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Effects of camptothecin or *TOP1* overexpression on genetic stability in *Saccharomyces cerevisiae*

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Abstract

Topoisomerase I (Top1) removes DNA torsional stress by nicking and resealing one strand of DNA, and is essential in higher eukaryotes. The enzyme is frequently overproduced in tumors and is the sole target of the chemotherapeutic drug camptothecin (CPT) and its clinical derivatives. CPT stabilizes the covalent Top1-DNA cleavage intermediate, which leads to toxic double-strand breaks (DSBs) when encountered by a replication fork. In the current study, we examined genetic instability associated with CPT treatment or with Top1 overexpression in the yeast *Saccharomyces cerevisiae*. Two types of instability were monitored: Top1-dependent deletions in haploid strains, which do not require processing into a DSB, and instability at the repetitive ribosomal DNA (rDNA) locus in diploid strains, which reflects DSB formation. Three 2-bp deletion hotspots were examined and mutations at each were elevated either when a wild-type strain was treated with CPT or when *TOP1* was overexpressed, with the mutation frequency correlating with the level of *TOP1* overexpression. Under both conditions, deletions at novel positions were enriched. rDNA stability was examined by measuring loss-of-heterozygosity and as was observed previously upon CPT treatment of a wild-type strain, Top1 overexpression destabilized rDNA. We conclude that too much, as well as too little of Top1 is detrimental to eukaryotic genomes, and that CPT has destabilizing effects that extend beyond those associated with DSB formation.

Keywords

Topoisomerase I; camptothecin; mutagenesis; recombination; yeast

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1. Introduction

Topoisomerase I (Top1) is a Type IB enzyme that resolves torsional stress associated with DNA unwinding that occurs during transcription and replication (reviewed by [1]). The enzyme uses an active site tyrosine to cleave one DNA strand and covalently associates with the 3' end of the nick via a phosphotyrosyl bond; a 5'-OH flanks the other side of the nick. Following controlled strand rotation, attack of the phosphotyrosyl bond by the 5'-OH re-seals the DNA backbone and releases the enzyme. Camptothecin (CPT) is a planar molecule that reversibly intercalates into the Top1 cleavage complex (Top1cc) and blocks nucleophilic attack by the 5'-OH, thereby stabilizing the covalent intermediate. CPT and its derivatives (topotecan and irinotecan) are widely used in the treatment of malignancies, with encounter between the Top1cc and a replication fork leading to toxicity through double-strand break (DSB) formation [1]. Top1 is overexpressed in many types of cancer ([2]; see also proteomics.org), which presumably reflects a greater demand to support increased replication and/or transcription. Because CPT sensitivity correlates with Top1 level [3–4], tumors that overexpress Top1 may be particularly good therapeutic targets [5].

In addition to the well-characterized toxicity of CPT, mutagenic effects of the drug have been reported in Chinese hamster fibroblast and ovary cell lines [6–7]. Mutants that had lost hypoxanthine phosphoribosyl-transferase (Hprt) activity were selected, and subsequent molecular analysis indicated that the corresponding Hprt locus had suffered a deletion or rearrangement in 50–80% of the mutants analyzed. Such gross alterations are consistent with mutagenic DSB repair. In *Saccharomyces cerevisiae*, CPT-induced DSBs stimulate homologous recombination [8–9] and gross chromosomal rearrangements [10]. CPT is also weakly mutagenic at the yeast *CAN1* locus, but the molecular nature of induced mutations was not characterized [8].

In the absence of CPT, yeast Top1 is responsible for a distinctive, short-deletion signature, which was originally described in highly transcribed reporters [11–12]. This signature occurs at low copy-number, tandem-repeat hotspots, with the deletion size reflecting the size of the repeat unit. Furthermore, there are two distinct classes of Top1-dependent deletions: those that reflect cleavage at a ribonucleotide (ribo) embedded in DNA and those that reflect cleavage at a deoxyribonucleotide (ribo-independent; [13]). Ribo-independent deletions are stimulated in a *top1-T722A* background where the corresponding Top1cc is inherently more stable, which mimics the effect of CPT on the wild-type (WT) protein [14]. By contrast, ribo-dependent deletions are reduced in a *top1-T722A* background, suggesting a fundamental difference in the underlying molecular mechanism [13]. The ribo-dependent deletion process has been reconstituted *in vitro* using purified Top1 and involves formation of a small gap via sequential enzyme cleavage [15–17]. Although the precise mechanism of ribo-independent deletion formation is not known, the stimulatory effect of the *top1-T722A* allele suggests that a trapped Top1cc is a key intermediate [13]. Finally, overexpression of Top1 sensitizes yeast cells to DNA-damaging agents as well as to CPT [18].

