efficiency to ~50% and increased the growth rate to wild-type levels (Supplementary Table S1). These results suggest that deleting *SML1* in *mec1-21 rad52* decreases the number of lethal DNA double-strand breaks. We therefore determined whether hyper-recombination phenotypes of *mec1-21* would be suppressed by *SML1* deletions and correlate with dNTP levels. Unequal SCE was measured by selecting for His^+ recombinants in haploid strains containing two truncated *his3* gene fragments (20,21, Figure 1). Diploid strains were used to measure heteroallelic recombination between *ade2-a* and *ade2-n* (28) and ectopic recombination between *GAL1::his3-Δ5′* and *trp1::his3-Δ3′* (22, Figure 1).

Higher rates of spontaneous, homologous recombination in *mec1-21* are *SML1*-dependent

We reasoned that if lower dNTP levels correlate with higher recombination rates in *mec1* mutants, then *SML1* mutations would decrease homologous recombination in *mec1-21* mutants. We previously observed a 6-fold increase in the rate of spontaneous SCE in the *mec1-21* mutant (YB312) compared to wild-type (18). We observed no difference in the rates of spontaneous recombination between *sml1* (YB326) and wild-type (YB163); however the 6-fold increase in rates of SCE was reduced to wild-type levels in *mec1-21 sml1* (YB329, Table 2).

Rates of heteroallelic recombination and translocations in *mec1-21* were 10-fold higher and 23-fold higher than wild-type, respectively (Table 3). We determined whether *mec1-21* hyper-recombination between homologs and non-homologs also required *SML1*. Heteroallelic recombination between *ade2* alleles can be visualized using a colony pigment assay, where Ade^+ recombinants appear as white colony sectors (Figure 2). Heavily white sectored colonies indicated that more heteroallelic recombination occurred in *mec1-21* (YB325) than in wild-type (YB348), while the *mec1-21 sml1* mutant (YB375) exhibited less visible sectoring than *mec1-21* cells (Figure 2).

We then measured rates of translocations and heteroallelic recombination in cells grown on medium containing excess adenine (YPAD) so that there is no growth advantage for Ade^+ cells. While the rates of heteroallelic recombination and translocations in the *sml1* (YB323) and wild-type (YB348) were similar (Table 3), *mec1-21 sml1* diploid mutant (YB375) exhibited 8- and 4-fold lower rates of heteroallelic recombination and translocations, respectively, compared to *mec1-21*. However the rates of recombination in *mec1-21 sml1* were still between 2- and 5-fold higher than those observed in wild-type (Table 3). Thus, *SML1* deletion in *mec1-21* diploid partially suppresses spontaneous heteroallelic recombination and translocations.

Rate of spontaneous SCE in *mec1-21* are further increased by *dun1*

The *MEC1*-mediated pathway for regulating dNTP levels involves Rad53 activation, which in turn leads to Dun1 activation (7,9,29). By western blots, we observed that Rad53 is partially activated to P-Rad53 in the *mec1-21* hypomorph, compared to wild-type, after HU and MMS exposure (Supplementary Figure S1). We observed ~40% of the Rad53 activation (P-Rad53/Rad53) in *mec1-21*, compared to wild-type, while in *mec1-Δ*, the level of activation was <30% ($n = 2$). These data are consistent with observations that *mec1-21* exhibits partial checkpoint activation after exposure to agents that cause DNA replication stress (30).

Considering that *DUN1* is required for transcriptional induction of the *RNR* genes (7) and is suggested to maintain basal dNTP levels (9), we asked whether *mec1-21 dun1* mutants would exhibit higher levels of spontaneous recombination, compared to *mec1-21*. Although there is no difference between spontaneous rates of SCE in *dun1* (YB370) and wild-type, we observed a 15-fold increase in the rate of SCE in the *mec1-21 dun1* double mutant, compared to wild-type (Table 2). *SML1* mutations conferred significantly lower rates of SCE in *mec1-21 dun1* (YB369), but were still 3-fold higher than wild-type. These data indicate that *DUN1* suppresses recombination in *mec1-21*, but a *SML1* deletion only partially suppresses the hyper-recombination phenotype of the *mec1-21 dun1* double mutant.

