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Does interference between replication and transcription contribute to genomic instability in cancer cells?

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Abstract

We have recently reported that topoisomerase 1 (Top1) cooperates with ASF/SF2, a splicing factor of the SR family, to prevent unscheduled replication fork arrest and genomic instability in human cells. Our results suggest that Top1 execute this function by suppressing the formation of DNA-RNA hybrids during transcription, these so-called R-loops interfering with the progression of replication forks. Using ChIP-chip, we have shown that γ-H2AX, a marker of DNA damage, accumulates at gene-rich regions of the genome in Top1-deficient cells. This is best illustrated at histone genes, which are highly expressed during S phase and display discrete γ-H2AX peaks on ChIP-chip profiles. Here, we show that these γ-H2AX domains are different from those induced by camptothecin, a Top1 inhibitor inducing double-strand DNA breaks throughout the genome. These data support the view that R-loops promote genomic instability at specific sites by blocking fork progression and inducing chromosome breaks. Whether this type of transcription-dependent fork arrest contributes to the replication stress observed in precancerous lesions is an important question that deserves further attention.

Keywords: DNA replication – Transcription – Genomic instability – Cancer – ChIP-chip
Introduction

Genomic instability is a hallmark of cancer cells. It is most often manifested in chromosomal instability, which refers to changes in chromosome structure and number \(^1,^2\). A growing body of evidence indicates that genomic instability plays an active role in tumorigenesis by providing the genetic alterations that underlie the multistep process of cancer development \(^3,^4\). It has been recently reported that DNA damage arises in precancerous lesions as a consequence of aberrant oncogene activation. This would induce a constitutive DNA damage response that would select for p53 inactivation and would allow pre-tumoral cells to bypass anti-cancer barriers such as apoptosis and senescence \(^5\). Replication stress has been proposed to be at the origin of spontaneous DNA damage in precancerous lesions \(^6\). However, the links between oncogene activation, replication defects and genomic instability remain poorly understood.

Replication forks frequently stall when they encounter DNA lesions or natural pause sites \(^7\). Arrested forks are fragile structures that are prone to breakage and unscheduled recombination. Studies in model systems such as budding yeast have shown that transcription represents a prominent source of replication fork arrest and genomic instability, especially when DNA and RNA polymerases converge \(^8\)–\(^11\). It is now well established that this instability is mainly caused by RNA-DNA hybrids (R-loops) that form when assembly of mRNA-particle complexes (mRNPs) is perturbed \(^2,^12\). Similar transcription-associated recombination (TAR) processes are also believed to occur in human cells \(^13\) but their incidence in cancer development remains largely unexplored. In a recent report, we have shown that DNA topoisomerase I (Top1) suppresses interference between replication and transcription in mouse and in human cells \(^14\). The mechanism by which Top1 plays this crucial role, the nature of the DNA lesions induced by TAR and their potential implication in the cancer process are discussed in this review.

**Top1 regulates fork progression and pausing**

Top1 is a ubiquitous enzyme that acts at the interface between replication, transcription and RNA maturation. Besides its well-characterized DNA relaxation activity \(^15\), metazoan Top1 contains a protein kinase domain that has been implicated in the phosphorylation of splicing factors of the SR family \(^16,^17\). These activities qualify Top1 as a potential regulator of TAR. However, although Top1 prevents genomic instability in human cells \(^18\), its implication in the coordination between replication and transcription was never directly addressed.
Using a single-molecule approach, we have recently monitored the progression of individual replication forks in human and murine cells expressing low to undetectable levels of Top1. These include human colon carcinoma HCT116 cells stably transfected with a shRNA directed against Top1 and Top1-deficient murine B lymphoma-derived cells. Asynchronous cultures were pulse-labeled with BrdU and individual DNA fibers were stretched on silanized coverslips by DNA combing. This analysis revealed that forks are ~50% slower in Top1-deficient cells, which confirmed that topoisomerase 1 is required for normal fork progression. Interestingly, we also observed a concomitant increase in origin firing in Top1-deficient cells, indicating that cells compensate for slow forks by activating dormant origins.

