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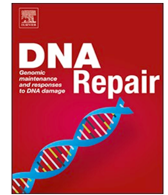

Hypermutation in single-stranded DNA

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ABSTRACT

Regions of genomic DNA can become single-stranded in the course of normal replication and transcription as well as during DNA repair. Abnormal repair and replication intermediates can contain large stretches of persistent single-stranded DNA, which is extremely vulnerable to DNA damaging agents and hypermutation. Since such single-stranded DNA spans only a fraction of the genome at a given instance, hypermutation in these regions leads to tightly-spaced mutation clusters. This phenomenon of hypermutation in single-stranded DNA has been documented in several experimental models as well as in cancer genomes. Recently, hypermutated single-stranded RNA viral genomes also have been documented. Moreover, indications of hypermutation in single-stranded DNA may also be found in the human germline. This review will summarize key current knowledge and the recent developments in understanding the diverse mechanisms and sources of ssDNA hypermutation.

1. Introduction

DNA is double-stranded (dsDNA) most of the time, however many intermediates of DNA replication and repair are single-stranded (ssDNA). Lesions in dsDNA are prevented from becoming mutations by templated excision repair [1]. However, lesions in ssDNA are not reparable through any excision repair pathway because of the lack of the requisite template strand. While certain kinds of base alkylation in ssDNA can be repaired via direct reversal repair (DRR) [2,3], this repair mechanism cannot reverse all ssDNA lesions, which would result in mutations once copied by error-prone trans-lesion synthesis (TLS) polymerases. Yeast studies have shown that the density of mutations stemming from lesions in ssDNA can exceed 100-1,000-fold over the rest of the genome [4–6] (also reviewed in [5,7] and summarized on Figs. 1 and 2).

Conceivably, hypermutation of ssDNA might occur not only due to the lack of lesion repair but also from ssDNA-specific lesions. In both cases, subsequent replication often involving error-prone TLS would fix these lesions as mutations. Hypermutation in a ssDNA stretch can often result in the generation of a tightly spaced group of mutations called a mutation cluster. Mutation density in clusters is far greater than the density of mutations seen in the rest of the genome, where lesions either do not occur or are repaired by excision repair pathways before copying the damaged strand. If a cluster is formed by a single source of DNA damage, lesions as well as the resulting mutations would be similar in the progeny of the damaged strand, and are commonly referred to as strand-coordinated clusters [6]. Notably, strand-coordinated clustering only implies the simultaneous occurrences of similar mutations. In case of acute mutagenesis, the density of lesions in dsDNA across the genome can be very high. If high-density lesions are not repaired in small section(s) of a genome, they will again result in a mutation cluster. Subsequent DNA replication will separate clustered mutations of the top (Watson) strand and bottom (Crick) strand into different cells, so a cluster found in a single-cell clone will be strand-coordinated (Fig. 3).

Strand-coordinated clusters stemming from regions in damaged dsDNA, where repair did not occur before DNA replication was demonstrated in *E. coli* mutagenized by EMS [8]. Recently Martin Taylor and colleagues found that similar mechanisms operate in cancers, resulting in strand-coordinated “phasing” of mutations on the chromosome scale [9]. This phenomenon may reflect transient genome-wide bursts of mutagenesis due to the lack of damage repair in a single cell cycle. A similar phenomenon involving bursts of mutagenesis in cultured cancer cells were recently reported by [10]

In summary, assignment of a mutation source to hypermutable ssDNA cannot only rely on mutation clustering but should include additional knowledge about ssDNA preference and DNA motif specificity for a mutagenic factor, as well as the potential sources of ssDNA in a biological context. Taking this into account, this review will focus on the recent developments in understanding the diverse mechanisms and sources of ssDNA hypermutation

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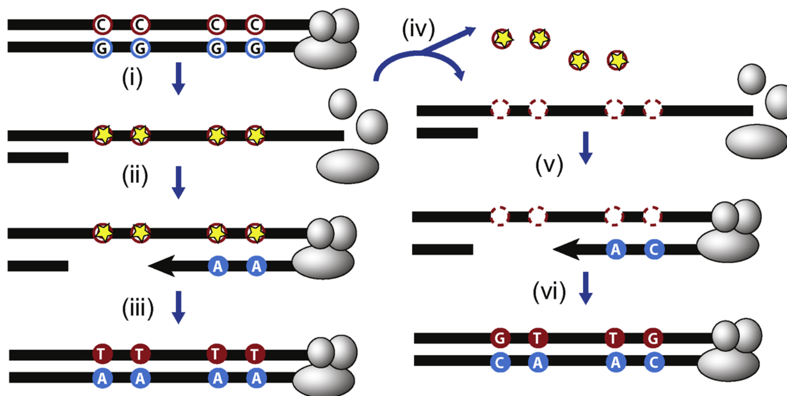

