

Video Article

Genetic Studies of Human DNA Repair Proteins Using Yeast as a Model System

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Abstract

Understanding the roles of human DNA repair proteins in genetic pathways is a formidable challenge to many researchers. Genetic studies in mammalian systems have been limited due to the lack of readily available tools including defined mutant genetic cell lines, regulatory expression systems, and appropriate selectable markers. To circumvent these difficulties, model genetic systems in lower eukaryotes have become an attractive choice for the study of functionally conserved DNA repair proteins and pathways. We have developed a model yeast system to study the poorly defined genetic functions of the Werner syndrome helicase-nuclease (*WRN*) in nucleic acid metabolism. Cellular phenotypes associated with defined genetic mutant backgrounds can be investigated to clarify the cellular and molecular functions of *WRN* through its catalytic activities and protein interactions. The human *WRN* gene and associated variants, cloned into DNA plasmids for expression in yeast, can be placed under the control of a regulatory plasmid element. The expression construct can then be transformed into the appropriate yeast mutant background, and genetic function assayed by a variety of methodologies. Using this approach, we determined that *WRN*, like its related RecQ family members BLM and Sgs1, operates in a Top3-dependent pathway that is likely to be important for genomic stability. This is described in our recent publication [1] at www.impactaging.com. Detailed methods of specific assays for genetic complementation studies in yeast are provided in this paper.

Video Link

The video component of this article can be found at <http://www.jove.com/video/1639/>

Protocol

1. Yeast Strains

Strains with wild-type *SGS1 TOP3* (WT; W303-1A, genotype, *MATa ade2-1 can1- 100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) [2], a *sgs1* mutant (W1292-3C; genotype *MATa SUP4-o::URA3 sgs1-25 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*) and a *sgs1 top3* mutant (W1058-11C, genotype, *MATa SUP4-o::URA3 sgs1-25 top3-2::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*) have been characterized [3] and were kindly provided by Dr. Rodney Rothstein (Columbia University).

2. Plasmid DNA constructions

The *WRN* gene was cloned in YEp112SpGAL following the scheme as discussed.

1. Site-directed mutations of *WRN* (E84A exonuclease dead, K577M helicase dead, R834C, and K1016A) in the plasmid YEp195SpGAL-*WRN* [4] were constructed using mutagenic primers and a standard protocol from Quickchange II XL site-directed mutagenesis kit (Stratagene) by Lofstrand labs (Gaithersburg, MD).
2. DNA fragments encoding *WRN* and its associated variants were gel purified from the respective YEp195SpGAL-*WRN* constructs after double digestion with *Sall* and *MluI*.
3. Gel purified fragments were then cloned into the *Sall* *MluI* sites of vector YEp112SpGAL, a 2 μ m multi-copy plasmid containing a *TRP1* selectable marker to construct YEp112SpGAL *WRN* or *WRN* variants under the control of a gal-inducible promoter as shown in Figure 1.
4. The constructs so obtained were named as YEp112SpGAL-*WRN*, YEp112SpGAL-*WRN* E84A, YEp112SpGAL-*WRN* K577M, YEp112SpGAL-*WRN* K1016A, and YEp112SpGAL-*WRN* R834C.

3. Transformation

Yeast cultures were grown using standard protocol and transformations were performed using a Lithium Acetate-based protocol by Gietz *et al* [5]. Briefly, the following steps were performed.

1. Cultures of the above mentioned strains were grown overnight in 5 ml YPD (yeast extract-peptone-dextrose) medium. Cultures were reinoculated in 50 ml YPD medium and were allowed to grow until an OD₆₀₀ of 1.0 - 1.2 was reached.
2. Cultures were harvested, washed with 20 ml water, and resuspended in 1 ml of 100 mM lithium acetate (LiAc). Cells were incubated at 30°C for 10 min.
3. Cell pellet was resuspended in 100 mM LiAc to a final volume of 500 µl, which is enough to do ten transformations. 50 µl of the cell suspension was aliquoted and cell pellet was collected by centrifugation.
4. To the cell pellet was added in the following order: 250 µl 50% Polyethylene glycol (PEG), 36 µl of 1 M LiAc, 25 µl of 2 mg/ml ssDNA (salmon sperm DNA), 5 ml of plasmid DNA (100 ng 1000 ng), and 50 µl water. Cell pellet was vortexed for 1 min.
5. Cells were then incubated at 30°C for 30 min followed by a heat shock at 42°C for 20 min.
6. Cells were harvested and resuspended in 1 ml water. Since the plasmid YEp112SpGAL contains *trp* as a selectable marker, all the mutant strains harboring the construct would be *trp* proficient. Therefore, transformants were selected by plating the suspension on SC (Synthetic complete) glucose media lacking *Trp* and incubating the plates at 30°C for 3-4 days until the colonies appear.