To determine whether forks pause or stall more frequently in the absence of Top1, control and Top1-deficient cells were labeled with successive pulses of IdU and CldU and progression of sister replication forks was analyzed by DNA combing. In control cells, sister forks progressing from a given origin move at a constant rate and generate symmetrical patterns of IdU and CldU incorporation. In contrast, we detected a 3-fold increase of the frequency of asymmetrical patterns in Top1-cells, indicating that ~25% of the forks pause or stall in the absence of Top1. This increased rate of fork stalling accounts for the higher genomic instability of Top1-deficient cells and correlates with the accumulation of γ-H2AX foci in S phase, which are indicative of DNA damage.

Top1 acts with splicing factors to prevent genomic instability in S phase

In contrast to higher eukaryotes, deletion of Top1 is not lethal in S. cerevisiae and does not significantly affect S-phase progression. Using DNA combing, we found that yeast top1Δ cells also displayed slow forks and increased initiation rate as their mammalian counterparts. However, yeast top1Δ mutants do not show increased fork stalling or genomic instability. These data suggest that a non-canonical function of Top1 that is not conserved in yeast prevents unscheduled fork arrest in mammalian cells. One attractive candidate for this function is the elusive kinase activity of Top1, which is found only in metazoae. In higher eukaryotes, Top1 phosphorylates the alternative splicing factor ASF/SF2 to promote the formation of messenger ribonucleoprotein particles (mRNPs) and the maturation of nascent RNAs. Since mRNP biogenesis prevents the formation of R-loops and since R-loops are toxic for replication forks, we reasoned that fork arrest in mammalian Top1- cells could reflect interference between replication and transcription.
In agreement with this model, we found that chemical inhibition of Top1 kinase with Diospyrin\textsuperscript{43} or depletion of the alternative splicing factor ASF/SF2 by RNA interference mimic the phenotype of Top1-deficient cells (Fig. 1). Conversely, inhibition of transcription elongation with Cordycepin or degradation of R-loops with RNase H suppressed replication defects and DNA damage in Top1-deficient cells (Fig. 1). Together, these data indicate that Top1 promotes the maintenance of genome integrity during S phase by preventing the formation of R-loops in an ASF/SF2-dependent manner\textsuperscript{14}. This is reminiscent of the function of the THO/TREX complex, which suppresses TAR in budding yeast by facilitating mRNP biogenesis during transcription\textsuperscript{12}. Interestingly, a recent genome-wide siRNA screen performed by the Cimprich lab also identified several RNA processing factors as suppressors of genomic instability\textsuperscript{24}. Together with earlier work from Manley and colleagues\textsuperscript{23}, these data suggest the existence of a relationship between defective mRNP biogenesis, replication fork arrest and genomic instability.

Active genes are enriched in $\gamma$-H2AX in the absence of Top1

Top1-deficient human and murine cells accumulate DNA breaks and $\gamma$-H2AX foci in S phase. To test whether these breaks occur at specific sites in the genome, we monitored the distribution of this phosphorylated histone variant by ChIP-chip\textsuperscript{14}. We measured a 3-fold increase in the overall number of loci enriched in $\gamma$-H2AX in Top1-deficient cells relative to control cells. Statistical analysis also revealed a strong correlation between the distribution of $\gamma$-H2AX domains and the position of active genes in Top1-deficient cells. This is best illustrated with the 64 replication-dependent histone genes, which are highly transcribed during S phase and accumulate $\gamma$-H2AX proportionally to their level of expression\textsuperscript{14}. These data support the view that interference between replication and transcription increases in the absence of Top1 and induces DNA damage.