In the experiments reported here, we examined the effects of CPT treatment on the short-deletion signature previously associated with Top1 activity. We additionally analyzed the effect of Top1 overexpression on short-deletion hotspots and on recombination at the rDNA

tandem-repeat locus. Either CPT treatment or Top1 overexpression increased 2-bp deletions at previously characterized hotspots and in each case, a novel deletion hotspot was detected. In addition, recombination at the rDNA locus was stimulated by Top1 overexpression. These results demonstrate that either excess Top1 or Top1-targeting chemotherapeutic drugs drive genome instability and are likely to contribute to tumor evolution.

2. Methods

2.1. Strain and plasmids

A complete list of strains is given in Table S1. All haploid yeast strains were derived by sequential transformation of YPH45 (*MATa ura3-52 ade2-101_{oc} trp1⁻*; [19]). Each contained a highly-transcribed *pTET-lys2* reporter with either the Top1-dependent (AT)₂, (TC)₃ or (AG)₄ hotspot [20]. Briefly, the *LYS2* locus was deleted from its endogenous location on chromosome II and inserted next to *ARS306* on chromosome III. The *LYS2* promoter was then replaced with a doxycycline-repressible *pTET* promoter; all analyses were done under high-transcription conditions. In the strains used here, *LYS2* transcription was in the same direction as the replication fork emanating from *ARS306*. Finally, ~20 bp hotspot-containing fragments (from the *CAN1* gene) were inserted into the *pTET-LYS2* allele, creating –1 frameshift alleles that can revert via a 2-bp deletion at the relevant hotspot [11].

To increase the uptake of CPT from the growth medium, we deleted the *ERG6* gene using a PCR-generated fragment. Primers ERG6KANF (5′ - AAAACAAGAATAAAATAATAATATAGTAGGCAGCATAAGATGAGTGAAACAGAA TTGAGCAGCTGAAGCTTCGTACG) and ERG6KANR (5′ - TTATCTGCATATATAGGAAAATAGGTATATATCGTGCGCTTTATTTGAATCTTATTGAT CAGGCCACTAGTGGATCTG) were used to amplify a *TRP1* or *URA3K1* cassette from pUG6 [21] or pUG72 [22], respectively, as appropriate. The *TOP1* gene was similarly deleted using a fragment amplified from pUG6 [11].

The diploid yeast strains used in the recombination studies (JSC25 and SLA46) were previously described [9], and were derived by crossing derivatives of W303-1A [23] and YJM789 [24].

TOP1 expression was regulated using the *pCUP-TOP1* plasmid pWJ1491, a centromeric plasmid containing *URA3* as a selectable marker [25]. A *pCUP-top1-Y727F* plasmid was constructed by replacing the *MluI/EagI* fragment of pWJ1491 with the corresponding *MluI/EagI* fragment from plasmid YCpSCtop1-Y727F [26].

2.2. Mutation frequencies

For CPT experiments, 1-ml YPD (1% yeast extract, 2% yeast peptone, 2% dextrose; 2% agar for plates) cultures were inoculated with a single colony, and a 100-fold dilution was incubated overnight at 30°C. At least three independent cultures of each strain were used in a given experiment. From each overnight culture (cell density between 5×10⁶ and 5×10⁷ cells/ml), two 10-ml cultures containing 10⁵ cells/ml were established in fresh YPD. One culture was supplemented with 100 μM CPT (Sigma; 4mg/ml stock dissolved in DMSO)

and the other with an equivalent amount of DMSO (untreated control). Following further incubation for six hours, appropriate dilutions were plated on YPD and on synthetic complete dextrose plates lacking lysine (SD-Lys) to determine total numbers of viable and Lys⁺ cells, respectively. Median Lys⁺ frequencies are reported and frequency distributions were compared using the Mann-Whitney U test (vassarstats.net).