4. Genetic analysis of the slow growth phenotype restoration in *WRN* transformed *sgs1 top3* strain.

1. Streak analysis:

1. To examine the effect of *WRN* expression on the growth of wild-type parental (W303-1A), *sgs1*, or *sgs1 top3* strains, the corresponding strains with YEp112SpGAL or YEp112SpGAL-*WRN* were streaked onto SC minus *Trp* plates containing either 2% glucose (glu) or 2% galactose (gal). As a control, *sgs1 top3*/YEp112SpGAL-SGS1 was included. Plates were incubated at 30°C for 2 days.
2. To determine the ability of *WRN* to affect growth rate of *sgs1 top3* strain at lower *WRN* protein expression levels, the *sgs1 top3* strain transformed with YEp112SpGAL, YEp112SpGAL-*WRN*, or YEp112SpGAL-SGS1 was streaked onto SC minus *Trp* plates containing varying concentrations of gal from as low as 0.005% to as high as 2%. Plates were then incubated at 30°C for 2 days.
3. To assess the functional requirements of *WRN* to restore the slow growth phenotype in the *sgs1 top3* background, *sgs1 top3* transformed with YEp112SpGAL-*WRN* (E84A, K577M, R834C, and K1016A) were streaked onto SC minus *Trp* plates containing either glu or gal at the indicated concentrations. For controls, *sgs1 top3* strain transformed with YEp112SpGAL, YEp112SpGAL-*WRN* or YEp112SpGAL-SGS1 was included. Plates were then incubated at 30°C for 2 days.

2. Liquid culture analysis:

1. To evaluate the growth of transformed *sgs1 top3* strains in liquid culture, yeast cells were grown in SC raf minus *Trp* at 30°C overnight. Cultures were reinoculated in SC raf minus *Trp* and grown at 30°C to early log phase (OD₆₀₀ of ~ 0.5). Cultures were then reinoculated at OD_{0.05} in SC media containing 2% gal and growth was followed by measuring absorbance at the indicated time intervals.

5. Genetic analysis of hydroxyurea or methylmethane-sulfonate sensitivity in *WRN* transformed yeast strains.

1. To determine the effect of *WRN* expression on the MMS and HU sensitivity of *sgs1* or *sgs1 top3* strains, strains transformed with YEp112SpGAL or YEp112SpGAL-*WRN* were grown in SC raf minus *Trp* at 30°C.
2. Cultures were reinoculated in SC raf minus *Trp* and grown at 30°C to early log phase (OD₆₀₀ of ~0.6 to 0.8).
3. Ten-fold serial dilutions of these strains were prepared using SC medium lacking *Trp*. From an exponentially growing culture, 5 x 10⁶ cells were taken and diluted serially up to 10,000-fold. Four microliters of each dilution was spotted onto SC gal minus *Trp* plates containing the indicated concentrations of HU or MMS. Plates were incubated at 30°C.
4. As a control, wild-type parental strain (W303-1A) transformed with YEp112SpGAL or YEp112SpGAL-*WRN*, or *sgs1 top3*/YEp112SpGAL-SGS1 was treated as described above.

