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Constitutively Active Artemis Nuclease Recognizes Structures Containing Single-Stranded DNA Configurations

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Abstract

The Artemis nuclease recognizes and endonucleolytically cleaves at single-stranded to double-stranded DNA (ss/dsDNA) boundaries. It is also a key enzyme in the non-homologous end joining (NHEJ) DNA double-strand break repair pathway. Previously, a truncated form, Artemis-413, was developed that is constitutively active both in vitro and in vivo. Here, we use this constitutively active form of Artemis to detect DNA structures with ss/dsDNA boundaries that arise under topological stress. Topoisomerases prevent abnormal levels of torsional stress through modulation of positive and negative supercoiling. We show that overexpression of Artemis-413 in yeast cells carrying genetic mutations that ablate topoisomerase activity have an increased frequency of DNA double-strand breaks (DSBs). Based on the biochemical activity of Artemis, this suggests an increase in ss/dsDNA-containing structures upon increased torsional stress, with DSBs arising due to Artemis cutting at these ss/dsDNA structures. Camptothecin targets topoisomerase IB (Top1), and cells treated with camptothecin show increased DSBs. We find that expression of Artemis-413 in camptothecin-treated cells leads to a reduction in DSBs, the opposite of what we find with topoisomerase genetic mutations. This contrast between outcomes not only confirms that topoisomerase mutation and topoisomerase poisoning have distinct effects on cells, but also demonstrates the usefulness of Artemis-413 to study changes in DNA structure.

Keywords

DNA repair; recombination; topoisomerases; DNA double-strand breaks; *Saccharomyces cerevisiae*; Artemis

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1. Introduction

Artemis is an important DNA end processing factor in the non-homologous end-joining (NHEJ) repair pathway and in hairpin opening during V(D)J recombination (1,2). These nuclease functions of Artemis are due to recognition by Artemis of any single-stranded to double-stranded DNA (ss/dsDNA) boundary at contact points at such boundaries (Fig. 1A) (3–7). The endonuclease function of Artemis is activated by autophosphorylated DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (6–8); thus, this nuclease activity of Artemis would typically only be present after a DSB had already been made. A C-terminal truncation of the full-length, 692 residue Artemis has been made that consists of residues 1 to 413 (Artemis-413, also known as ARM37). This truncation confers constitutive endonuclease activity to Artemis, and thus bypasses the need for DNA-PKcs activation (9). We have recently adapted this for use in *S. cerevisiae* (10). Yeast lacks both a functional homolog of Artemis (11,12) and DNA-PKcs (13) to activate mammalian Artemis. Therefore, use of this mutant provides an opportunity to more easily study the DNA substrates that Artemis can cleave in vivo. The goal of this study is to explore the possibility that increased torsional stress may lead to more structures with ss/dsDNA boundaries that would be substrates for Artemis cleavage.

Processes that change the local supercoiling of DNA, such as transcription and replication, or that require the separation of interlinked DNA single strands or duplexes, such as chromosome separation during mitosis, necessitate the need for topoisomerases (14). These enzymes cut the DNA, allow for addition or removal of turns to the DNA helix, then accurately rejoin the DNA (15–20). Eukaryotes possess two classes of topoisomerases that can relieve both positive and negative supercoiling. The type IB topoisomerase, encoded by *TOP1* in *S. cerevisiae*, acts by nicking one strand of DNA while the type II class, encoded by *TOP2*, acts through a DNA double-strand break (DSB) intermediate (21). The ability of Top2 to pass an intact DNA strand through a DSB makes it essential for catenane removal (22) during replication, whereas Top1 appears to have a major role in processing the positive and negative supercoiling that occurs in the wake of transcription (15,23,24).

Due to the importance of both types of topoisomerases in rapidly dividing cancer cells, each are targets of various chemotherapeutic agents (25). The plant alkaloid camptothecin (CPT) targets Top1 by blocking re-ligation of the nicked DNA and enzyme release; thus, the DNA remains nicked and Top1 becomes covalently trapped on the DNA at the protein-DNA adduct intermediate step of the topoisomerase reaction. The epipodophyllotoxin and anthracycline classes of drugs similarly target Top2, with the important distinction that, in addition to a covalently trapped Top2 adduct, both DNA strands remain broken. While these drugs are often referred to as “topoisomerase inhibitors”, a more accurate mechanistic term is “topoisomerase poisons” (26) as they interrupt the enzymatic process and convert the broken DNA and covalently bound enzyme into a cytotoxic lesion (27,28). The covalently trapped Top1 caused by treatment with CPT, for example, leads to a DSB during DNA synthesis (29,30), likely due to the presence of a DNA nick and an inability of the replication machinery to bypass the lesion (31).

Here, we use a genetic recombination assay as a DNA DSB readout to determine the effect of Artemis-413 expression following either mutation of the topoisomerase encoding genes or treatment with the Top1 poison CPT. We found that *top1 top2* double mutants had a greater increase in recombination frequency with Artemis-413 expression, most clearly in dividing cells where efficient topoisomerase activity is critical. In contrast, Artemis-413 leads to a decreased recombination frequency with CPT treatment. This work confirms and extends previous data contrasting the effects of CPT treatment and *TOP1* gene deletion on genome stability (30) by demonstrating different Artemis-dependent effects under each condition. The results illustrate the value of using constitutively active Artemis to detect ss/dsDNA boundaries after changes in topological tension.

2. Materials and methods

2.1. Yeast strains

S. cerevisiae strains used in this study are listed in Supplementary Table 1 and are isogenic to the W303 background (32) except that the *rad5-G535R* allele was corrected to *RAD5*. Standard procedures for the genetic and molecular manipulation of yeast were done (33,34). The *top1::LEU2* and *top2-1* alleles have been described previously (35). Details of the Artemis-413 construction have been described (10). Briefly, the DNA encoding the first 413 amino acids of human Artemis was synthesized by Integrated DNA Technologies (IDT, Coralville, IA) to be codon optimized for *S. cerevisiae*. The Artemis-413 sequence was then cloned into the pESC-TRP vector (Agilent, Santa Clara, CA) under the control of the GAL1/10 promoter. pNP25 carries Artemis-413 in the coding orientation while pNP24 is a control that carries Artemis-413 in the non-coding orientation.

The Artemis-413_{H115A} mutant was generated by first having a 1182 bp double-stranded DNA fragment synthesized by IDT. This fragment carries a C-to-G transversion at nucleotide 343 of the coding sequence and an A-to-C transition at nucleotide 344 of the coding sequence that will result in the H115A missense mutation. The 5’ end of the fragment has an *Nde*I site and the 3’ end has an *Xho*I site, both of which are present in the wild-type coding sequence. The mutated *Nde*I/*Xho*I fragment was cloned into pNP25, replacing the wild-type *Nde*I/*Xho*I fragment to create the Artemis-413_{H115A} expression vector pNP177.