For *TOP1* overexpression experiments, the *pCUP-TOP1* plasmid pWJ1491 was transformed into *top1* cells and Ura⁺ transformants were selected on standard SD-Ura medium. As controls, WT and *top1* cells were transformed with the parent *CEN-URA3* plasmid pRS415 [19]. For Lys⁺ frequency measurements, independent SD-Ura cultures were directly started from transformants. Copper-free SD-Ura medium was made using yeast nitrogen base lacking CuSO₄ (Sunrise Science Products); where indicated, SD-Ura was supplemented with CuSO₄ to a concentration of 100 µM. Following three days of selective growth, appropriate dilutions were plated on standard SD-Ura and SD-Ura-Lys media to determine the total number of cells that maintained plasmid and the number of Lys⁺ cells, respectively, in each culture. The median Lys⁺ frequencies and associated 95% confidence intervals [27] were determined and the Mann-Whitney U test was used to compare frequency distributions.

2.3. Mutation spectra

Mutation spectra were generated by sequencing independent Lys⁺ revertants. For Sanger sequencing, an 800 bp fragment was PCR-amplified using primers LYSWINF (5′ - GCCTCATGATAGTTTTCTAACAAATACG) and LYSWINR (5′ - CCCATCACACATACCATCAAATCCAC). PCR products were sequenced by Eton Bioscience (Research Triangle Park, NC) using primer MO18 (5′ - GTAACCGGTGACGATGAT). Mutation spectra were compared using a Fisher exact probability or chi-square test.

2.4. Quantitative real time RT-PCR

Total RNA was extracted from 5 ml of cells growing exponentially in standard SD-Ura, copper-free SD-Ura and in copper-free SD-Ura supplemented with 100 µM CuSO₄. RNA extraction was performed as previously described [20]. Quantitative real-time PCR (qPCR) was performed using the Quantitect One Step SYBR Green RT-PCR kit (Qiagen) and the amount of DNA amplified during each cycle was measured using the StepOne Plus Real-Time PCR System (Applied Biosystems). As a normalization standard, qPCR was done in parallel for *RFC1* transcripts. *TOP1*-specific primers were TOP1F1 (5′ - TCGTCGTCATTACCATCGCC) and TOP1R1 (5′ - TCCTCCTTCGCTTTCTTGTC) and *RFC1*-specific primers were RFC2134F (5′ - CGCTTCTGATGTTTCGCTC) and RFC2295R (5′ - CCTCCGCTCATACCATCAAC). The 2^{-C_T} method was used to determine relative transcript levels [28].

2.5. Detection of crossovers initiating in the rDNA locus

A region 21 kb centromere-distal to the rDNA locus on chromosome XII was amplified from genomic DNA of sub-cultured diploid clones as previously described [9]. Briefly, a 750-bp PCR product containing a *SpeI* polymorphism was generated using primers ChrXIIF490730 (5′ - CTGATGAGTTCTGCATCTGTCC) and ChrXIIR491473 (5′ -

TCCGTTACCATTCGATACAGAA). *SpeI* digestion resulted in three fragments if both W303-1A and YJM789 alleles were present, two if only the YJM789 allele was present and one fragment if only the W303-1A allele was present. To confirm loss of heterozygosity through recombination rather than chromosome loss, a centromere-proximal region 60 kb from the rDNA locus was examined using a *PspXI* polymorphism. Following the amplification of a 780 bp PCR product (primers ChrXIIuprDNA391662F, 5'-GACGCAGAGGAAGAAACAAATC, and ChrXIIuprDNA392432R, 5'-TCCCACAGCCAGTTCAATATC), presence of three fragments following *PspXI* digestion was confirmation that there had been no chromosome loss.

2.7. Top1 cleavage assay

An *in vitro* Top1 cleavage assay was performed as previously described [16]. A 45-nucleotide fragment containing the (TC)₃ sequence (5'-TTTGAAATACCGTGGCATCTCTCGTGACGAGTTACCATTTAAAGC) was labeled at the 3' end prior to annealing to a complementary fragment (5'-GCTTTAAATGGTAACCTCGTCACGAGAGATGCCACGGTATTTCAA) at 1:1 ratio to produce the intact duplex substrate. The marker fragment (5'-GACGAGTTACCATTTAAAGC) was also 3'-end labeled. Human TOP1 (140 nM) was added to a reaction mixture containing 20–500 nM end-labeled DNA substrate, 10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 15 µg/ml BSA and 10% DMSO. 10 µM CPT was added to the reaction as indicated. Following incubation at 25°C for 1 hour, reactions were terminated by addition of 0.5% SDS. Samples were analyzed on a 20% polyacrylamide gel and labeled fragments were detected using a PhosphorImager.