6. Cell cycle distribution of *WRN* transformed *sgs1 top3* strain.

1. To study the cell cycle distribution of *WRN* transformed *sgs1 top3* strain, cultures of *sgs1 top3* transformed with YEp112SpGAL, YEp112SpGAL-*WRN*, or YEp112SpGAL-SGS1, and the vector-transformed wild-type parental strain (W303-1A) were grown in SC raf minus *trp* at 30°C for overnight.
2. Cultures were reinoculated at OD_{0.05} and allowed to grow til early log phase (OD₆₀₀ of 0.5 -0.6). *WRN* expression was induced by adding gal to 2% final concentration. Cultures were then allowed to grow at 30°C for 6 h.
3. Cultures were then processed for DAPI (4', 6-diamidino-2-phenylindole) staining.
 1. Cultures were harvested by centrifugation at 5800 x g for 10 min at room temperature (RT).
 2. Cells were washed once with 1X phosphate-buffered saline (PBS) and were then fixed in 70% ethanol for 20 min at RT.
 3. Fixed cells were washed twice with 1 X PBS, and finally resuspended in 1X PBS.

- Cells were placed on slide and mounted in Vectashield mounting medium with DAPI (1 µg/ml) (Vector, USA). Cells were examined with an Axiovert 200 M microscope (Zeiss; 100x lens) and composite differential interference contrast (DIC). Fluorescence images were analyzed using AxioVision, version 3.0 program (Zeiss).

7. Western blot analyses.

1. To determine the protein expression of *WRN* or the *WRN* variants in transformed *sgs1* or *sgs1 top3* strains, lysates were prepared by the following method and proteins analyzed by immunoblotting.

- Transformed *sgs1* or *sgs1 top3* strains were grown in SC raf minus Trp at 30°C for overnight.
- Cultures were reinoculated and incubated at 30°C until early log phase (OD_{600} of 0.5 - 0.8) and then induced by adding gal to 2% final concentration. Cultures were then allowed to grow at 30°C for 6 h.
- Cells (3 ml) were collected by centrifugation and washed with PBS. Cell pellet was resuspended in alkaline lysis buffer [50 mM NaOH, pH 10.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and protease inhibitors (Roche Molecular Biochemicals)], boiled for 5 min, clarified by centrifugation, and neutralized with 1 M HCl.
- Proteins from equivalent amounts of cell lysate were resolved on 8-16% polyacrylamide SDS gels.

2. To determine the level of expression of *WRN* protein under the influence of varying concentrations of galactose, cultures were processed as follows.

- Transformed *sgs1 top3* strains were grown in SC raf minus Trp at 30°C for overnight.
- Cultures were reinoculated and incubated at 30°C until early log phase (OD_{600} of 0.5 - 0.8) and then induced by adding gal to 2% final concentration. Cultures were then allowed to grow at 30°C for 6 h.
- Cells were harvested by centrifugation and resuspended in buffer (50 mM Tris-Cl, pH 7.5, 1% sodium deoxycholate, 1% triton X-100, 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitors (0.05% PMSF, 0.05 µg/ml leupeptin).
- Glass beads (425 to 600 µm) were added to the mixture, and the cells were broken by vortexing.
- The resulting homogenate was centrifuged for 5 min at 15000 x g and the supernatant fraction was collected.
- Protein in the lysate fractions was estimated by Bradford method.
- Equal concentration of protein from different lysate fractions was resolved on 8-16% polyacrylamide SDS gels.

3. Expression of *WRN* or *WRN* mutant proteins was determined by Western blot using a *WRN* mouse monoclonal antibody directed against an epitope in a purified C-terminal fragment of *WRN* [6] (1:1000, Spring Valley Labs).

4. Primary antibody incubation was followed by incubation with a secondary (horseradish peroxidase-conjugated) antibody (1:5000). Blots were processed with an ECL Plus Western detection system per the manufacturer's protocol.

5. For quantitative Western blot analysis, increasing concentrations of purified recombinant His-tagged full-length *WRN* protein were included on gels such that upon quantification of the blot a standard linear curve could be generated. Further, cell extract from equivalent amount of cells or 20 µg of the yeast lysate samples were loaded on gels to estimate the concentration of *WRN*.

6. ImageQuant analysis was then performed on gels and standard linear curve was generated.

7. Concentration of *WRN* protein at varying gal concentrations was estimated using standard linear curve generated above.