2.2. Direct repeat recombination (DRR) assay

The version of the DRR assay used here is the third in a series of related DRR assays developed in the lab. The original was created by first cloning the 190 bp *Hind*III fragment of *HIS3* from pUC18-HIS3 (36) into the yeast-integration vector YIp5 (37), to create pNP1. Linearized pNP1 was transformed into a haploid yeast strain and targeted to the *HIS3* locus. Integration of pNP1 disrupts the *HIS3* gene and results in a 190 bp direct repeat of internal *HIS3* sequence flanking the intact *URA3* gene present on YIp5. We found that the DRR frequency was higher than expected, which we attributed to the integrated YIp5 sequence containing several guanine clusters and also several “TATA-like” sequences that may direct spurious transcription (38), both of which may contribute to making the YIp5 integration unstable. A second version of the assay was then created to eliminate the majority of the

YIp5 sequence. Two halves of the *LEU2* gene, *leu2-Δ3'* and *leu2-Δ5'*, that share 500 bp of overlapping sequence were generated by PCR in such a way to introduce silent mutations into the *LEU2* sequence to eliminate *Eco*RI and *Bst*XI sites from the original sequences. The two *leu2* fragments were then cloned into pNP1 to create pNP13. pNP13 was linearized and transformed into haploid yeast and targeted to the *HIS3* locus. This creates a nested construct where the *URA3* gene and most of the YIp5 sequence is flanked by *leu2-Δ3'* and *leu2-Δ5'*, which is flanked by the 190 bp *HIS3* direct repeats. Selecting for colonies resistant to 5-fluoroorotic acid and able to grow on medium lacking leucine, we obtained a *LEU2*-marked DRR assay. Finally, in the third version (Fig. 2A), the *LEU2* gene is replaced with *URA3* by directing a PCR fragment amplified from pUC18-URA3 that carries the *URA3* gene flanked by 60 bp of sequence that will direct the transformed DNA to the *LEU2* gene at the *HIS3* locus, thus creating the *URA3*-marked DRR assay.

The GAL-DRR assay was constructed by excising the *Kpn*I/*Eco*RI fragment from pESC-TRP carrying the *GAL1/GAL10* divergent promoters and cloning it into the *Kpn*I/*Eco*RI sites of pUC18-URA3 to create pNP31. As above, pNP31 was used as a template in a PCR to generate the GAL-URA3 fragment with 60 bp of sequence to target the fragment to the *HIS3* locus.

The assay was performed using established methods (39). Briefly, colonies growing on synthetic complete with 2% dextrose (SC-dextrose), minus uracil plates were picked and disbursed into 1 mL of SC-dextrose, minus uracil. Uracil selection was maintained to prevent jackpots. Cultures where grown to saturation at 30°C. Dilutions were plated to SC-dextrose, minus histidine to select for recombinants and to YPD (1% yeast extract, 2% peptone, 2% dextrose) to determine the number of viable cells in the culture. These numbers were used to calculate the median DRR frequency from a minimum of ten independent cultures. A 95% confidence interval was determined using Microsoft Excel software. Microsoft Excel was also used to generate the box and whisker plots. All reported p-values are the result of a Mann-Whitney U test and were calculated using the Past v3.13 software (40).

Cells carrying the plasmids pNP24, pNP25, or pNP177 were grown on SC-dextrose, minus uracil and tryptophan media to maintain both the unrecombined DRR assay and the *TRP1*-marked plasmids. Individual colonies were disbursed in SC-galactose (synthetic complete, 2% galactose), minus uracil and tryptophan with the remainder of the assay performed as described above or in the text.

2.3. Camptothecin treatment

Camptothecin was obtained from Sigma-Aldrich (CAT# C9911, CAS: 7689-03-4). A 3 mM stock was prepared in DMSO (VWR, CAS: 67-68-5). Camptothecin was added to a concentration of 30 μM to 1 mL of either SC-dextrose, minus uracil or SC-galactose, minus uracil and tryptophan prior to disbursal of the cells being tested. Cultures where then grown to saturation at 30°C. Cells were washed in 1 volume of ddH_2O then resuspended in 1 volume of ddH_20. Dilutions were plated to SC-dextrose, minus histidine or YPD to determine DRR frequency.

3. Results

3.1. Experimental Rational

Extensive biochemical analysis has demonstrated that activated Artemis can endonucleolytically cleave a range of DNA structures that possess ss/dsDNA boundaries (Fig. 1A) (3–7). The endonuclease function of Artemis is activated by autophosphorylated DNA-dependent protein kinase catalytic subunit (DNA-PKcs) following DNA DSB formation (6,7). In response, the C-terminus of Artemis is phosphorylated (8,41). While a crystal structure is still lacking, the C-terminus of Artemis appears to be a regulatory region that blocks the endonuclease activity of the enzyme (1) with phosphorylation causing a conformational change to the endonucleolytically active state. Previously, a truncation of the Artemis nuclease that lacks the C-terminal residues 414 to 692, Artemis-413, was shown to have constitutive endonuclease activity independent of DNA-PKcs in in vitro biochemical assays (9). Additionally, a form of this truncation is active in vivo (11).

Use of Artemis-413 in yeast is ideal since yeast do not possess DNA-PKcs to activate full-length Artemis. Furthermore, due to the constitutive activity conferred by the truncation, Artemis-413 can scan the genome for DNA structures that have ss/dsDNA boundaries. Recently, we constructed a form of Artemis-413 codon optimized for *S. cerevisiae* and under the control of a galactose-inducible promoter (10). Expression of Artemis-413 resulted in formation of a stable protein product with measurable biological activity in a genetic assay measuring the rate of DNA DSB formation.

Using Artemis-413, we can probe the genomic DNA for structures with ss/dsDNA boundaries. Not only can this be none under normal cellular conditions, but also under conditions that stress the DNA. Increased torsional stress could greatly potentiate formation of structures with ss/dsDNA boundaries. We theorize that mutating *TOP1* and *TOP2* will greatly impair the cell's ability to mitigate topological tension and that an increase in structures with ss/dsDNA boundaries will be detectable by an increase in Artemis-413 activity resulting in DNA breaks (Fig. 1B).

We hypothesize that, at some frequency, DNA structures with ss/dsDNA boundaries (e.g., symmetrical bubbles or heterologous loops) (Fig. 1A) (3–7) form and are substrates for Artemis-413, with endonuclease activity on each strand resulting in a DSB. Another possibility is that thermally-driven structural fluctuations (i.e., breathing) (42) can transiently occur around a DNA nick, leading to strand separation and generation of a transient ss/dsDNA boundary. This was demonstrated, in principle, in previous studies using FEN-1 (Rad27 in yeast) to cut when a nick breathes into a transient flap. FEN-1 cuts the 5' flap before the flap re-anneals back to the nick state, accounting for the nick translation activity of FEN-1 when combined with a DNA polymerase (43–46). Flap structures, and subsequent gaps, are also substrates for the DNA-PKcs-independent, constitutively active Artemis-413 where cutting can result in a DSB.