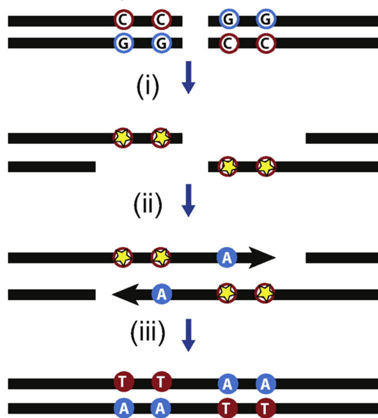

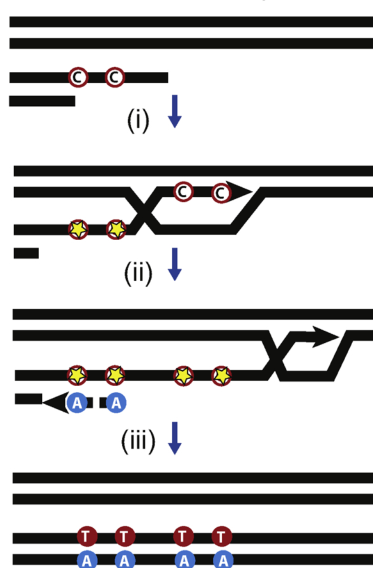

Fig. 1. Hypermutation in ssDNA formed during DSB repair. (The color code and symbols explained in the legend to panel A are used throughout in all panels of Figs. 1–3). The figures are adapted from [7].
A. 5′ to 3′ DNA resection at uncapped telomeres. (i) In the temperature sensitive *cdc13-1* yeast strains, yeast telomeres are uncapped upon shift of the strains to 37 °C. 5′ to 3′ resection ensues and approximately 30 kb of the telomeric ends are rendered single stranded. The undamaged nucleotides are depicted as red (cytosines) or blue (guanine) circles with white background with the nucleotide shown as a black letter inside a circle. (ii) Exposure to a ssDNA-specific mutagen will lead to DNA damage in the un-resected single strand (yellow stars). In this figure, we depict DNA damage in cytosines in ssDNA as an example. (iii) Upon switch to permissive temperatures the resected strand is resynthesized. In the absence of excision of the damaged base by a glycosylase, DNA replication over the lesions will yield mutations. Mutated nucleotides are shown as filled circles. Red solid circles correspond to mutant nucleotides originated from damaged cytosines and blue solid circles to nucleotides that replaced damaged guanines. (The same color code and symbols are used in throughout all panels of Figs. 1–3). As an example, replication of unrepaired base damage in cytosines such as deamination will be expected to yield strand coordinated clusters (stretches of C→T changes). (iv) On the other hand, certain DNA glycosylases have been shown to function in ssDNA. As such, the removal of the damaged base (depicted as red circles with a yellow star) in ssDNA may lead to abasic sites in ssDNA (dotted red circles). (v, vi) Bypass of such abasic sites by translesion polymerases during resynthesis of the resected strand will lead to insertions of A or C opposite the abasic sites. In the example shown in the figure, bypass of abasic sites generated upon excision of damaged cytosines in ssDNA will yield C→T and C→G changes.
B. Bidirectional resection at double strand breaks. (i) A two ended break is symmetrically resected on both sides of the break to generate 3′ overhangs. ssDNA-specific base damage in the overhangs and (ii, iii) bypass of the damaged base leads to single-switch clustered mutations. An example of ssDNA-specific damage leading to mutations in cytosines is shown here. DNA damage accumulated in the single stranded overhangs created during repair lead to clustered mutations in cytosines to the left of the break-point followed by clustered mutations in guanines to the right of the break-point.
C. Break-induced replication. (i) A one-sided DNA double strand break is repaired via 5′ to 3′ resection of the broken end and (ii) a one-ended invasion into a homologous donor template. Uncoupling of the leading and lagging strands during break-induced replication yields long ssDNA intermediates that accumulate DNA damage. (iii) Synthesis of the second strand using the damaged template strand followed by excision repair of the lesions fixes the mutations in the newly synthesized DNA molecule. As an example, damage and mutations in cytosines is depicted here. Note that unlike for bi-directional resection, cytosines of the top strand are mutated on both sides of a DSB.