dNTP are lower in *mec1-21, dun1* and *mec1-21 dun1* but are increased in *mec1-21 sml1* and *mec1-21 dun1 sml1* mutants compared to wild-type

We expected that the higher and lower rates of homologous recombination exhibited by S-phase checkpoint mutants would inversely correlate with dNTP levels. We measured dNTP levels in wild-type, *sml1*, *mec1-21*, *dun1*, *mec1-21 sml1*, *mec1-21 dun1* and *mec1-21 dun1 sml1* strains (Figure 3). In comparison to wild-type, the S288c *sml1* strains exhibited increased dNTP levels over 100%, while the S288c *dun1* strains exhibited ~50–70% reduction in dNTP levels. The *mec1-21* levels of dNTPs were ~50–70% of those observed in wild-type strains.

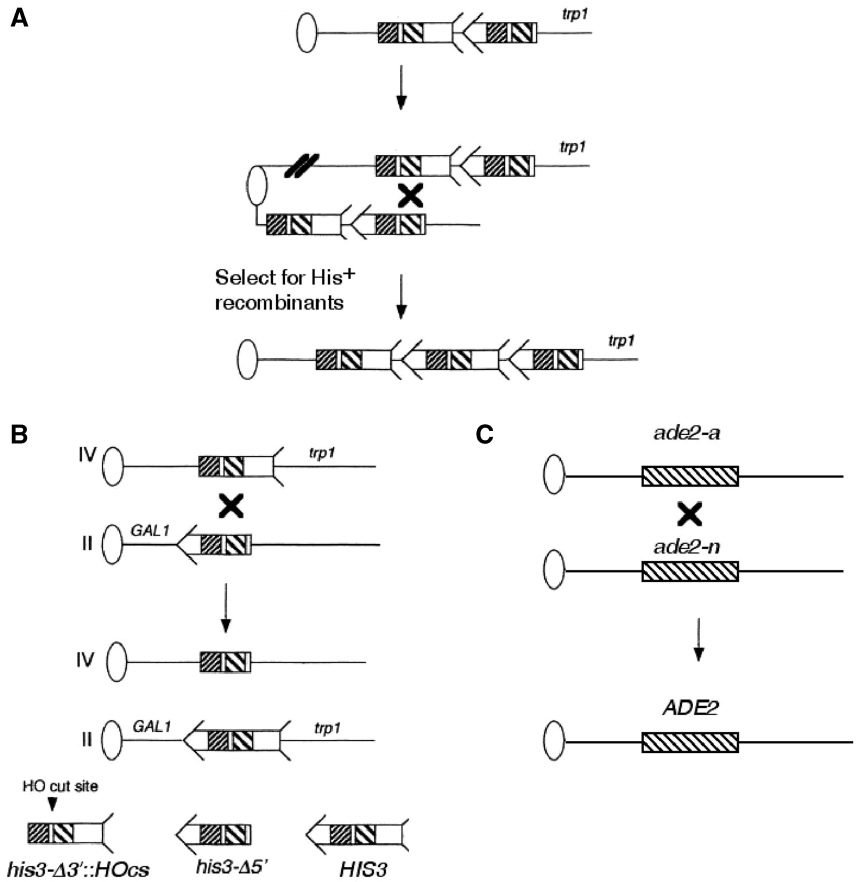

Figure 1. Unequal SCE, translocation and heteroallelic recombination assays used in this study. Ovals represent centromeres and lines represent chromosomes. For simplicity, the left arms of chromosomes are not included. An arrow and feathers together denote *HIS3*. As indicated in the bottom left of the figure, the 5′ deletion lacks the feather and the 3′ deletion lacks the arrow. The two regions of the sequence identity shared by the *his3* fragments are indicated by decorated boxes; closely-spaced diagonal-filled boxes indicate a region of 167 bp and the broadly-spaced diagonal line-filled boxes indicate a region of ~300bp. The 117-bp HO cut site (*HOcs*), as indicated by an arrowhead, is located between these sequences within the *his3-Δ3′::HOcs* fragment. (**A**) The *his3*-truncated fragments are integrated into the *trp1* locus to measure unequal SCE events. (**B**) Translocation events result from recombination between the same *his3* fragments located each on chromosomes II and IV. Positions of the *GAL1* and *trp1* are shown on chromosomes II, IV and the reciprocal translocation. (**C**) Heteroallelic recombination between *ade2-a* and *ade2-n* generates *ADE2*. *ADE2* and *ade2* alleles are represented as boxes; *ade2-a* and *ade2-n* are separated by ~1 kb.

Table 2. Rates of spontaneous SCE in *mec1* mutants

Strain	Genotype[a]	Rate ($\times 10^6$)[b]	Ratio[c]
YB163	*MEC1*	1.1 ± 0.1	1.0
YB326	*sml1*	1.1 ± 0.2	1.0
YB312	*mec1-21*	6.3 ± 0.9	5.7
YB329	*mec1-21 sml1*	1.1 ± 0.2	1.0