3.2. Expression of Artemis-413 increases DRR frequency

We have constructed a haploid direct repeat recombination (DRR) assay to monitor the frequency of DNA DSBs in cells expressing constitutively active Artemis-413 (Fig. 2A).

The majority of such events occur through the single-strand annealing (SSA) DSB repair process (Fig. 2B) (39,47–51). Thus, an increase in DRR frequency is indicative of increased DSBs. The human Artemis gene sequence was used to create the *S. cerevisiae* codon optimized Artemis-413 allele. Artemis-413 was cloned into the pESC-TRP vector in a non-coding orientation to create a control vector (pNP24) or in the coding orientation under the control of a galactose-inducible promoter (pNP25) (Supplementary Fig. 1). All episomes were maintained under tryptophan selection and validated for stability. Previously, we verified production of a stable gene product upon galactose induction by Western blot (10).

Comparing Artemis-413 to the control, we measured a significant 5-fold increase in DRR frequency (2.2E-5 vs. 11E-5, p=0.00032) (Fig. 2C). This confirms that the Artemis-413 gene product has biological activity. If we compare this frequency to past work measuring the frequency of DRR following DSB induction by homing meganucleases (48,52,53), which for 190 bp of homology would be expected to be above 50%, we can see that the level of DRR stimulation by Artemis-413 is much lower. This is not unexpected as DSB formation by Artemis-413 depends on the transient formation of ss/dsDNA boundaries, the distance between nicks generated by Artemis-413, and the efficiency that these nicks are converted into a DSB. While DRR, therefore, likely underestimates Artemis-413 activity, the fact that Artemis-413 recognizes a specific type of DNA structure and that we can detect an increase in DRR makes this a valuable assay in assessing formation of ss/dsDNA structures in the genome.

In addition to the control plasmid, we also wanted to confirm that the increase in DRR is due to the nuclease activity of Artemis-413. The conserved histidine at amino acid position 115 appears important for binding of the divalent cations required for catalysis (54). Previous work by us in collaboration with others has shown that a missense mutation that changes histidine 115 to alanine (H115A) generates an Artemis protein that lacks detectable nuclease activity. Therefore, we generated a form of the Artemis truncation with the H115A mutation (Artemis-413_{H115A}) to determine if loss of nuclease activity impacts DRR. Indeed, cells expressing Artemis-413_{H115A} exhibit no increase in DRR with a frequency that is not significantly different from cells bearing the control plasmid (Fig. 2C). Thus, Artemis nuclease activity is required for generating increased DNA DSBs.

Interestingly, attempts to express full-length Artemis in yeast did not result in a stable gene product and did not increase the DRR frequency. Thus, the constitutively active Artemis-413 truncation represents the best method, so far, to test Artemis activity at ss/dsDNA boundaries in the in vivo yeast genetic system.

3.3. *top1 top2* double mutants show a synergistic increase in DRR frequency when cells are grown at 30°C

Next, we were interested in knowing the effect of Artemis-413 expression in torsionally stressed cells. If positive and negative supercoiling are allowed to accumulate, DNA structures with ss/dsDNA boundaries may form, resulting in increased Artemis-413 cleavage. Mutating the genes encoding the two major topoisomerases in yeast, *TOP1* and *TOP2* (21,55), would decrease the cell's ability to manage torsional stress, possibly resulting

in more ss/dsDNA structures. Even without Artemis-413 expression, such mutants are known to affect genome stability (30,35,56–58).

TOP1 encodes the Top1 protein (59), a type IB topoisomerase that relieves both positive and negative supercoiling by nicking one strand of the DNA. Deletion of the *TOP1* gene is not lethal, allowing for use of a complete genetic knockout (*top1Δ*). We measured the DRR frequency in *top1Δ* mutants bearing either a control plasmid or the Artemis-413 expression plasmid. With no Artemis-413 expression, we measured a significant 2-fold increase in DRR in *top1Δ* cells above wild-type (WT) cells (2.2E-5 vs. 4.4E-5, p=0.00037) (Fig. 3). A nearly 4-fold increase in DRR is measured in *top1Δ* cells expressing Artemis-413 compared to expression in wild-type (WT) cells (11E-5 vs. 39E-5) (Fig. 3). The 9-fold effect in *top1Δ* cells with or without Artemis-413 (4.4E-5 vs. 39E-5) appears indicative of a combined 2-fold effect from loss of *TOP1* and the 5-fold effect from Artemis-413 expression.

That a greater effect of *TOP1* deletion is not measured may be due to compensation by the type II topoisomerase encoded by the *TOP2* gene. Like Top1, Top2 also relieves both positive and negative supercoiling, but through a DSB intermediate. Since a *TOP2* deletion is lethal, the temperature sensitive *top2-1* allele was utilized. *top2-1* mutants exhibit reduced growth at 25°C, but normal DNA and RNA synthesis (60). *top2-1* cells do not survive temperatures in excess of 37°C due to an inability to resolve the catenated DNA that arises during replication (27,61). At the semi-permissive temperature of 30°C, the mutant Top2 protein retains enough function to allow DNA replication; however, it exhibits resistance to the Top2 drug etoposide as well as reduced vitality and increased mutagenesis (27).

Similar to our previously published strategy (35), the data in Fig. 3 are from cells grown at the semi-permissive 30°C where replication is possible, but Top2 activity is greatly reduced. The *top2-1* mutation alone did not confer a significant increase in DRR frequency, and the 4-fold effect measured with Artemis-413 is comparable to the increase observed in WT cells. The *top1Δtop2-1* double mutant, however, resulted in a synergistic increase of 15- to 29-fold higher than either single mutant (67E-5 vs. 4.4E-5; 67E-5 vs. 2.3E-5), indicating that loss or impairment of both topoisomerases is highly detrimental, resulting in more DSBs. This is similar to previously reported recombination frequencies in single and double topoisomerase mutants (58). We also obtained equivalent results when the DRR assay was performed in cells not maintaining a plasmid (Supplementary Fig. 2).

Next, we tested if Artemis-413 expression led to more DSBs in the *top1Δ top2-1* double mutant. Interestingly, we found that there was only a small increase in DRR frequency in the *top1Δ top2-1* mutants with Artemis-413 expression versus without (67E-5 vs. 102E-5, p=0.0029) (Fig. 3).