3. Results

3.1. CPT stimulates 2-bp deletions at hotspots

Top1-dependent 2–5 bp deletion hotspots were originally identified as forward mutations at *CAN1* following replacement of the endogenous promoter with the highly active *GAL1* promoter [11–12]. To further study representative 2-bp deletion hotspots in isolation, ~20 bp fragments spanning each were transferred into a region of a highly transcribed *pTET-LYS2* gene that tolerates heterologous amino acids. Fragments were inserted out-of-frame, with reversion to lysine prototrophy requiring a net +1 frameshift mutation, among which are 2-bp deletions. Three hotspots were examined: a ribo-independent (AT)₂ hotspot, a ribo-dependent (AG)₄ hotspot, and a (TC)₃ hotspot, which accumulates both ribo-dependent and -independent events [13]. Prior studies demonstrated that most Lys⁺ revertants contain a 2-bp deletion at the introduced hotspot and that these events are completely Top1 dependent. In addition, to improve the uptake of CPT from the growth medium we deleted the *ERG6* gene [8], which encodes a membrane sterol transmethylease, from strains containing the *pTET-lys2::hotspot* alleles. We confirmed that, as previously reported [8], only strains with functional Top1 were sensitive to CPT (data not shown).

Median Lys⁺ reversion frequencies were measured in growing cultures treated for six hours either with CPT dissolved in DMSO, or with an equivalent amount of DMSO (Figure 1A and Table S2). Under these conditions, CPT treatment resulted in a 2-fold reduction in cell

viability. With the *pTET-lys2::(AT)₂* reporter, the Lys⁺ frequency increased 2.8-fold when cells were treated with CPT ($p=0.009$ by Mann-Whitney U test). Molecular analysis of revertants confirmed that most mutations were 2-bp deletions in the (AT)₂ hotspot: 45/48 and 46/48 in the control and CPT-treated cells, respectively. With the (TC)₃-containing reporter, CPT treatment similarly increased the frequency of Lys⁺ colonies 2.8-fold ($p=0.002$). In this case, however, the reversion spectrum was different in the presence versus absence of CPT. In the absence of CPT, 31/48 revertants had a 2-bp deletion at the (TC)₃ hotspot, and the remaining events were randomly scattered across the region where compensatory frameshifts occur. Although 2-bp deletions remained clustered at the hotspot in the presence of CPT (21/48), a novel 2-bp deletion hotspot was present immediately adjacent to the (TC)₃ sequence (Figure 1B). The new hotspot accounted for 12/48 reversion events that occurred in the presence CPT but only 2/48 in the absence of drug ($p=0.007$). It is unusual in that a GT (or TG) was deleted from the sequence GTG, which is only 1.5 repeat units; all other hotspots characterized to date have contained at least two repeat units. Finally, CPT had a somewhat smaller, 1.9-fold effect on the reversion frequency of the reporter containing the (AG)₄ ribo-dependent hotspot, but the effect was still significant ($p=0.016$). As with the other hotspots, most mutations were 2-bp deletions at the hotspot: 8/11 and 13/14 in the absence and presence of CPT, respectively.

3.2. The (TC)₃ and GTG hotspots contain TOP1 cleavage sites

To map Top1 cleavage sites near the deletion hotspots in the *pTET-lys2::(TC)₃* reporter, an *in vitro* assay was performed using human TOP1 and a 45-bp fragment in which the (TC)₃-containing strand was labeled at its 3'-end. Transient cleavage intermediates were stabilized by adding CPT to the reaction. TOP1 cleavage was detected at both the terminal (3') thymine of the (TC)₃ repeat and at the thymine of the novel GTG deletion hotspot (Figure 2). Both sites were enhanced by inclusion of CPT in the reactions. We note that neither of these sites was previously detected [29]. This may have been due to the shorter, 32-bp duplex used in the earlier study, as Top1 binding close to DNA ends may be reduced by breathing of the strands.