YB370	*dun1*	1.2 ± 0.3	1.1
YB369	*mec1-21 dun1*	17 ± 4.0	15.0
YB380	*mec1-21 dun1 sml1*	3.1 ± 0.7	2.8
YB327	*mec1-Δsml1*	1.2 ± 0.2	1.2

[a]All strains derived from S288c. For complete genotype (Table 1).
[b]Rate represents the number of events per cell division; $n \geq 3$.
[c]Ratio represents rate of SCE in mutant/rate of SCE in wild–type.

Interestingly, the dNTP levels of *mec1-21 dun1* strains were similar to *mec1-21*. These results are consistent with observations that *MEC1* is epistatic to *DUN1* in controlling dNTP levels (6,8). We do not know the lowest dNTP level that maintains viability in yeast; thus, it is possible that basal dNTP levels cannot be reduced further than those observed in *mec1-21 dun1*.

The basal dNTP levels were similar in *sml1*, *mec1-21 sml1* and *mecl-21 dun1 sml1*, and increased ~2-fold, relative to wild-type (Figure 3). The basal level of each dNTP was elevated between 2- and 4-fold in both the *mec1-21 sml1* and the *mec1-21 dun1 sml1* strains, relative to *mec1-21* and *mec1-21dun1*, which exhibit lower dNTP levels than wild-type (Figure 3). These data indicate that deleting *SML1* in both checkpoint mutants and wild-type leads to similar dNTP levels. These results are consistent with observations that *MEC1* and *DUN1* both function in a pathway to degrade Sml1 (8,9). Thus, higher dNTP levels correlate with suppression of homologous recombination in *mec1-21* mutants.

Spontaneous mutagenesis rates are lower in the *mec1-21 dun1* mutant but increased in *mec1-21 dun1 sml1* mutants

One hypothesis is that higher dNTP levels facilitate DNA replication at stalled replication forks, preventing

Table 3. Rates of spontaneous translocations and heteroallelic recombination events in *mec1* mutants

Strain	Genotype[a]	Translocation ($\times 10^8$)[b]	Ratio[c]	Heteroallelic ($\times 10^6$)[b]	Ratio[c]
YB348	*MEC1*	3.0 ± 0.8	1.0	0.9 ± 0.02	1.0
YB323	*sml1*	2.3 ± 0.3	0.8	0.7 ± 0.2	0.8
YB325	*mec1-21*	68.0 ± 16.0	23.0	9.1 ± 1.9	10.1
YB375	*mec1-21 sml1*	13.0 ± 1.0	4.3	2.2 ± 0.4	2.4

[a]For complete genotype (Table 1).
[b]Rate represents the number of events per cell division; $n \geq 3$.
[c]Ratio represents rate of recombination in mutant/rate of recombination in wild–type.