3.4. An Artemis-dependent increase in DRR occurs upon loss of Top2 in dividing cells

Our hypothesis is that loss of Top1 and Top2 would result in structures with ss/dsDNA boundaries that would be targets for Artemis-413 and increase the DRR frequency. Since we do not see a greater effect with Artemis-413 expression in *top1Δ top2-1* double mutants grown at 30°C, we wondered if the remaining activity of the *top2-1* gene product was enough to prevent an excess of such structures. Thus, we next wanted to test the effect of

Artemis-413 expression in the context of complete loss of both Top1 and Top2 by performing the assay with cells grown at 37°C, which would inactivate the *top2-1* gene product.

It has been previously shown that shifting *top1 top2* double mutants from the permissive 25°C to the non-permissive 37°C results in a rapid cessation of DNA synthesis (60). This work also showed that RNA synthesis is affected in mutants, though, importantly, galactose induction and production of mRNA from a galactose promoter occurs efficiently in the double mutant; thus, Artemis-413 expression in our system can continue following a shift to 37°C.

Here, we grew *top1Δ top2-1* mutants in galactose-containing medium lacking uracil and tryptophan at 25°C before shifting the cells to 37°C and measuring the DRR frequency at set timepoints. In addition to measuring the DRR frequency in cells immediately prior to the shift (0-hour timepoint), we also measured the DRR frequency 1, 5, and 10 hours following the shift to 37°C, with the 1-hour time point being most critical. As DNA synthesis is rapidly failing, there will be increased formation of DNA structures with ss/dsDNA boundaries as torsional stress accumulates to stop the progress of replication forks. A measurement at the 1-hour timepoint would represent DSBs occurring between the initial shift to 37°C and the first hour of the time course. That is, cells collected at this timepoint that are plated to selective medium and incubated at 25°C will yield recombinant colonies that represent the accumulation of DSBs during the first hour following the shift to 37°C and inactivation of Top2. At later timepoints, cell viability, and possibly the ability to repair DSBs, decreases as chromosome separation is affected by loss of Top2 and the ability to separate tangled DNA.

In the first time-course, *top1Δ top2-1* cells with or without Artemis-413 expression were grown to late-log/stationary phase at 25°C before the shift to 37°C (Fig. 4A). We measured a DRR frequency approximately 5-fold higher for cells expressing Artemis-413 compared to cells with a control plasmid at each timepoint. Thus, we see the usual effect of Artemis-413, but no greater accumulation of DSBs upon loss of Top2. Top2 is critical in replicating cells and, since most cells in late log/stationary phase are no longer replicating, there is less of a critical need for Top2 activity.

Next, we repeated the time-course, but this time shifted the cells to 37°C when a majority were in mid-log phase and replicating (Fig. 4B). Here we see a dramatic difference with a highly significant increase in DRR recombination between the 0- and 1-hour timepoints. *top2-1* single mutants were also tested in this context (Fig. 4C). Though there is still a significant increase between the 0- and 1-hour time points, the effects are not as dramatic as the *top1Δ top2-1* double mutant. Finally, *top1Δ top2-1* cells expressing the nuclease-deficient Artemis-413_{H115A} mutation were tested by shifting cells from 25 to 37°C at either mid-log or late-log/stationary phase. Under both conditions, the DRR frequencies are diminished to the levels measured with the control plasmid (Fig. 4D), again indicating that the Artemis nuclease activity is required to measure an increase in DSBs. These frequencies are not due to large changes in cell viability during the time-courses as these numbers remain relatively stable (Supplementary Figs. 3A–3D).

From these results, particularly those of Fig. 4B, we conclude that there is an Artemis-dependent increase in DRR in dividing cells upon concurrent loss of Top1 and Top2. With regards to the model shown in Fig. 1, loss of Top1 and Top2 during rapid cell division leads to an increase in torsional stress and DNA structures with ss/dsDNA boundaries that Artemis-413 can cleave to generated two-ended DSBs. The dependence on replication also raises the possibility that single-stranded nicks generated by Artemis during torsional stress can collapse a replication fork when encountered by the replicative DNA polymerases and result in a one-ended DSB (Fig. 4E). Future experiments will attempt to determine which mechanism occurs more frequently, but the data clearly indicated that the presence of active Artemis in replicating cells undergoing torsional stress can lead to genome rearrangements.

3.5. Artemis-413 expression decreases DSB formation in camptothecin-treated cells

With Artemis-413, we saw an additional opportunity to compare the effect of a *TOP1* genetic deletion to pharmacological targeting of the Top1 protein by camptothecin (CPT). CPT specifically acts on the Top1 protein and the toxicity of CPT is eliminated upon disruption of the *TOP1* gene (28). While CPT is often referred to clinically as a Top1 inhibitor, it is more accurately called a Top1 poison (26). When Top1 nicks the DNA, the 3'-end of the DNA forms a covalent bond with the tyrosine in the Top1 active site (21), trapping the protein by preventing sealing of the nick and Top1 release from DNA (Fig. 5B). This Top1-trapping can result in a DSB when a replication fork fails to bypass the adduct (25,31). In contrast, in a *top1Δ* mutant, no Top1 protein is present to bind and nick supercoiled DNA, thus leading to increased torsional stress. It is not clear that CPT treatment would lead to increased torsional stress, since, in CPT-treated cells, Top1 binding to DNA and nicking occurs in the normal manner and formation of the covalent Top1 complex makes this nick persistent. If DSBs that lead to DRR events are occurring by two different mechanisms in *top1Δ* mutants and CPT-treated cells, we can differentiate them using Artemis-413.

CPT treatment of WT cells results in a striking 57-fold increase in DRR compared to untreated cells (Fig. 5A). CPT treatment, therefore, is clearly more detrimental in terms of generating DSBs than the *top1Δ* mutant, which only resulted in a 2-fold increase in DRR (Fig. 3). The increased torsional stress upon Top1 loss can be compensated for by Top2, but covalently bound Top1 adducts need to be actively removed following CPT treatment or the cell will suffer DSBs during replication.

When Artemis-413 is expressed in CPT-treated cells, we see that the increase in DRR frequency caused by CPT treatment is reduced approximately 3-fold (Fig. 5A). If CPT treatment greatly increased supercoiling, we may expect to see a further increase in DRR, similar to our time-course in *top1Δ top2-1* mutants. Instead, this reduction in DRR suggests that the presence of Artemis-413 can reduce the number of DSBs due to covalent Top1 adducts. Tyrosyl DNA phosphodiesterase I (Tdp1) is the major enzyme that processes these Top1 lesions (62,63). Whether Artemis-413 acts independently, with other DNA end processing factors, or in conjunction with Tdp1 to remove Top1 adducts is an active area of investigation. Regardless, use of Artemis-413 further contrasts the effects of *TOP1* mutation

with pharmacological poisoning and indicates that using camptothecin to target Top1 activity with the intention of studying increased supercoiling is problematic.

