

Fork rotation and DNA precatenation are restricted during DNA replication to prevent chromosomal instability

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Supporting Information

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SI Materials and Methods

Yeast Strains. Yeast containing *top2-td* was derived from W303-1a (*MATa ade2-1 ura3-1 his3-11, trp1-1 leu2-3, can1-100*) (14); *top2-4* cells were derived from ref. 15. Full genotypes are listed in Table S1.

Media and Cell-Cycle Synchronization. *Top2-td* cell cultures for alpha factor release experiments were prepared as described previously (2). For plasmid experiments with *top2-4* strains, yeast cells were grown in minimal media, 2% glucose selecting for the plasmid (–ura or –trp) to log phase at 25 °C before transferring to YP, +ade, 2% glucose and grown to midlog phase. Cells were then arrested in G1 with 10 µg/mL alpha factor until 90% of cells were unbudded (120 min). The culture was incubated at 37 °C for 1 h and cells were released from the block into YP, +ade, 2% glucose. Time 0 was designated as the time of the addition of the first wash. Nocodazole was added to cultures at 10 µg/mL. Samples were taken at the indicated time points, pelleted, and frozen on dry ice.

Flow Cytometry Analysis. For FACS analysis, cells were prepared as described previously (14).

DNA Preparation. DNA was extracted as described in Baxter et al. (13).

Gel Electrophoresis for Detection of Plasmid Catenation. For catenation 2D gels, the DNA was nicked with either Nb.BsmI or Nb.BsrDI (NEB) according to the manufacturer's instructions.

Nicked catenanes were separated in the first dimension on a 0.4% agarose (MegaSieve; Flowgen) gel in 1× TBE at 1.2 V/cm for 13–17 h at room temperature. The respective lanes were excised and embedded into a 0.8–1.2% (depending on plasmid size) agarose (MegaSieve; Flowgen) gel and run at 2–4.8 V/cm in 1× TBE (in the cold room if more than 2 V/cm was used). Nonradioactive Southern blotting and detection were carried out as described (3). Blots were probed with either DNA amplified

from sequences of pRS316 including the URA3 sequences or from pRS314 including the TRP1 gene as appropriate. Nonsaturating exposures of the blot were acquired by ImageQuant LAS 4000 (GE Healthcare), and densitometry analysis was carried out using ImageQuant TL software. Overexposed images were taken to clearly identify the CatAn = 1 signal, which was often weak in nonsaturating exposures.

Protein Extraction and Analysis. Whole-protein extracts were prepared by alkaline lysis followed by TCA precipitation. Cells were resuspended in lysis buffer (1.85 M NaOH, 7.5% β-mercaptoethanol) and incubated on ice for 15 min. Proteins were precipitated with TCA (6.4% final concentration) on ice for 10 min. Precipitates were resuspended in HU buffer (8 M urea, 5% SDS, 200 mM Tris-HCl, pH 6.8, 1 mM EDTA, 1.5% DTT, 0.1% bromophenol blue) and separated by 15% SDS/PAGE. Phosphorylation of S129 of H2A and PGK1 expression were detected using an antibody against H2AP (Abcam; ab15083) and anti-PGK1 (Invitrogen; 459250). Detection of PCNA ubiquitylation was as described in Karras and Jentsch (34) using an anti-PCNA antibody [5E6/2] (Abcam; ab70472).

Rad53 Kinase Assay. In situ autophosphorylation assay was carried out as described (26).

Chromatin Immunoprecipitation of H2A S129 Phosphorylation. Synchronized cultures were fixed with 1% formaldehyde for 10 min at room temperature before immunoprecipitation with an anti-phospho-H2A S129 antibody (Abcam; ab15083). ChIP assays were performed essentially as in ref. 36. Immunoprecipitated and input DNA was quantified by quantitative PCR. Enrichment was analyzed at tRNA loci tI(AAU)N1 and tA(UGC)L and within the euchromatic loci *AS11* and *HXT7* (primer sequences are available upon request). ChIP signal was normalized to amplification from input DNA and expressed relative to wild-type. Data shown are the mean enrichment ±1 SD.

Supplemental information for Introduction;
Topological stress generation and relaxation
during elongation of DNA replication