3.3. Top1 overexpression increases 2-bp deletions at hotspots

TOP1 previously was fused to the copper-inducible *CUP1* promoter (*pCUP-TOP1*) on a *CEN-URA3* plasmid [25], and this construct was used here to modulate gene expression. The *pCUP-TOP1* plasmid was transformed into *top1* strains containing the (AT)₂, (TC)₃ or (AG)₄ hotspot within the *pTET-lys2* reporter; as controls, we transformed the parental empty vector into the corresponding *TOP1* strains. Relative to transcript produced by the endogenous *TOP1* gene, presence of the *pCUP-TOP1* plasmid in a *top1* background elevated the level 5-fold even in the absence of exogenously added copper. Upon addition of 100 μ M copper, the transcript level was elevated over 200-fold relative to that of the endogenous gene. Although protein levels were not measured, results below indicate that there were corresponding (though not necessarily proportional) increases.

With expression of endogenous *TOP1*, the median frequency of Lys⁺ colonies in the *pTET-lys2::(AT)₂* strain was 2.4×10^{-6} , and all revertants sequenced (28/28) had a 2-bp deletion at the hotspot (Figure 3 and Table S3). Relative to endogenous *TOP1*, the presence of the

pCUP-TOP1 plasmid (*top1*⁻ background) elevated the median Lys⁺ frequency 4.3-fold ($p<0.001$), and 22/23 reversion events examined were 2-bp deletions at the (AT)₂ hotspot. Addition of copper to the medium resulted in much larger, 15-fold increase in the Lys⁺ frequency ($p<0.001$) and all but one of the events sequenced (22/23) was at the (AT)₂ hotspot. Addition of copper had no effect on the Lys⁺ frequency in the strain expressing endogenous *TOP1*.

For the (TC)₃ reporter, presence of the *pCUP-TOP1* plasmid (*top1*⁻ background) elevated the Lys⁺ frequency 2.4-fold and 18-fold in the absence and presence of copper, respectively, relative to that associated with endogenous *TOP1* expression ($p<0.001$ in each case; Figure 3 and Table S3). The proportion of 2-bp deletions at the (TC)₃ hotspot was similar when *TOP1* was expressed endogenously or from *pCUP1* in the presence or absence of copper (31/41 or 31/44, respectively; $p=0.63$), but there was a proportional reduction in these events (36/81; $p=0.008$ relative to no CuSO₄) when copper was added to the medium (Figure 3B). Deletions at two additional positions were evident upon copper addition: the GTG hotspot (GTG>G) previously associated with CPT treatment, and a new GAG hotspot (GAG>G) not previously observed (21/81, $p=0.0009$ and 10/81, $p=0.023$, respectively). To confirm that the increased mutagenesis associated with *TOP1* overexpression requires Top1 catalytic activity, we examined reversion of the *pTET-lys2::(TC)₃* allele in the presence of the *top1-Y727F* allele, which encodes a catalytically dead protein [26]. When expressed from the *CUP1* promoter, the median Lys⁺ frequency was the same as in a *top1*⁻ strain (data not shown).

To assess the effect of *TOP1* expression level on a ribo-dependent hotspot, we examined reversion of the *pTET-lys2::(AG)₄* allele. In the presence of RNase H2 and endogenous *TOP1*, most events (20/27) were 2-bp deletions at the hotspot. When the *pCUP-TOP1* plasmid was introduced into a *top1*⁻ background, the Lys⁺ frequency increased 1.4-fold ($p<0.001$) in the absence of exogenous copper, and 20-fold when copper was added ($p<0.001$; Table S3). The proportions of events at the hotspot were similar to that observed in the strain expressing endogenous *TOP1*: 18/26 and 19/27 in the *pCUP-TOP1* strain in the absence and presence of copper, respectively. Loss of RNase H2 (*rnh201*⁻), which initiates ribo removal, in the *TOP1* background was associated with a 390-fold increase in the median Lys⁺ frequency ($p<0.001$) and all events examined were 2-bp deletions at the (AG)₄ hotspot (28/28). Following introduction of the *pCUP-TOP1* plasmid into an *rnh201*⁻ *top1*⁻ background, there was an additional 1.7-fold increase in Lys⁺ frequency when cells were grown in the absence of copper, but no further increase when exogenous copper was added to the growth medium ($p=0.008$ and $p=0.38$, respectively). This suggests that the system is saturated with respect to Top1 incision, and that the excess enzyme removes the vast majority of ribos that persist in the absence of RNase H2.