2. Break-induced spontaneous mutation

2.1. Stress-induced spontaneous mutagenesis in bacteria

The bacterial phenomenon initially named as adaptive mutagenesis was described as accumulating Lac^{+} reversion mutants with a selective growth advantage in non-dividing populations of *E. coli* carrying the F'-episome [11–13]. In a series of studies by Rosenberg and colleagues, it was established that this phenomenon is initiated by DSB repair and possibly involves ssDNA formed as a result of break-induced conjugational transfer of bacterial F'-episome or chromosome as well as with repair of an episome-independent DSB [14–19]. Adaptive mutagenesis relies on error-prone DNA synthesis that is activated by stress caused by DNA break(s) and is often referred to as stress-induced mutagenesis (reviewed in [20]). Stress-induced mutagenesis in *E. coli* relies upon a large gene-network and may be responsible for up to 50 % of spontaneous mutagenesis in starving *E. coli* [21] as well as for mutagenesis in strains treated with clastogens [22].

2.2. Break-induced spontaneous mutagenesis in yeast

Strathern and colleagues observed a strong increase in mutagenesis in a reporter gene placed in the vicinity of a site-specific DSB in yeast likely due to hypermutation in ssDNA formed at the break site [23–25]. In a similar study, the region in the immediate vicinity of a site-specific DSB was found to be hypermutated upon breakage [26,27]. DSB-associated hypermutation in the vicinity of the break points [28,29] as well as up to 30 kb away from the break points [30], has also been detected at fragile motifs in the yeast genome. Mutagenesis at loci up to 30 kb away from the fragile motifs was dependent upon the propensity of the fragile site for breakage, as well as resection and repair synthesis utilizing TLS polymerases using the intact sister chromatid as the template [30].

Various studies on yeast model systems have also demonstrated mutagenesis in long and persistent ssDNA which requires restoration to dsDNA for viability. One way to generate long and persistent ssDNA is to use yeast strains with a temperature sensitive allele of the *CDC13* gene. Upon the shift of the *cdc13-1* mutant to high non-permissive temperature yeast telomeres are uncapped and 5′→3′ resection ensues