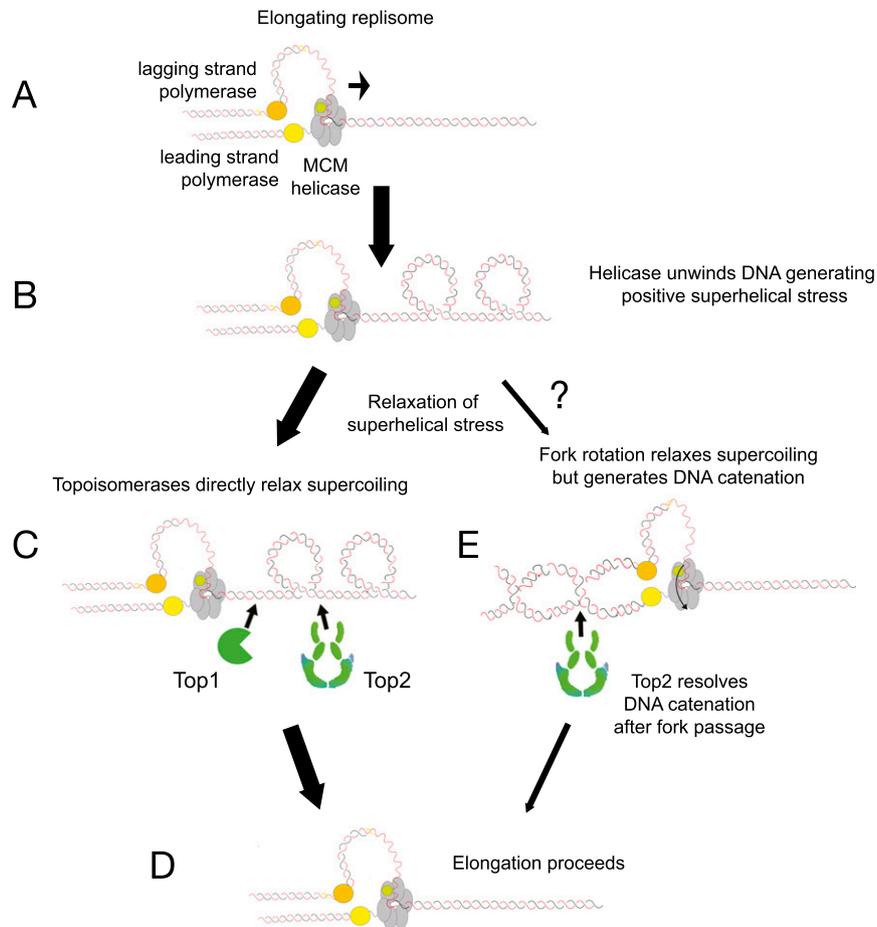
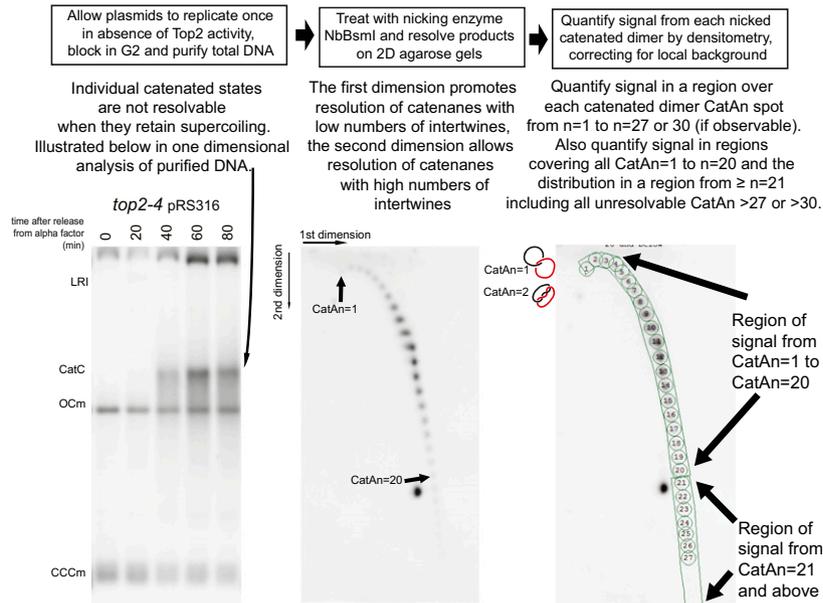


Fig. S1. Model of topological stress generation and relaxation during elongation of DNA replication. (A) During elongation, unwinding of the parental template separates the parental strands but does not resolve the linkages that exist between the two strands. (B) The linkages between the strands are displaced into the region ahead of the fork, leading to this becoming overwound, that is, positively supercoiled. (C and D) This tension is normally resolved by the action of either topoisomerase I (Top1) or topoisomerase II (Top2) (C), which act effectively as "swivelases" ahead of the fork to generate a relaxed replication region (D). (E) However, Champoux and Been (4) proposed a second mode of topological stress unwinding where the helical tension is relaxed by rotation of the fork to generate DNA precatenation behind the fork. Although these linkages should not arrest forward elongation of replication, it is essential that the type II topoisomerase resolve all DNA catenation before the completion of cell division.

Analysis of replication fork rotation by resolution of individual catenation states and quantification of relative strengths of the signal from each state



- Calculate 1: Relative intensities of all signals associated with region 1 to 27 correcting for background
- 2: Quantify relative amount of all signal in arc including regions 1 to 20 and 21 and above correcting for background.
- 3: Calculate median of entire distribution by relating signal in regions 1 to 20 to total signal of large region 1-20 and determining position along arc where 50% of total signal (total signal in regions 1 to 20 and 21 and above) has accumulated.
4. Take average of at least 3 experiments for quantifying differences in DNA catenation of plasmids and at least twice for quantifying differences between different genetic backgrounds (See Table S2 for exact number of replicates for each experiment).