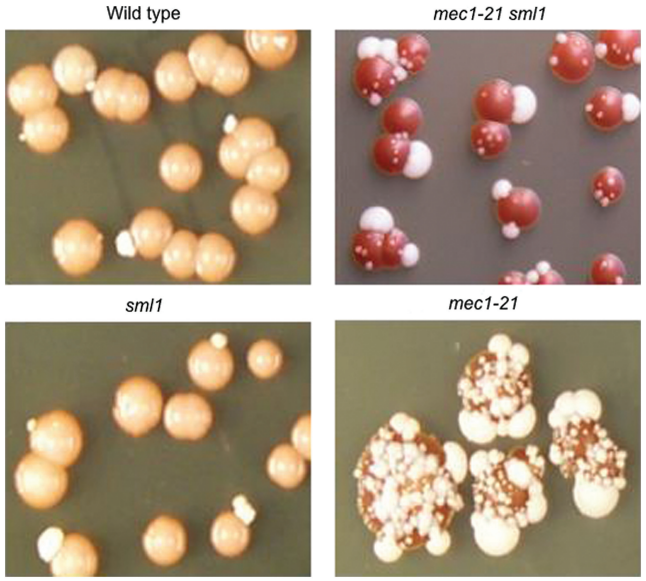

Figure 2. Heteroallelic recombination in *mec1-21* and *mec1-21 sml1* is exhibited by a colony sector assay in diploid strains. *ADE2* results from heteroallelic recombination between *ade2-a* and *ade2-n* generates; red sectors are Ade^- and white sectors are Ade^+(18). Colony phenotypes are shown for *MEC1* (wild-type, YB348), *sml1* (YB323), *mec1-21*(YB325) and *mec1-21 sml1*(YB375). Cells were plated on YPD medium and colonies were photographed after 10-day incubation.

replication fork regression or the formation of double-strand breaks. Higher dNTP levels can also lead to higher rates of 4NQO-associated mutagenesis (12). We therefore measured rates of spontaneous mutation at *CAN1* in wild-type, *mec1-21*, *sml1, mec1-21 sml1*, *mec1-21 dun1*, *dun1* and *mec1-21 dun1 sml1* strains (Table 4). The rate of spontaneous mutation in wild-type (YB163) was 4×10^{-7}, in agreement with Datta *et al.* (11). In comparison to wild-type, the mutation rate is the same in *sml1* and *mec1-21 sml1* but lower in *mec1-21 dun1* ($P < 0.05$). The rates of spontaneous mutation are increased ~2-fold in *mec1-21 dun1 sml1* ($P < 0.05$), in comparison *mec1-21 dun1*. Thus, higher dNTP levels correlate with the higher rate of spontaneous mutagenesis in *mec1-21 dun1 sml1* but not in *sml1* strains.

DISCUSSION

The ATR (ATM) yeast homolog *MEC1* has a pivotal role in stabilizing stalled DNA replication forks and is required to maintain vital levels of deoxyribonucleotides to ensure that the genome is faithfully replicated. The viability of *mec1* hypomorphic mutants, such as *mec1-21*, is significantly reduced in *rad52* mutants (17), suggesting that double-strand breaks are spontaneously generated in *mec1* mutants. In this study, we observed that deleting *SML1* suppressed the *mec1* hyper-recombination phenotypes while increasing the rate of spontaneous mutation in *mec1-21 dun1*. We compared measurements of dNTPs in *SML1* and *sml1* deletion strains that contained combinations of the *mec1-21* and *dun1* mutations, and derived the following conclusions: (i) lower dNTP levels in *mec1-21* correlate with an increase in recombination rates, while higher dNTP levels in both *mec1-21* and *mec1-21 dun1* correlate with a decrease in recombination rates, (ii) *DUN1* can suppress homologous recombination in *mec1-21* independent of its function in maintaining dNTP levels and (iii) higher dNTP levels increase spontaneous mutagenesis in *mec1 dun1* strains. We suggest that increasing dNTP levels reduces the formation of recombinogenic DNA lesions in *mec1* hypomorphic and *dun1* mutants. This is the first study to measure dNTP levels in *mec1-21* and *dun1* mutants and to demonstrate that *DUN1* is required for maintaining basal dNTP levels.