8. Representative Results

All strains used in this study are Trp auxotrophs. Therefore, *sgs1*, *sgs1 top3* and wild type W303-1A strains transformed with YEp112SpGAL or YEp112SpGAL-*WRN* were selected on the basis of their ability to grow in presence of SC minus Trp media. To examine the effect of *WRN* expression on the growth of transformed *sgs1 top3* mutant cells, the cultures were streaked onto plates containing SC minus Trp media with 2% gal to induce *WRN* expression. Top3 decatenates intertwined DNA molecules generated by Sgs1 helicase during replication [1, 2]; therefore, in the absence of Top3, torsional stress is not relieved resulting in slow growth and hyper-recombination. The genetic function of *sgs1* is to suppress the slow growth phenotype of a *top3* mutant. If *WRN* could substitute for *SGS1* in genetic interaction with Top3, restoration of *top3* slow growth phenotype in *sgs1 top3* would be expected.

As shown in Figure 2A, the *WRN* transformed *sgs1 top3* strain grew significantly more slowly compared to the vector transformed *sgs1 top3* strain. *sgs1 top3* transformed with the YEp112SpGAL-*SGS1* plasmid was included as a positive control (Figure 2A), demonstrating that wild-type Sgs1 expressed in the *sgs1 top3* mutant was able to genetically complement the growth phenotype. The transformed *sgs1 top3* strains grew similarly in the absence of gal as shown for 2 days (Figure 2A). Expression of *WRN* had no effect on the growth of parental wild-type (W3031A) or *sgs1* strains (Figure 2B), indicating that the effect of *WRN* on cell growth was specific to that observed in the *sgs1 top3* mutant background. Genetic studies performed using *WRN* variants demonstrated that *WRN* helicase but not exonuclease activity was required for the restoration of *top3* growth phenotype (Figure 3B). A naturally occurring missense polymorphism in *WRN* that interferes with helicase activity abolished its ability to restore *top3* slow growth phenotype.

top3 mutant strains are delayed in the late S/G2 phase of the cell cycle [1], a characteristic that may account for their slow growth. Mutation of the *SGS1* gene in the *top3* background suppresses the delay in the S/G2 phase of the cell cycle. If *WRN* expression could restore the delay in the S/G2 phase of the cell cycle in *sgs1 top3*, an elevated population of large budded cells with undivided nuclei would be expected for *sgs1 top3* mutant cells expressing *WRN*. As shown in Figure 4, the percentage of large budded cells was higher for *sgs1 top3/WRN* than *sgs1 top3/vector*, suggesting the restoration of the S/G2 delay characteristic of *top3*.

The *sgs1 top3* double mutant is less sensitive to MMS or HU than the *top3* single mutant [2]. Since *WRN* affected cell growth of *sgs1 top3*, we next examined its effect on sensitivity to methylmethane sulfonate (MMS, an alkylating agent) and hydroxyurea (HU, a replication inhibitor). *sgs1 top3 / WRN* displayed sensitivity to both the drugs comparable to *sgs1 top3/ SGS1* (Figure 5). Genetic analysis of *WRN* variants revealed that *WRN* helicase /ATPase, but not *WRN* exonuclease activity, was required for its effect on sensitivity to MMS and HU.

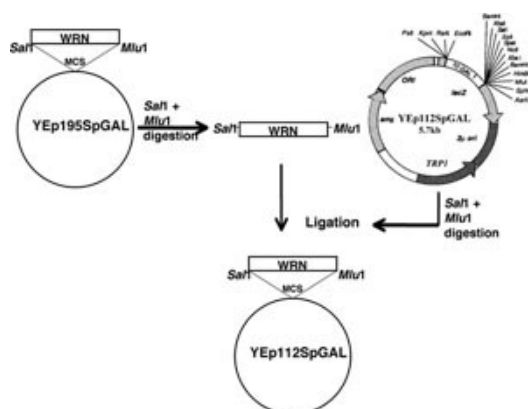


Figure 1. Schematic presentation for cloning *WRN* / *WRN* variants in YEp112SpGAL vector. Please [click here](#) for a larger version of figure 1.