3.4. rDNA is destabilized by *TOP1* overexpression

The diploid strain JSC25 was derived by mating sequenced-diverged haploids (W303-1A and YJM789) and has been used extensively to monitor mitotic loss of heterozygosity (LOH; e.g. [30–31]). We previously used this strain to demonstrate that either loss of *TOP1* or treatment of *TOP1* cells with CPT increases recombination within the ribosomal DNA (rDNA) tandem-repeat locus on chromosome XII [9]. To examine whether increased *TOP1*

expression affects recombination in the rDNA array, we sub-cultured WT (JSC25 containing the vector control plasmid), *pCUP-TOP1* (JSC25 *top1* /*top1* containing the *pCUP-TOP1* plasmid) and *top1* (JSC25 *top1* /*top1* containing the control vector plasmid) diploids. For the sub-culturing, independent colonies were streaked on copper-free SD-Ura plates to maintain plasmid selection, or on SD-Ura medium supplemented with 100 μ M CuSO₄. After two days, a single colony was streaked again onto the same medium; this was repeated 10 times and is equivalent to ~250 generations.

The rDNA gene cluster is a 1–2 Mb region that comprises ~10% of the yeast genome and contains 100–200 tandem copies of a 9.1 kb repeat [32]. LOH on the arm of chromosome XII containing the array was monitored using a polymorphic, centromere-distal *SpeI* restriction site [9]. There were no LOH events among the WT colonies sub-cultured in the absence or presence of 100 μ M CuSO₄, respectively (Figure 4A and Table S4). The presence of the *pCUP-TOP1* plasmid in the *top1* background, however, was associated with increased rDNA instability in the presence or absence of copper ($p < 0.001$ for each comparison). Interestingly, there was a strong bias to lose the W303-1A allele in the absence of copper, but a bias to lose the YJM789 allele in the presence of copper when Top1 was overexpressed ($p < 0.001$). The rDNA instability associated with *TOP1* overexpression was similar to the elevated level of LOH observed in *top1* isolates in the presence or absence of copper ($p = 1$ and $p = 0.53$, respectively). There was no bias for loss of the YJM789 versus W303-1A allele in the absence of Top1 ($p = 0.42$ and $p = 0.21$ in the presence and absence of copper, respectively).

4. Discussion

Stabilization of the Top1 cleavage complex (Top1cc) by CPT or through expression of the *top1-T722A* allele, which encodes a protein with reduced resealing activity, stimulates both recombination and gross chromosomal rearrangements in yeast [9]. While this type of instability primarily reflects conversion of the nicked intermediate into a DSB, the nick itself can initiate mutagenesis at short-deletion hotspots. Ribo-dependence of hotspots is operationally defined by whether deletion rates increase upon loss of RNase H2, which incises and initiates repair of single ribos embedded in duplex DNA [33]. The Top1-T722A protein stimulates the activity of ribo-independent hotspots, but has the reverse effect on ribo-dependent hotspots [13]. In the current study, we examined the effect of CPT treatment on three deletion hotspots – (AT)₂, (TC)₃ and (AG)₄ – in strains expressing WT Top1. Although our expectation was that CPT would similarly have a stimulatory effect only on those hotspots classified previously as being ribo-independent, it also stimulated activity of the ribo-dependent (AG)₄ hotspot. There are two possible, not necessarily mutually exclusive, explanations for this. First, the results may reflect use of an RNase H2-defective background for the previous Top1-T722A experiments with the (AG)₄ hotspot [13], but the performance of the CPT experiments reported here in the presence of RNase H2. In the presence of functional RNase H2, it may be that most deletions at the (AG)₄ hotspot reflect Top1 incision at a deoxyribonucleotide rather than at a ribonucleotide. A second explanation is that intercalation of CPT into the Top1cc [34] blocks attack of the phosphotyrosyl bond by the 5'-OH and thereby increases attack by the vicinal 2'-OH of ribose, which is the first step in ribo-dependent deletion formation.