These observations may seem to contradict published reports that frequencies of gross chromosomal rearrangements (GCRs) are highest in *mec1-Δ*, which is deleted for *SML1* compared to *mec1-21* (31,32). However, *mec1-Δ* is deficient in both S-phase and G2 checkpoint functions, while the *mec1-21* hypomorph retains partial G2 checkpoint function and is less X-ray sensitive compared to the null mutant (17,18,33). We speculate that replication fork collapse is more severe in *mec1-Δ*, compared to *mec1-21*. Thus, the combination of replication fork collapse and the deficiency in recombinational repair of double-strand breaks in G2 likely results in the higher rate of GCRs in the *mec1-Δ* compared to *mec1-21*.

Lower dNTP levels contribute to the higher rates of spontaneous recombination

Both *mec1* and *dun1* mutants exhibit lower dNTP levels, compared to wild-type, but only *mec1-21* exhibits higher SCE recombination rates. We suggest that the higher recombination rates result from replication forks stalling due to inadequate dNTP levels. *MEC1* functions to prevent replication fork collapse, especially at slow zones of replication (3), and when ribonucleotide reductase is inhibited by HU (34). We suggest that the *MEC1*

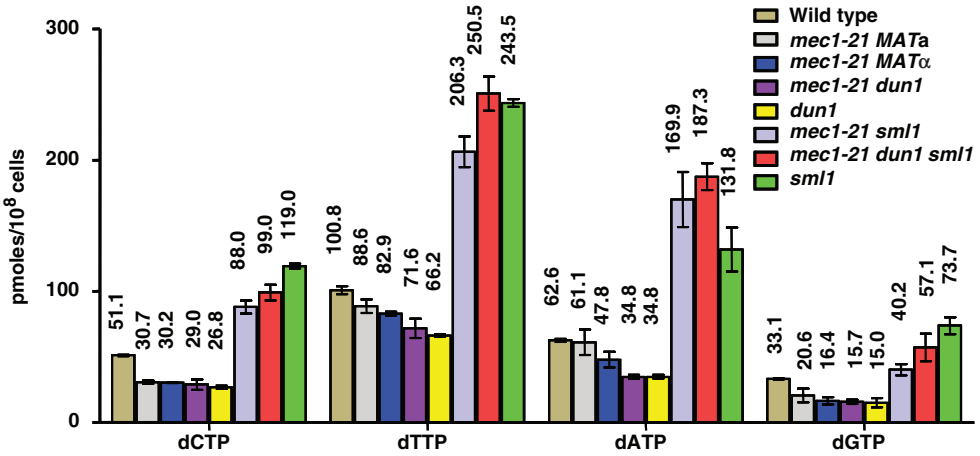

Figure 3. dNTP levels measured in wild-type (YB163), *dun1* (YB370), *sml1* (YB326), *mec1-21 dun1* (YB369), *mec1-21 dun1 sml1* (YB380) and *mec1-21* (YB312 and YB311) mutants. Two *mec1-21* strains were used; YB311 *MAT***a**-*inc* and YB312 *MATα* (Table 1).

Table 4. Rates of spontaneous canavanine resistance in *mec1* mutants

Strain	Genotype[a]	Rate ($\times 10^7$)[b]	Ratio[c]
YB163	*MEC1*	4.0 ± 0.6	1.0
YB326	*sml1*	4.3 ± 0.5	1.1
YB312	*mec1-21*	5.3 ± 1.4	1.3
YB329	*mec1-21 sml1*	3.8 ± 0.1	1.0
YB370	*dun1*	2.8 ± 1.9	0.7
YB369	*mec1-21 dun1*	1.8 ± 0.6	0.5
YB380	*mec1-21 dun1 sml1*	3.2 ± 0.5	0.8

[a]All strains derived from S288c. For complete genotype (Table 1).
[b]Rate represents the number of events per cell division; $n \geq 2$.
[c]Ratio represents rate of SCE in mutant/rate of SCE in wild-type.