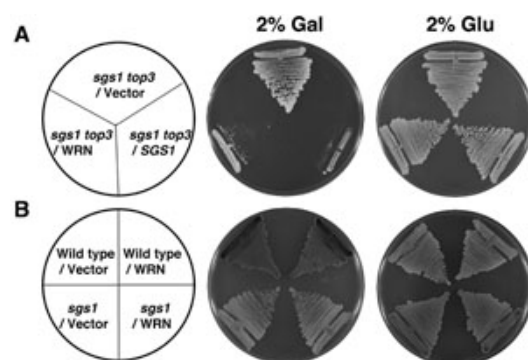


Figure 2. *WRN* expression in *sgs1 top3* restores the slow growth phenotype of *top3*. Panel A, *sgs1 top3* strain transformed with YEp112SpGAL or YEp112SpGAL *WRN* were streaked on an SC-Trp plate containing either 2% glu or 2% gal. As a control *sgs1 top3* strain transformed with YEp112SpGAL *SGS1* was streaked on both the plates. Plates were incubated at 30°C for 2 days and then photographed. Panel B, Wild type parental strain W303-1A or *sgs1* strain transformed with YEp112SpGAL or YEp112SpGAL *WRN* were streaked on an SC-Trp plate either containing 2% glu or 2% gal. Plates were incubated at 30°C for 4 days and then photographed. Composition of the plates was as in Panel A and Panel B respectively. Please [click here](#) to see a larger version of figure 2.

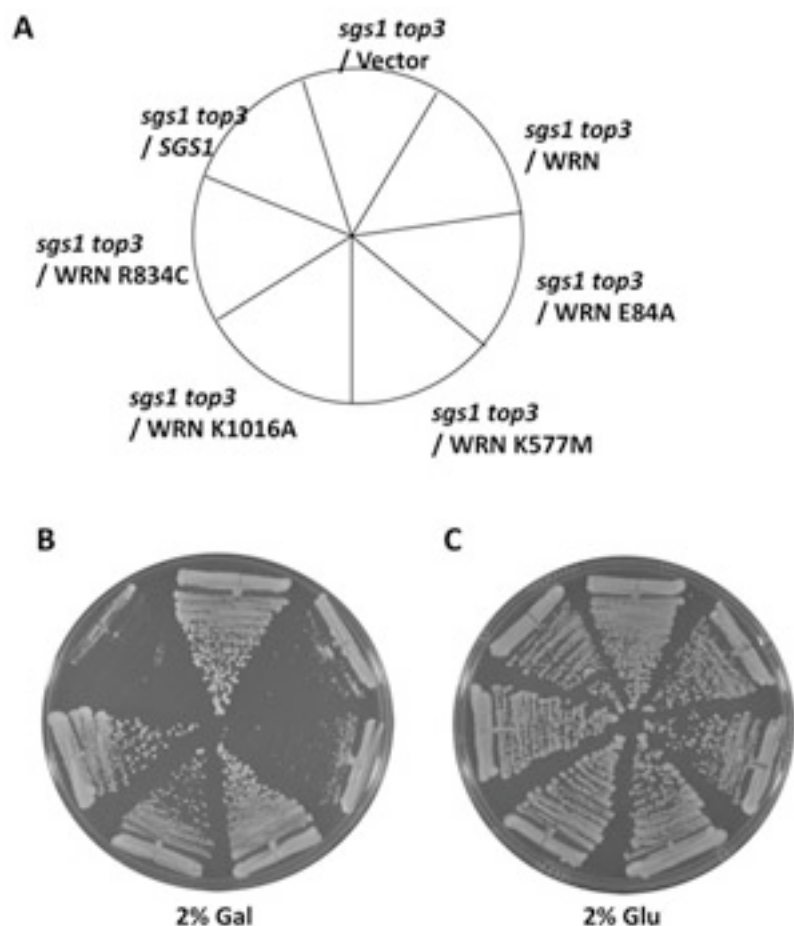


Figure 3. WRN ATPase/helicase, but not exonuclease activity, is required to restore the slow growth phenotype of *top3* in *sgs1 top3* background. *sgs1 top3* strain transformed with ATPase/helicase-dead (YEp195SpGAL *WRN* K577M), exonuclease-dead (YEp195SpGAL-*WRN* E84A), RQC mutant (YEp195SpGAL-*WRN* K1016A), or polymorphic mutant (YEp195SpGAL-*WRN* R834C) was streaked on SC-Trp plates containing either 2% glu (Panel C) or 2% gal (Panel B). Plates were incubated at 30°C for 2 days and then photographed. Composition of the plates was as in Panel A. Please [click here](#) to see a larger version of figure 3.