4. Discussion

In this study, we utilize the Artemis-413 truncation to study increases in DNA structures with ss/dsDNA boundaries in vivo. After establishing that Artemis-413 leads to an increase in DRR, we wanted to determine the effect of having this constitutively active, structure-specific nuclease present under various conditions of topoisomerase deficiency. Increased torsional stress can physically distort the DNA and result in more ss/dsDNA structures that would be cleaved by Artemis-413. Additionally, we wanted to compare genetic mutation of topoisomerase encoding genes with pharmacological targeting of topoisomerase proteins. Interestingly, we found that Artemis-413 expression had opposing effects under these conditions.

Upon genetic mutation of topoisomerase genes, the level of DRR increases with Artemis-413 expression, suggesting Artemis-413 leads to more DNA DSBs . This is most clear in our time-course study where we can measure the effect of complete loss of Top1 and Top2. In dividing cells, where topoisomerase activity is critical in relieving torsional stress and allowing for progression of the replication machinery, loss of both topoisomerases appears to cause an accumulation of structures with ss/dsDNA boundaries. Some structures, such as symmetrical bubbles, (Fig. 1A), would allow for Artemis-413 cutting of each DNA strand and a DSB.

It is possible that increasing transcription, even in non-dividing cells, would create additional torsional stress that would require topoisomerase activity. We attempted to test this by inserting the *GAL1/GAL10* galactose-inducible promoters upstream of the *URA3* gene in the DRR assay (Supplementary Fig. 4), however, we found that the DRR frequency did not change in the cells tested at late-log/stationary following a shift to 37°C. Other labs have demonstrated that transcription can increase recombination frequencies, but only in the context of replication, with transcription interfering with replication fork movement leading to more frequent fork stalls (64,65). Thus, the lack of replication in the late-log stationary cells may be why we do not measure a greater recombination frequency. When the assay is repeated in mid-log phase cells, the replication transcription conflicts appear severe enough that the cells lose viability as the recombination frequency increases at later time points following loss of topoisomerase activity (Supplementary Fig. 4D and 4E). Future work in which the level and location of transcription is varied may provide further insight into the interplay of transcription, replication, and torsional stress in genome stability.

We also compared the effects of a *TOP1* genetic deletion with poisoning of the Top1 protein with the drug camptothecin (CPT). Even without Artemis-413 expression, it is clear that CPT treatment is much more detrimental to cells than Top1 loss. The drug causes a nearly 60-fold increase in DRR frequency whereas only a 2-fold increase is measured in *top1Δ* cells. This finding is in agreement with recent studies showing that mitotic recombination events in a *top1Δ* mutant were minimal compared to the genome-wide loss of heterozygosity measured in CPT-treated cells (30). The small effect of the *top1Δ* allele may be due to the

Top2 enzyme compensating for the loss and, indeed, a synergistic increase in DRR frequency is observed in a *top1Δ top2-1* double mutant (Fig. 3).

Expression of constitutively active Artemis-413 further underscores the difference between poisoning Top1 verses complete lack of the enzyme through genetic ablation. While Artemis-413 expression leads to more DSBs in topoisomerase mutants, we actually measure fewer DSBs in CPT-treated cells expressing Artemis-413 (Fig. 5A). Top1 creates a nick by using the active-site tyrosine to break the DNA backbone, leaving the enzyme covalently bound to the DNA via a 3' phosphate. CPT prevents the resealing of this nick, leaving Top1 covalently trapped on the DNA (25). The large steric barrier presented by this interrupted enzymatic process can lead to DSBs as replication attempts to traverse the area. The presence of Artemis-413 appears to prevent DSBs, perhaps by processing the DNA in such a way as to remove the Top1 adduct.

Artemis is an important DNA end processing factor in the NHEJ repair pathway (1) and, among its roles, has been shown to work in concert with Tdp1 to remove 3'-phosphoglycolate adducts (66). Currently, reports are conflicting on the result of CPT treatment in human cells with an Artemis knockout (67,68). It is known that other DNA repair enzymes can function to resolve Top1 adducts. Such lesions not only form following CPT treatment, but also upon Top1 interactions with DNA aberrations such as nicks, abasic sites, or damaged DNA. Indeed, overexpression of *TOP1* results in increased DNA DSBs (69,70), presumably due to more abundant Top1 adduct formation. In addition to Tdp1, which can specifically hydrolyze the bond between the active-site tyrosine of Top1 and the 3'-phosphate of the DNA (71,72), the Rad1-Rad10 endonuclease complex (XPF/ERCC1 in mammals) offers another pathway to repair covalent Top1 lesions (73) as do the DNA DSB repair enzymes Mre11 and Sae2 (CtIP in mammals) (74–77). Therefore, such a role for Artemis may be possible and we are currently testing this hypothesis. Most likely, Artemis is not acting directly at the covalent adduct, but cleaving at a ss/dsDNA boundary upstream of the adduct. This would allow for release of the DNA strand with the covalently-bound Top1 protein and repair of the subsequent DNA gap.

Understanding the effect of drugs that target topoisomerases is important in developing new strategies to treat cancer. The majority of drugs that target topoisomerases are poisons (25,78). Even though these compounds are quite specific for the Top1 and Top2 enzymes, they are not without side-effects, which include therapy related leukemias (79) and cardiovascular damage (80). There are, however, compounds that act as catalytic topoisomerase inhibitors (25,81). Instead of converting topoisomerases to toxic lesions, these drugs act in various ways to prevent nucleophilic attack of the DNA backbone, thus avoiding formation of covalent topoisomerase adducts.

A number of the described catalytic topoisomerase simultaneously inhibit both Top1 and Top2 (25,81). Therefore, they would most likely mimic the effects of the *top1 top2* double mutants utilized in this study and act to increase torsional stress in the genomes of rapidly dividing cells. Thus, further work with these mutants in yeast may help with the development of combination therapies. In one possible scenario, preventing topoisomerase action would increase topological tension and lead to formation of transient non-B DNA

structures. In addition to being susceptible to Artemis-413, we have shown that such structures are likely to be targeted by reactive oxygen species (ROS) and cytidine deaminases (10,82). Thus, combining catalytic topoisomerase inhibitors with treatments that increase ROS or treating cancer cells that overexpress cytidine deaminases (83), could potentially lead to novel, specific cancer treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Expression of constitutively active Artemis-413 increases recombinogenic deletions.
- Topoisomerase mutations result in an Artemis-413-dependent increase in recombination.
- Dividing cells are more sensitive to topoisomerase loss and Artemis-413 expression.
- Artemis-413 expression prevents recombination following camptothecin treatment.