Fig. S2. Quantification of fork rotation during DNA replication. In wild-type cells, Top2 resolves both precatenanes and catenanes formed during DNA replication. Therefore, to assay how often fork rotation and precatenation occur during replication on a plasmid replicon, we allowed one round of replication in the absence of Top2 activity and collected DNA from the cells blocked in G2 by nocodazole 80 min after release into the cell cycle (plasmid products are shown; *Left*). The catenated products of replication in this background are negatively supercoiled due to nucleosome deposition in the sister chromatids. This supercoiling of deproteinized plasmids normally compacts all catenated states into an unresolvable population. To resolve individual catenated states, the purified DNA is treated with a site-specific nicking enzyme to remove supercoiling but maintain the catenated nodes. The nicked DNA is resolved by agarose gel electrophoresis in two separate dimensions to be able to resolve both low- and high-catenated states before Southern blotting and probing for plasmid sequences (*Middle*). The relative intensities of each state were then calculated by densitometry in two ways. The first was by quantifying signal in equally sized regions centered on each catenated signal state on states 1–27 (or 1–30 for hypercatenated samples where the signal >27 was definable) and correcting for local background, and then expressing the relative intensity as a percentage of the total signal in all regions. The second measure was to compare the signal from the arc related to states 1–20 to states 21 and above. For this measure, regions were drawn around all states 1–20 and from the remainder of the arc relating to 21 and above. Then, each set of states is expressed as a percentage of the sum of both. This measure has the advantage of being able to quantify the signal from the individually unresolvable catenated states that produced detectable signal.

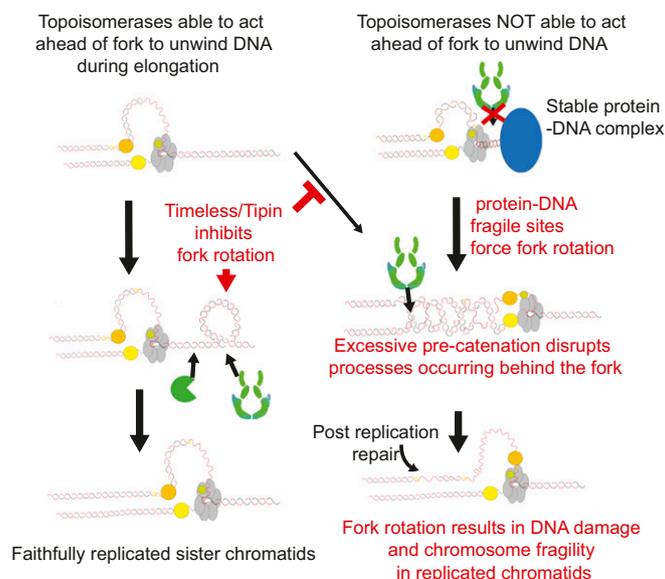


Fig. S9. Model of causes and consequences of fork rotation and precatenation. During normal DNA replication the Tof1/Csm3 structured replisome inhibits fork rotation, and topoisomerases act ahead of the fork (*Left*). In certain genomic contexts, such as stable protein–DNA structures, topoisomerases are impeded from acting ahead of the fork (*Right Top*). Here fork rotation aids unwinding, resulting in elevated precatenation. Excessive precatenation could impede several processes, including Okazaki fragment maturation, resulting in gaps in the newly replicated chromosomes that require repair and leave chromatids fragile.

Table S1. Summary of DNA catenation quantification experiments

Strain	Plasmid size, bp	Number of replicates	Median CatAn	% >20
<i>top2-4 pRS316</i>	4,887	5	13	14 ± 4
<i>top2-4 8kb pRS316</i>	7,936	3	12	14 ± 1
<i>top2-4 12kb pRS316</i>	12,391	3	13	21 ± 1
<i>top2-4 pR426</i>	5,726	5	12	8 ± 2
<i>top2-4 tRNApRS316</i>	6,060	7	16	28 ± 4
<i>top2-4 1ori Ylplac</i>	5,640	4	12	12 ± 2
<i>top2-4 7ori Ylplac</i>	6,144	5	16	35 ± 6
<i>tof1Δ top2-4 pRS316</i>	4,887	2	>30	89 ± 2
<i>tof1Δ top2-4 pRS426</i>	5,726	2	>30	93 ± 1
<i>tof1Δ top2-4 tRNApRS316</i>	6,060	2	>30	95 ± 2
<i>csm3Δ top2-4 pRS316</i>	4,887	2	>30	89 ± 2
<i>mrc1Δ top2-4 pRS316</i>	4,887	2	12	10 ± 2
<i>ctf4Δ top2-4 pRS316</i>	4,887	2	12	17 ± 7
<i>rrm3Δ top2-4 pRS316</i>	4,887	2	14	23 ± 6
<i>rrm3Δ top2-4 pRS426</i>	5,726	2	12	10 ± 6
<i>rrm3Δ top2-4 tRNApRS316</i>	6,060	2	18	38 ± 2
<i>sgs1Δ top3Δ top2-4 pRS316</i>	4,887	2	12	17 ± 1
<i>top2-4 pRS316 + 200 mM HU</i>	4,887	2	11	11 ± 1

The median was calculated as explained in Fig. S2.