CPT had a similar, 2–3 fold stimulatory effect on each of the hotspot-containing reporters used here. Although this primarily reflected deletions at the hotspots, there were multiple occurrences of a novel, 2-bp deletion within a GTG sequence adjacent to the (TC)₃ hotspot. This additional hotspot was part of the 20-bp *CAN1* sequence inserted into the *pTET-LYS2* reporter, and hence was not present in either the (AT)₂ or (AG)₄ construct. We mapped a Top1 cleavage site to the central thymine of the GTG hotspot, a position consistent with enhanced, CPT-stabilization of the Top1cc *in vitro* when there is a guanine at the +1 position [34]. Figure 5 presents a ribo-based, sequential-cleavage model for 2-bp deletions at the GTG hotspot. In this model the initial Top1 cleavage occurs at the ribo T of the hotspot; attack of the 3′-phosphotyrosyl bond by the 2′ of ribose releases the enzyme and generates a 2′,3′-cyclic phosphate. This is followed by a second Top1 cleavage at the cytosine located 2 nt upstream. Spontaneous release of the oligo between the Top1-generated nicks creates a gap, and traps the enzyme. Sliding of the downstream 5′-OH towards the Top1cc [16,Cho, 2016 #2589] converts the 2-nt gap to a nick and extrudes a 2-nt loop. A single, terminal G:C base pair is sufficient to stabilize a realigned deletion intermediate [15]. Finally, the remaining nick is sealed when the phosphotyrosyl bond of the Top1cc is attacked by the properly aligned 5′-OH. Although ribo-dependence of the GTG hotspot has not been established, similar models have been proposed for ribo-independent events [35].

It should be noted that the use of *LYS2*-based frameshift reporters in the current study allowed only ~170 bp of sequence to be monitored. It is likely that additional CPT-dependent short-deletion hotspots and/or novel types of CPT-associated events will be evident with a larger target, such as the ~2 kb *pGAL-CAN1* reporter used previously [11]. The weak stimulation of canavanine resistance by CPT reported previously [8] is expected to be greatly enhanced by highly transcribing the *CAN1* gene.

TOP1 is essential in mammalian cells [36], but its loss in a diploid yeast strain seems only to affect stability of the repetitive rDNA and *CUP1* loci [9]. Similar to loss of *TOP1*, we previously reported that expression of *pCUP-TOP1* in standard SD-Ura medium is associated with LOH that initiates in the rDNA locus [9]. Although the simplest explanation was that *TOP1* was poorly expressed in the absence of exogenous copper, an alternative possibility was that instability reflected *TOP1* overexpression. This was particularly intriguing because TOP1 is frequently overexpressed in human tumors. In the current study, we found that, even in copper-free synthetic medium, there is ~5-fold more *TOP1* transcript produced from the *pCUP-TOP1* fusion than from endogenous *TOP1* promoter. This indicates that the elevated LOH seen previously was due to too much rather than too little Top1, and demonstrates that there is a delicate balance between too little and too much enzyme in maintaining rDNA homeostasis. Although the strong increase in transcript associated with addition of exogenous copper did not further increase rDNA instability, the bias for which chromosome arm became homozygous was reversed. In the absence of copper the YJM789 arm most often became homozygous; in the presence of copper, the W303-1A arm was usually homozygous. We have no explanation for this anomaly, but note that the bias for W303-1A homozygosity is similar to that observed in the absence of Top1.

Just as excess Top1 destabilized the rDNA locus, it also increased reversion of each of the *pTET-lys2::hotspot* alleles examined. Mirroring the transcription of *pCUP-TOP1*, there was

a stimulation in Lys⁺ frequency in the absence of exogenous copper, and an even greater stimulation when copper was added to the growth medium. It is possible that the level of Top1 relative to transcription complex is limiting, and that the additional enzyme may be recruited by its interaction with the phosphorylated C-terminal domain of elongating RNA polymerase II [37]. The increased mutagenesis could reflect additional removal of transcription-associated supercoils or random nicking of DNA. One interesting aspect of Top1 overexpression concerns the mutation spectrum obtained with the (TC)₃-containing reporter in copper-containing medium. There was a proportional decrease in 2-bp deletions within the (TC)₃ hotspot, from ~75% of events in the absence of copper to ~45% in the presence of copper. Two positions with proportional increases were notable: the GTG hotspot observed upon CPT treatment of the WT strain, and a GAG hotspot not previously noted. It thus appears that the activity of the GTG hotspot can be increased either by enhanced stabilization of the Top1cc intermediate or by increasing the amount of Top1; the GAG hotspot was only affected by the latter. Although the GAG hotspot is part of the *LYS2* ORF, it was not evident in the reversion spectra for the (AT)₂- or (AG)₄-containing reporters. In the former case, its absence can be explained by the 10-fold greater frequency of 2-bp deletions at the (AT)₂ than at the (AG)₄ hotspot. In the case of the (AG)₄-containing reporter, which reverts at a frequency comparable to the (TC)₃ reporter, 3-fold fewer revertants were sequenced. Comparing the proportion of events at the relevant GAG in the (AG)₄ and (TC)₃ reporters (1/27 and 10/81, respectively) revealed no significant difference (p=0.29).