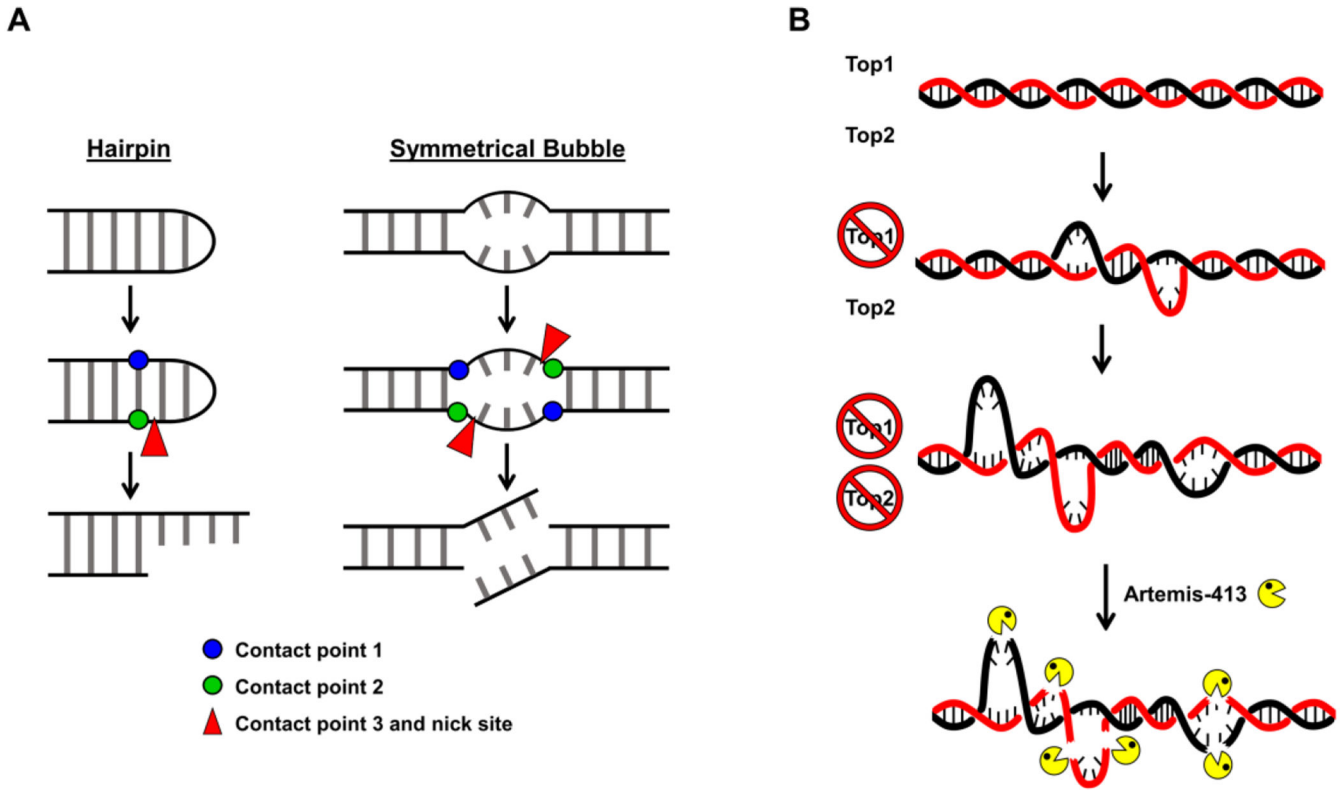

Fig. 1.

(A) Biochemical data demonstrates that after activation by DNA-PKcs, Artemis nicks many DNA structures with ss/dsDNA boundaries (3–7). Artemis endonuclease activity is essential for opening DNA hairpins created during V(D)J recombination to allow for DSB repair. Activated Artemis or constitutively active Artemis (due to C-terminal truncation, as in this study) also has activity at symmetrical bubbles, as these structures have ss/dsDNA boundaries. Endonucleolytic nicks on each strand can result in a DSB. **(B)** We hypothesize that by mutating both *TOP1* and *TOP2*, we can greatly increase the amount of torsional stress. Increased torsional stress may lead to an increase in DNA structures similar to symmetrical bubbles. Such structures would be detectable due to Artemis cleavage events that result in a DSB.

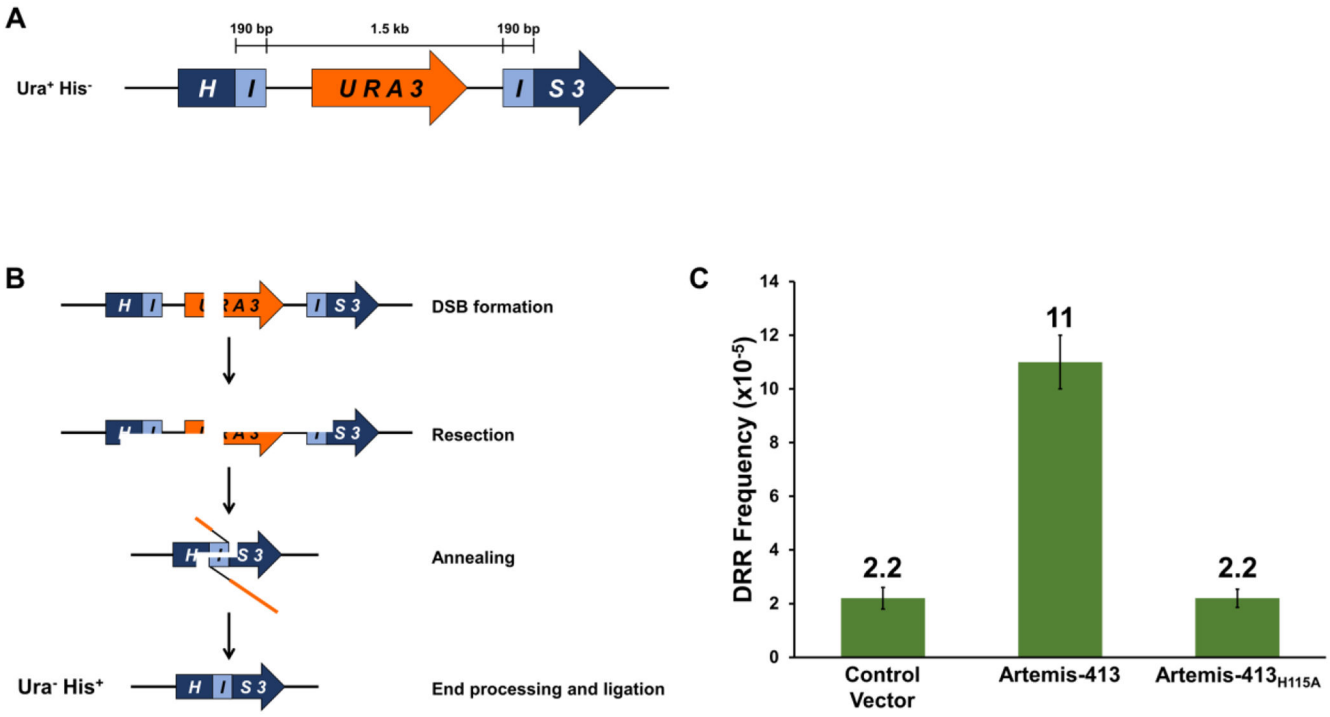

Fig. 2. Artemis-413 increases DSBs measured using a haploid direct repeat recombination (DRR) assay.