It has been reported that double-strand DNA breaks (DSBs) induce the formation of large $\gamma$-H2AX domains, encompassing several megabases in length\textsuperscript{21, 25}. In contrast, the $\gamma$-H2AX-enriched regions observed in Top1-deficient cells were several orders of magnitude smaller than those found at DSBs\textsuperscript{14}. These small domains are reminiscent of replication pause sites in budding yeast (our unpublished results and\textsuperscript{44}). We therefore propose that the $\gamma$-H2AX regions observed in Top1-deficient cells correspond to transient DSBs that are rapidly repaired or to stalled replication forks inducing a localized phosphorylation of H2AX by the ATR kinase. Although we cannot rule out the possibility that genomic instability in Top1-deficient cells
comes from persistent DSBs, we also believe that rearrangements also arise at stalled forks without formation of DSBs, as it was shown to be the case in other systems 26-30.

To further characterize the nature of γ-H2AX foci that accumulate in the absence of Top1, we exposed shTop1 and shCtrl cells to sublethal doses of camptothecin (CPT), a Top1 inhibitor that induces DSBs during DNA replication by blocking replication forks and the distribution of γ-H2AX-enriched regions was mapped by ChIP-chip on chromosome 1 and 6 as described previously 14. As expected, CPT exposure induced a sharp accumulation of γ-H2AX in control cells, and to a lesser extent in shTop1 cells (Fig. 2B). However, this treatment did not significantly increase the number of localized γ-H2AX domains in control cells (Fig. 2A, C), indicating that CPT-induced DSBs are randomly distributed throughout the genome. In contrast, CPT exposure induced a four-fold increase of γ-H2AX-enriched regions in shTop1 cells (Fig. 2A, C). We assume that this increase is due to further depletion of the residual Top1 pool in shTop1 cells.

Interestingly, although γ-H2AX was further enriched at active genes in shTop1 cells upon CPT treatment (Fig 2D), it was no longer detected at histone genes (Fig. 2E and 3). The expression of replication-dependent histone genes is temporally and functionally linked to DNA replication by transcriptional and posttranscriptional mechanisms 31, 32. It is also rapidly downregulated in response to DNA damage 33, which would explain why γ-H2AX levels drop at histone genes while they rise at neighboring non-histone genes (Fig. 3A). Together with our previous results 14, these data strongly support the view that replication fork stalling in Top1-deficient cells depends on gene expression.

**Unsolved issues and future directions**

The mechanism by which Top1 and ASF/SF2 suppress replication stress at active genes is currently unclear. Although the kinase activity of Top1 is required to prevent fork stalling, our data indicate that the DNA relaxation activity of Top1 is also involved in this process 14. It has been recently reported that Top1 suppresses class switch recombination (CSR) at immunoglobulin genes 34. CSR is induced by transcription and requires the formation of R-loops 35. Induction of DSBs during CSR also depends on AID, an enzyme that downregulates Top1 expression 34. Interestingly, AID is induced by DNA damage and is implicated in translocations at non-immunoglobulin genes 36, 37. Whether Top1 is also involved in AID-induced translocations is an important question that remains to be addressed.
Another important question concerns the link between Top1 and common fragile sites (CFSs). CFSs are regions of the genome that are particularly susceptible to break when replication is impeded and that contribute to gene amplification and chromosomal rearrangements\textsuperscript{38,39}. We have found that FRA3B, FRA16D and FRAXB, three major human CFSs, are frequently broken in human Top1-deficient cells\textsuperscript{14}. We believe that this increased fragility is not solely due to slower replication forks but also to altered mRNPs biogenesis at CFSs. The best-characterized CFSs are associated with very large genes\textsuperscript{40}, which is consistent with the view that breakage is induced by a TAR-related process. To test this hypothesis, the role of ASF/SF2 on CFS expression should be addressed in normal and Top1-deficient cells. If Top1 and ASF/SF2 act in the same pathway to prevent CFS expression, we expect the effect of their co-depletion not to be additive.

Finally, it has been proposed that overexpression of oncogenes in the early stages of the cancer process interferes with the proper assembly of pre-RCs and reduces the number of licensed replication origins\textsuperscript{41,42}. Since TAR depends on the relative orientation of replication and transcription, changes in the position of active origins are expected to affect the direction of fork progression relative to genes and therefore increase TAR. It is therefore tempting to speculate that transcription-associated replication stress acts synergistically with oncogene-induced alteration of the replication program to promote genomic instability in cancer cells.