function in preventing replication fork collapse reduces spontaneous SCE in *dun1* mutants. *dun1* mutants do exhibit higher rates of spontaneous heteroallelic gene conversion between homologs (10), and it is possible that sister chromatid gene conversions are not detected in our assay (35). However, in *mec1* hypomorphs, stalled replication forks would lead to more replication fork collapse and recombinogenic lesions. Thus, *MEC1* has two functions in suppressing recombinogenic lesions: (i) maintaining dNTP levels and (ii) preventing replication fork collapse.

When *SML1* is deleted in either *mec1-21* or *mec1-21 dun1* strains, we speculate that higher dNTP levels reduce replication fork stalling and thus fewer replication forks collapse. However, we observed that *mec1-21 sml1* mutants still exhibited some hyper-recombination between homologs or non-homologous chromosomes when dNTP levels were high. It is likely that we can detect higher levels of translocations in *mec1-21 sml1* mutants because there is a lower rate of spontaneous translocations, compared to heteroallelic and SCE, in wild-type. Thus, more recombinogenic lesions still occur in *mec1-21*, compared to wild-type, even at high dNTP levels.

DUN1 suppresses spontaneous homologous recombination in *mec1-21*

Although dNTP levels in *mec1-21*, *dun1* and *mec1-21 dun1* are similar, the rates of spontaneous recombination are synergistically increased in the double mutant, compared to the single mutants. We suggest that there may be two different reasons for these observations. First, although the overall dNTP levels are the same, the rate of dNTP production may be lower in *mec1-21 dun1*, compared to *dun1*, leading to more replication stalling. Second, Schollaert *et al.* (36) reported that *CHK1* is required for hydroxyurea resistance in *dun1* or *dun1 sml1* mutants, suggesting additional *DUN1* functions in promoting replication. This possibility is supported by the observation that *SML1* deletion only partially suppresses the hyper-recombination phenotype in *mec1-21 dun1*. We suggest that *MEC1* and *DUN1* may have redundant roles in suppressing recombinogenic breaks.

While *MEC1* may prevent replication fork collapse, a possible function of *DUN1* in preventing recombinogenic lesions is to promote translesion synthesis. *DUN1* functions in promoting spontaneous mutagenesis in both wild-type and in *pol3* mutants (11). We observed that the rate of spontaneous mutation was similar in *dun1* and *mec1-21 dun1*. Thus, it is possible that failure to promote translesion synthesis in *mec1-21 dun1* leads to more DNA replication stalling at sites of spontaneous damage, increasing the number of collapsed replication forks and recombinogenic lesions.

Spontaneous mutagenesis increases and spontaneous recombination decreases in *mec1-21 dun1* as dNTPs levels increase due to *SML1* deletion

4-NQO-associated mutagenesis increases with higher dNTP levels in strains deleted for genes that encode error-prone polymerases (12), suggesting that replicative polymerases function as translesion polymerases on non-bulky lesions when dNTP levels are higher (12). However, we did not observe higher levels of spontaneous mutagenesis in *sml1*, compared to wild-type. Considering that Sml1 is degraded in S-phase, the S-phase dNTP levels are likely similar in wild-type and in *sml1* mutants (8). We speculate that higher dNTP levels may promote translesion synthesis in cells where replication forks frequently stall, as in S-phase checkpoint mutants when the DNA polymerase encounters slow zones of

replication. Here, we speculate that higher dNTP levels in *mec1-21 dun1 sml1* promote more translesion synthesis and thus reduce recombinogenic lesions.

CONCLUSIONS

mec1-21 hyper-recombination phenotype correlates with low dNTP levels and can be suppressed by *SML1* mutations. We suggest that higher levels of dNTPs reduce recombinogenic lesions by promoting either replication fork progression or translesion synthesis. This is the first study to show that higher rates of recombination in a mutant deficient in wild-type dNTP levels can be suppressed by increasing dNTP concentrations.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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