(A) The *URA3* gene was inserted at the *HIS3* locus on chromosome XV to create 190 bp of directly repeated *HIS3* sequence flanking the insertion. **(B)** Increases in the frequency of DNA double-strand breaks (DSBs) are detected by selecting for the formation of a functional *HIS3* gene following DSB repair by single-stand annealing. **(C)** The DRR assay was performed in cells carrying either the pNP24 control vector, pNP25, which carries the galactose-inducible Artemis-413 truncation, or pNP177, which carries the Artemis-413 truncation with the H115A missense mutation that attenuates nuclease activity. The median frequency (x 10^{-5}) from at least 10 trials is indicated. Error bars represent a 95% confidence interval.

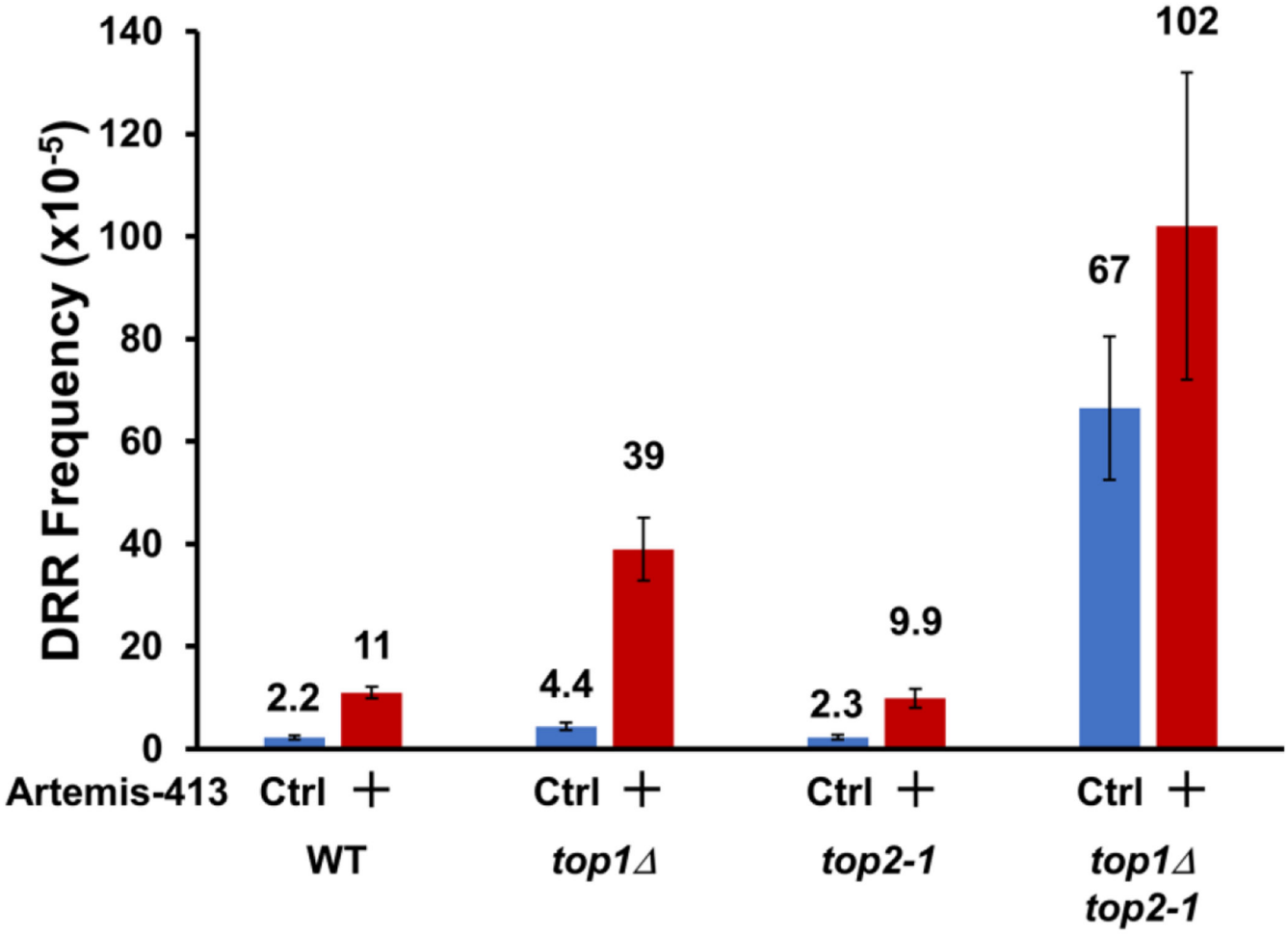

Fig. 3. Artemis-413 expression in *top1Δ top2-1* double mutants grown at 30°C.

The median DRR frequency ($x10^{-5}$) of cells grown at 30°C is indicated. Error bars represent a 95% confidence interval. DRR frequency in strains with the indicated mutations maintain either the pNP24 control plasmid (Ctrl) or the pNP25 Artemis-413 expression plasmid (+).