In summary, we have demonstrated that Top1-associated genetic instability is increased either by CPT-mediated stabilization of the cleavage intermediate or simply by increasing the expression of *TOP1*. Coupled with variable gene expression levels, and notably with the elevated expression of TOP1 in cancer cells, the effects of CPT treatment or Top1 overexpression are expected to vary considerably across the genome and to modify the mutation landscape. Although studies were done in the yeast system, results are expected to be applicable to any eukaryotic system that contains TOP1. This may be particularly relevant to genetic stability in tumors that overexpress TOP1 and/or are treated with CPT, and may contribute to disease recurrence and the evolution of more aggressive disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Top1 overexpression stimulates short deletions
- Camptothecin treatment is mutagenic in yeast
- Top1 overexpression elevates rDNA recombination

A. Effect of CPT on Lys⁺ frequency

B. Reversion spectrum of *pTET-lys2(TC)₃* allele

Figure 1. Effect of CPT on 2-bp deletion hotspots. **(A)** Circles represent Lys⁺ frequencies in individual cultures and the horizontal bar is the median. **(B)** A portion of the ~150 window where compensatory frameshift mutations that revert the *pTET-lys2(TC)₃* allele can be detected is shown. In the absence of CPT, 31/48 events were at the (TC)₃ hotspot; in the presence of CPT, only 21/48 events were at the hotspot. The novel 2-bp deletions at the GTG hotspot are in red. N, number of independent revertants sequenced.

Figure 2.

Top1 cleavage sites *in vitro*. The duplex fragment used matches the sequence of the *CAN1* locus; the (TC)₃ hotspot is bold black and the GTG hotspot is bold red. The lollipop corresponds to Top1 cleavage sites on the top strand, the asterisk to the labeled 3' end, and the filled triangle to the nick on the bottom strand. m, size marker.

A. Effect of *TOP1* overexpression on *Lys*⁺ frequency

B. Effect of *TOP1* overexpression on reversion events in the (TC)₃ reporter

Figure 3.

Effect of Top1 expression on 2-bp deletion hotspots. **(A)** Median *Lys*⁺ frequencies and 95% confidence intervals for strains containing the endogenous *TOP1* locus and empty vector or for *top1* strains containing the *pCUP-TOPI* plasmid (designated as *TOP1* or *CUP1* promoter, respectively). Experiments were done in copper-free synthetic medium with or without addition of 100 μ M CuSO₄ (+ or –, respectively). **(B)** The sequence window where compensatory frameshifts occur is shown; the 20-bp insert from *CAN1* that contains the (TC)₃ hotspot is shaded gray. The reversion spectra for the (TC)₃-containing reporter in the absence and presence of copper are above and below the sequence, respectively. The 2-bp deletions that are prominent only at the highest levels of Top1 expression are in red. N, number of independent revertants sequenced.

Figure 4.

Effect of Top1 overexpression on stability of the rDNA locus in W303-1A/YJM789 hybrid diploids. The indicated diploid strains were sub-cultured on copper-free synthetic medium with or without addition of 100 μ M CuSO₄. EV, empty vector. LOH on the chromosome XII arm containing the rDNA locus was monitored using a restriction site polymorphism and the percentage of colonies examined that were heterozygous (W303+YJM789; cross-hatched), homozygous for the W303 allele (black) or homozygous for the YJM789 (white) are indicated.

Figure 5.

Model for 2-bp deletions at the GTG hotspot. Top1 incises at the ribo-thymine (or ribo-uracil; in red) of the hotspot and is released when the 5'-OH attacks the phosphotyrosyl bond. This leaves a nick flanked by a 2'3'-cyclic phosphate (red triangle) and a 5'-OH. A second Top1 cleavage occurs (this could be the same enzyme or a different one) at the cytosine that is 2-nt upstream of nick. The oligo between the Top1-generated nicks is released, which creates a 2-nt gap and traps the enzyme. Realignment of the cleaved and intact strands converts the gap to a nick and extrudes a 2-nt loop; this intermediate is stabilized by a terminal G:C base pair. Attack of the covalent bond between Top1 and DNA by the 5'-OH releases the enzyme and seals the nick.