COMMENTARY

Stressed DNA replication generates stressed DNA

Hannah L. Klein[a,1]

Much of our understanding of eukaryotic replication dynamics, origin, and polymerase usage and replication factors has come from studies using the yeast *Saccharomyces cerevisiae*. From the definition of replication origins using plasmid transformation (1, 2) and two-dimensional gel electrophoresis (3), identification of replication factors using various genetic screens for cell cycle progression (4) and plasmid stability (5, 6) and genome-wide replication patterns using Okazaki fragment sequencing (7, 8) and strand-specific sequencing of incorporated ribonucleotides in DNA (9), we have learned much about DNA replication under normal and disrupted conditions. As many of the replication factors are conserved, the findings have been applicable to more complex genomes. Additionally, genome instability in yeast arising from perturbations to the normal replication program mirror those found in human cells, particularly the genome instability resulting in loss of heterozygosity (LOH), genome rearrangements, and mutagenesis found in tumor cells and other diseases with DNA repair defects (10, 11).

Perturbations in the DNA replication process can result in regions of single-stranded DNA (ssDNA) accumulating near the replication fork (12, 13). A useful tool in the *Saccharomyces* arsenal of in vivo genetic tricks is expression of the human APOBEC3B protein to mark the location of ssDNA (14–16). This protein is a member of the cytosine deaminase family and mutates cytosine residues on ssDNA to uracil, resulting in C-to-T mutations. APOBEC3B protein does not naturally occur in yeast but can be expressed from a plasmid introduced into yeast strains. The U residues that arise from C deamination are preserved by deleting the *UNG1* gene encoding uracil-DNA glycosylase, thereby allowing G-to-T mutations to accumulate. Consequently, the newly detected G-to-T mutations are a metric for ssDNA occurrence. This APOBEC3B signature has been exploited by several groups to demonstrate preferential accumulation of ssDNA on the lagging strand during replication (15), clustered

mutations in both yeast and human cancer cells during replication (16), as well as clustered mutations in break-repair intermediates (17, 18). Clustered mutations are indicative of extensive regions of ssDNA, which may form from R-loops, DNA double-strand break resection, or processivity of the APOBEC3B enzyme. In PNAS, Sui et al. (19) use the APOBEC3B tool to ask what happens when the replicative DNA polymerases are limiting. To increase the power of their studies, the authors perform the experiments in diploid yeast strains in which the haploid parents differ by 55,000 single-nucleotide polymorphisms (SNPs) (20). Previously, these authors had examined genomic instability in yeast under conditions of limiting replicative polymerases (13, 21), but had never queried the presence and genome location of ssDNA during replication. Combining mutation accumulation by Illumina whole-genome sequencing with SNP analysis by microarrays, the authors have been able to correlate ssDNA (mutagenesis) with LOH resulting from mitotic recombination. This one-two punch to the yeast genome has led to several insights regarding genome instability during replication stress.