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References


Figure Legends

Figure 1: Model for the role of Top1 in the regulation of replication/transcription interference. In control cells (Top1+), topoisomerase1 cooperates with ASF/SF2 to prevent the formation of DNA-RNA hybrids (R-loops). In Top1- cells, R-loops are formed and lead to replication fork stalling, phosphorylation of H2AX and genomic instability. Treatment of Top1+ cells with diospyrin (an inhibitor of Top1 kinase activity) or with a siRNA targeting ASF/SF2 mimics a Top1- phenotype. Treatment of Top1- cells with cordycepin (a transcription inhibitor) or transfection with RNaseH to degrade R-loops restores a wild type phenotype.

Figure 2: γ-H2AX is enriched at active genes in Top1- HCT116 cells. (A) Untreated (-CPT) or CPT-treated (40 µM, 1 hour; +CPT) HCT116 cells expressing control (shCtrl) or Top1 (shTop1) siRNAs were harvested by Trypsin treatment, washed with PBS, and fixed with 1% formaldehyde in PBS during 10 minutes at room temperature. Chromatin was immunoprecipitated with a phoso-specific antibody that recognizes γ-H2AX and DNA was hybridized on Affymetrix high-density tiling arrays (35 bp resolution) covering human chromosomes 1 and 6, as described previously 14. γ-H2AX enrichment is represented as heat maps, yellow areas corresponding to high enrichment. Local gene density (exons) is indicated above chromosome maps. R-bands: light grey, G-bands: dark grey. The position of the three major histone genes clusters is indicated in red. (B) Western blot analysis of the global amount of γ-H2AX in shCtrl and shTop1 HCT116 cells exposed or not to CPT. Equal amount chromatin samples used for ChIP-chip were analyzed. (C) Number of statistically-enriched γ-H2AX regions (P<0.01) on chromosome 1 and 6 of shTop1 and shCtrl cells treated or not with CPT. (D) Overlap between γ-H2AX loci and annotated genes in shTop1 and shCtrl cells exposed to CPT. Red: Observed overlap between γ-H2AX loci and annotated genes (+/- 1 kb). Black: Distribution of expected overlaps if γ-H2AX loci were randomly distributed. 500 random distributions were generated with the TAS v1.1 software (Affymetrix). (E) Proportion of γ-H2AX-enriched histone genes in shTop1 and shCtrl cells exposed or not to CPT.

Figure 3: Camptothecin suppresses the accumulation of γ-H2AX specifically at replication-dependent histone genes. Heat map of the location of γ-H2AX-enriched domains at 500 kb regions encompassing histone gene clusters in shTop1 (A) or shCtrl (B)
cells treated or not with camptothecin (+/- CPT). Histone genes are indicated in red and non-histone genes are in black.
Figure 1

**Top1+**
- Replisome
- Top1
- Promoter

- Diospyrin
  - Mimics Top1- phenotype
  - siRNA

**Top1-**
- Replisome
- γH2AX
- R-loop
- RNA polII
- RNaseH
- Cordycepin
  - Restores Top1+ phenotype

**Restores Top1+ phenotype**

**Mimics Top1- phenotype**
Figure 2

A

Chromosome 1

shTop1
- CPT
γ-H2AX genes
+ CPT
γ-H2AX genes

shCtrl
- CPT
γ-H2AX genes
+ CPT
γ-H2AX genes

Chromosome 6

shTop1
- CPT
γ-H2AX genes
+ CPT
γ-H2AX genes

shCtrl
- CPT
γ-H2AX genes
+ CPT
γ-H2AX genes

B

CPT
shCtrl shTop1
γ-H2AX

C

histo2 regions

D

Frequency

Overlap with genes (%)

E

Histone genes (%)

n = 64

P < 0.1

88.7

5.0

8.3

0.0

P value (-10 Log10)

20 30 40 50 10 20 30 40 50 10 20 30 40 50 10 20 30 40 50
Figure 3