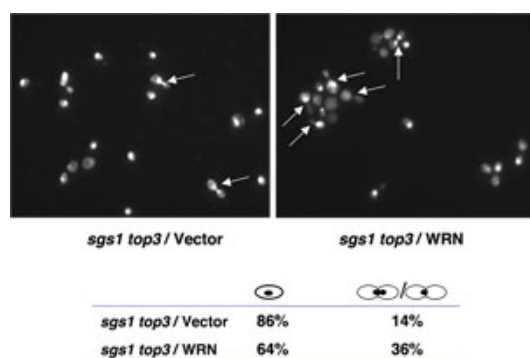


Figure 4. WRN expression induces S/G2 arrest in *sgs1 top3* cells. Logarithmically growing cultures of *sgs1 top3* strain transformed with YEp112SpGAL, YEp112SpGAL *WRN*, or YEp112SpGAL *SGS1*, and the vector-transformed wild-type parental strain were induced at 2% gal concentration for 6 h. Cultures were harvested, processed for DAPI staining as described in Materials and Methods and were observed using Axiovert 200 M microscope (Zeiss; 100x lens). Shown is the DAPI staining of the *sgs1 top3* transformed with YEp112SpGAL (upper left) and with YEp112SpGAL *WRN* (upper right). Arrows show cells with undivided nuclei. Distribution of the cells in G1 (single cells) and S/G2 (budded cells) is shown in lower panel. Please [click here](#) to see a larger version of figure 4.

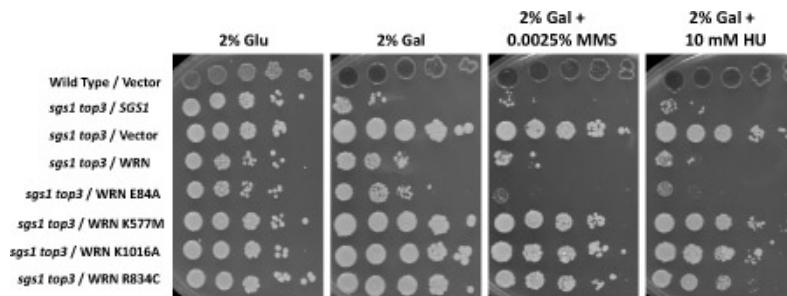


Figure 5. Effect of *WRN* expression on the MMS and HU sensitivity of *sgs1 top3* strain. Logarithmically growing cultures of *sgs1 top3* strain transformed with YEp112SpGAL, YEp112SpGAL *WRN*, exonuclease-dead (YEp112SpGAL-*WRN* E84A), ATPase/helicase-dead (YEp112SpGAL *WRN* K577M), RQC mutant (YEp112SpGAL-*WRN* K1016A), polymorphic mutant (YEp112SpGAL-*WRN* R834C), YEp112SpGAL *SGS1* and vector transformed wild type parental strains were spotted in a ten-fold serial dilutions onto SC-Trp plates containing glu or gal and either MMS or HU at the indicated concentrations. Plates were incubated at 30°C for 2 days (control plates) and 4 days (MMS and HU plates) and then photographed. Please [click here](#) to see a larger version of figure 5.

Discussion

One of the strengths of using yeast as a model system is the availability of mutants in defined DNA replication and repair pathways that are conserved between yeast and humans. Further selection of transformants harboring the specific genes is easy and reliable as the laboratory strains are auxotrophic mutants and vectors with auxotrophic markers are readily available. Using these vectors the expression of gene products can be regulated by placing them under the control of an inducible promoter (e.g., gal inducible promoter, etc.). Considering these advantages, we developed a yeast based model system to study the functional requirements of the human *WRN* gene defective in Werner Syndrome in a pathway that is potentially conserved between human and yeast. Using the described approaches, we provided the first evidence that *WRN* can function in a genetic pathway that affects *top3*-related phenotypes. Our observations prompt further investigation of the possibility that *WRN* functionally interacts with Top3 α in human cells during cellular DNA replication or recombination. Conceivably, in a BLM-impaired condition, *WRN* may partially substitute for BLM through its protein partnership with a topoisomerase.

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