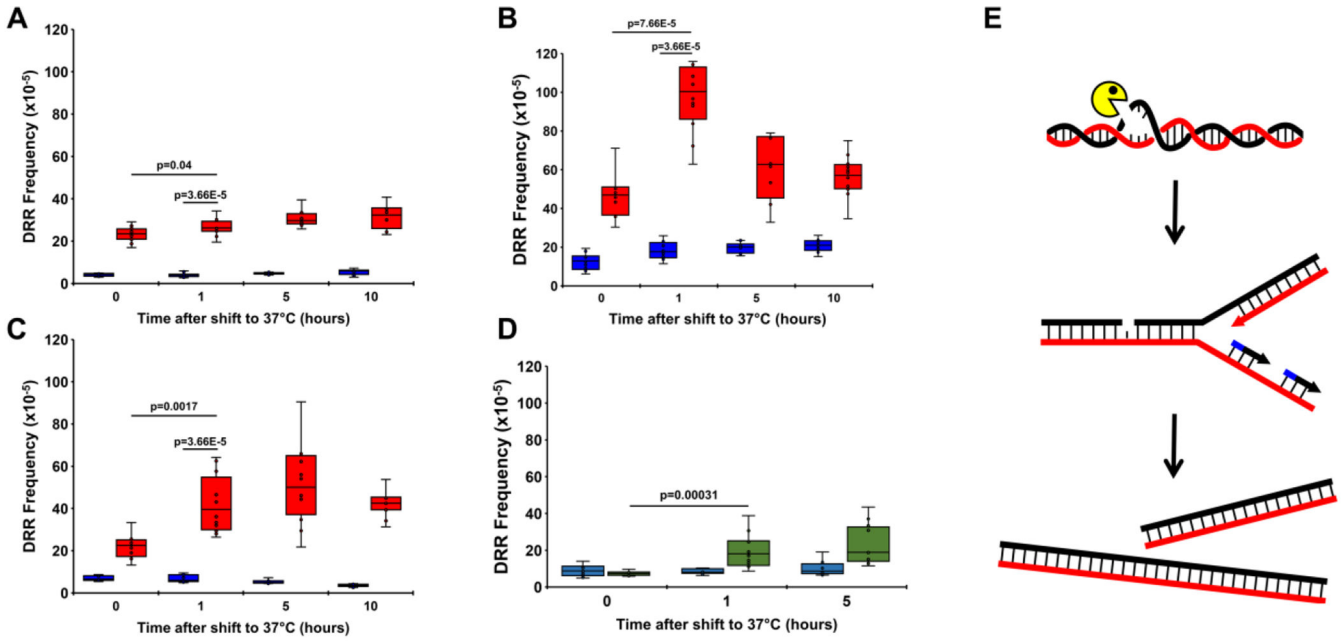

Fig. 4. Time-course of DRR frequency in *top1Δ top2-1* mutants with and without Artemis-413 expression following shift from 25°C to 37°C.

Cells were grown at 25°C before being shifted to 37°C to inactive the Top2 protein. DRR frequency was measured prior to (0) and 1, 5, and 10 hours following the shift to 37°C. **(A)** *top1Δ top2-1* double mutants grown to late log/stationary phase before shift to 37°C. Cells carry either a control plasmid (blue) or the Artemis-413 expression plasmid (red). **(B)** Same as (A) except *top1Δ top2-1* double mutants were grown to mid-log phase before the shift to 37°C. **(C)** *top2-1* single mutants grown to mid-log phase before the shift to 37°C. Cells carry either a control plasmid (blue) or the Artemis-413 expression plasmid (red). **(D)** *top1Δ top2-1* cells expressing the Artemis-413$_{H115A}$ missense mutant that encodes a nuclease-deficient enzyme. The DRR frequency was measured after the shift to 37°C in both late-log/stationary phase (blue) or mid-log phase (green) cells. The box and whisker plots show the distribution of DRR frequencies with the median frequency indicated by a black bar within each box. Distributions were compared by Mann-Whitney U tests to determine p-values. **(E)** The dependence on replication may indicate a mechanism where Artemis-induced single-stranded nicks result in replication fork collapse. Increased torsional stress upon loss of Top1 and Top2 will create non-B DNA structures with ss/dsDNA boundaries that Artemis (represented by a yellow Pac-Man) can cleave. This can create single-stranded nicks ahead of an approaching replication fork leading to fork collapse and formation of single-ended DNA DSBs. Depending on the mechanism of replication restart, this can lead to increased DRR frequencies.

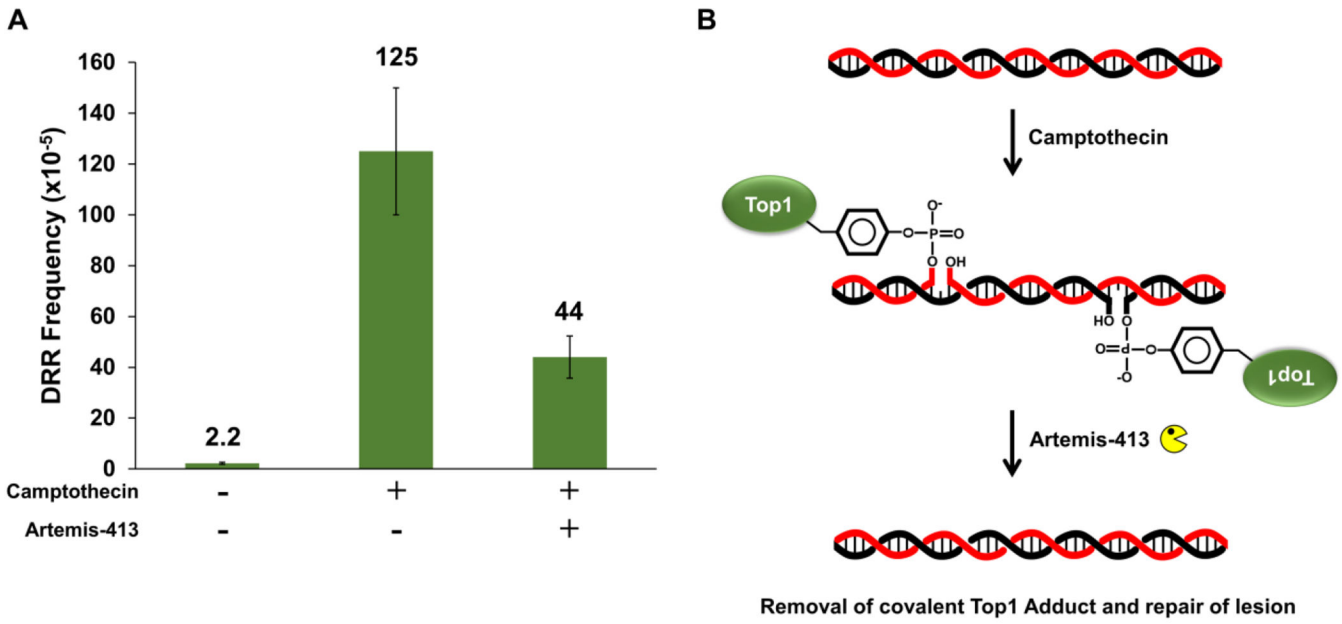

Fig. 5. Artemis-413 expression decreases DRR in camptothecin-treated cells.

(A) The median DRR frequency was determined in wild-type cells grown at 30°C and treated with 30 μM camptothecin either with or without Artemis-413 expression. The median DRR frequency ($x10^{-5}$) is indicated. Error bars represent a 95% confidence interval. **(B)** Treatment with the Top1 poison camptothecin causes Top1 to be trapped on the DNA. Following hydrolysis of the phosphate backbone by the active-site tyrosine, Top1 becomes covalently bound to the 3' phosphate with camptothecin preventing re-sealing of the nick and Top1 release. Artemis expression decreases the number of DSBs induced by camptothecin, suggesting that it can somehow process the DNA with the covalently trapped Top1 before a DSB occurs. Previous biochemical data (3–7) suggests this may occur by Artemis cleavage upstream of the lesion at a ss/dsDNA boundary. Whether this activity of Artemis occurs in cooperation with tyrosyl-DNA phosphodiesterase I (Tdp1) in removing the actual adduct or is part of an independent mechanism following adduct removal is not yet known.