The basic conclusions are that, first, reducing levels of the replicative DNA polymerases leads to an increase in ssDNA (Fig. 1). The strongest effect is seen on the lagging strand with either reduced DNA polymerase α or DNA polymerase δ. Reduction of DNA polymerase ε leads to increased ssDNA on both the leading and lagging strands. The analysis strongly suggests that the ssDNA is directly linked to the occurrence of chromosome rearrangements and LOH events. Second, the ssDNA occurs as a result of DNA replication stress directly and does not arise from processing of DNA double-strand breaks. Third, some mutation clusters and hence ssDNA regions are quite long, up to 10 kilobase pairs in length. Last, for most genes, the transcribed strand shows a slight preference for mutation in regions 5′ to the coding region as previously noted (22), but the tRNA genes differ in
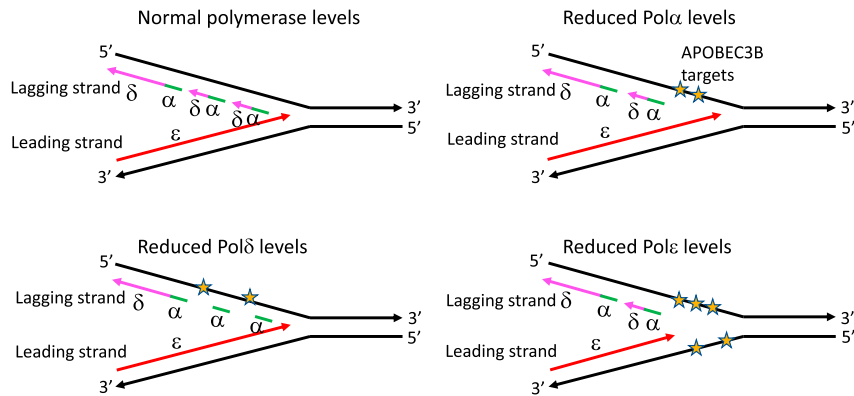
Fig. 1. Single-stranded DNA (ssDNA) at replication forks under conditions of reduced DNA polymerases. A schematic of a replication fork is shown with Polε replicating the leading strand and Polα and Polδ replicating the lagging strand. Under conditions of normal levels of all three polymerases, little ssDNA is formed, and hence a target for APOBEC3B mutagenesis (orange stars) is limiting. When Polα is limiting, gaps revealing ssDNA on the lagging-strand template arise due to reduced Okazaki fragment (Polα synthesis followed by Polδ elongation). These regions are targets for APOBEC3B mutagenesis. Similarly, limiting Polδ leads to reduced extension from the Polα primers, resulting in patches of ssDNA on the lagging-strand template. When Polε is limiting, ssDNA occurs at the fork on both leading- and lagging-strand templates, suggesting that failure to efficiently extend the leading strand alters the lagging-strand replication dynamics, possibly by the formation of an altered replication fork where Polδ may extend both strands.

that the nontranscribed strand is preferentially mutated, as previously described (23).

To execute these experiments, the authors use an oft-used method in yeast studies and drive the DNA polymerases expression from an inducible promoter (*GAL1*) that is responsive to the carbon source. However, in this case instead of using galactose to induce and overexpress the genes, the authors use a repressive regimen of low galactose with raffinose. These carbon sources sustain yeast growth but reduce DNA polymerase α or DNA polymerase δ expression to 10% of normal levels, determined by Western blotting. For DNA polymerase ε, the analysis is a bit more complex as the protein could not be detected by Western analysis. However, RT-PCR analysis indicated that under the repressive regimen DNA polymerase ε levels were one-third of that seen under normal growth conditions using the native promoter.

As expected for newly induced APOBEC3B-mediated mutations in diploid cells, most mutations were C-to-T changes, heterozygous, and in the APOBEC3B signature motif. The most important analysis, dividing the DNA strands into leading/lagging, transcribed/nontranscribed, and importantly, by transcript orientation relative to the nearest replication origin, the authors could determine mutagenicity due to replication and transcription separately. This level of detailed analysis has only been possible in yeast with the well-defined replication origin map. The biases for lagging-strand ssDNA and transcribed-strand ssDNA are significant and indicate significant amounts of ssDNA are formed during replicative DNA polymerase limitation.

The mutation clusters that are large in size are indicative of some regions that are more prone to accumulating ssDNA. The studies have shown that the single-stranded regions in low DNA polymerase δ conditions occur in one cell cycle and are recurrent, indicating that some genomic regions are more prone to forming ssDNA. The precise nature of this is not known but could reflect DNA structure, protein-bound factors, or other features that alter replication progression.

The studies with limiting DNA polymerase ε gave unexpected results. While it was expected that there would be increased ssDNA and hence mutagenesis on the leading strand, the increased mutagenesis on the lagging strand might not have been anticipated. The simplest interpretation is that reducing or delaying synthesis on the leading strand affects coordination of the replication complex with a compensatory delay on the lagging strand, despite the normal levels of DNA polymerases α and δ. As DNA polymerase δ has been reported to replicate the leading strand when DNA polymerase ε is defective (24, 25), an alternative replication fork with DNA polymerase δ replicating both strands might have altered dynamics and accumulate ssDNA on both templates.

These studies combining APOBEC3B-induced mutagenesis as a metric for ssDNA, whole-genome sequencing, and SNP array analysis to map LOH events onto a pattern of ssDNA accumulation have led to a picture of a distressed genome with fragile sites under stress conditions at a level of resolution not previously achieved. From such a small genome, such mighty conclusions are seen that can reach into the genomes of cancer cells.

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