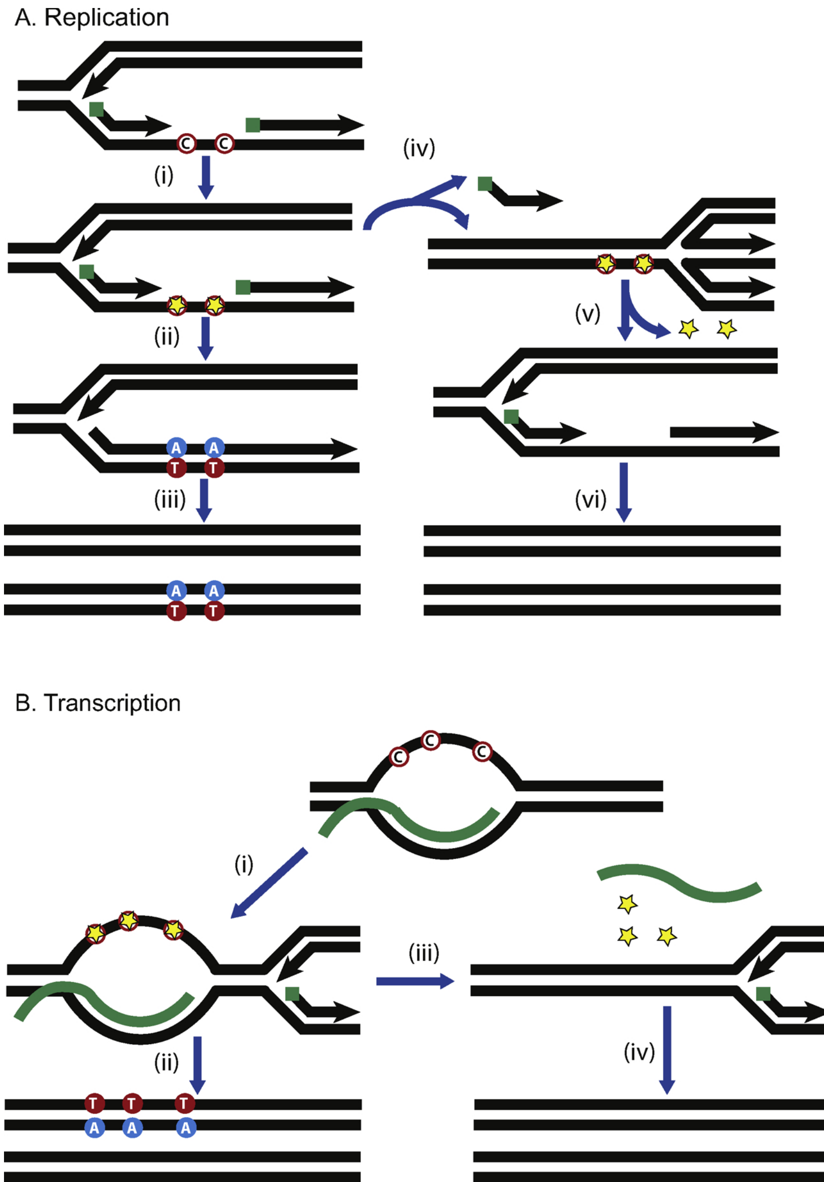

Fig. 2. Hypermutation in ssDNA formed during DNA replication and transcription.
A. DNA damage in ssDNA formed on the lagging strand during DNA replication. A replication fork is shown. The arrows depict the direction of DNA synthesis. The green blocks are primers added by Polα. (i) In the presence of an ssDNA-specific DNA damaging agent, ssDNA gaps in the lagging strand accumulate DNA damage. As an example, DNA damage accumulated in cytosines is portrayed here. (ii, iii) Bypass of the lesions without excision repair fixes the mutations in the daughter DNA and leads to the formation of replication-strand biased mutation clusters. (iv, v) Alternatively, removal of the un-ligated Okazaki fragment by fork regression would place the damaged nucleotides in dsDNA allowing the damage to be repaired and replication would proceed over the undamaged template [122]. (vi) The daughter DNA molecules are not mutated.
B. Perturbation of transcription may lead to stabilized R-loops leading to DNA damage accumulation in the single-stranded non-transcribed strand. The nascent RNA in the R-loop is shown in green. (i) As an example, ssDNA-specific mutagenesis in cytosines is shown in this figure. (ii) In the absence of repair, replication over the lesions leads to transcriptional-strand biased mutagenesis. Shown as C→T changes where the mutated cytosines are in the non-transcribed strand. (iii, iv) Resolution of the R-loops provides the undamaged template for excision repair and prevents mutagenesis.

from the telomere ends. Cells with multi-kilobase resection at multiple telomeres get arrested in G2 until the switch to the permissive temperature where re-synthesis of the second strand is allowed (Fig. 1). A 100-fold increase in mutation frequencies in a reporter placed in this ssDNA region was seen compared with the same reporter in the area of continuously double-stranded DNA [4,31]. DSB-associated spontaneous hypermutation in ssDNA was also observed when only one end of a DSB participated in recombinational repair. In this case, invasion of a free DNA end into a homologous template triggers DNA synthesis via break-induced replication (BIR), which can go as far as the chromosome end [32]. Strand invasion creates a displacement loop which proceeds along the chromosome by DNA polymerase extending the 3′-end of the invading strand. Importantly, second strand synthesis can be triggered much later. This results in persistent long ssDNA regions upstream of the migrating bubble [33,34]. This DSB-associated synthesis was also found to be error-prone with increased rates of base substitutions and small insertions and deletions (indels) (Fig. 1C) [35,36]. BIR initiated by a single-strand nick also causes spontaneous hypermutation around the nick site. Long stretches of nick-associated hypermutation has been shown to be prevented by the activity of Mus81, a Holliday junction resolution protein [37].

Importantly, all these studies demonstrated that ssDNA formed during DNA break repair is hypermutable. However, unlike many cancer genomes or model systems treated with DNA damaging agents, the level of spontaneous mutagenesis at ssDNA is not sufficient to generate clustered mutations.

3. Acute and chronic ssDNA damage in yeast

When DNA damage was applied to yeast strains engineered for controlled formation of ssDNA at DSBs or uncapped telomeres (Fig. 1A and 1B), hypermutation was increased by 10–100 fold, resulting in clusters of mutations near the telomeres (subtelomeric) often showing strand-coordination [4,5,31,38]. Since resection at uncapped telomeres only renders up to 30 kb DNA at the ends single-stranded, most of the genome (mid-chromosome regions) was double-stranded with very low mutation densities. Various studies demonstrated that DNA damage in such induced ssDNA led to clustered mutations [6,39–42].

3.1. UV light

UV-irradiation of yeast strains that form ssDNA at a site-specific DSB or at uncapped telomeres resulted in strand-coordinated clusters of mutated pyrimidines (See example in Fig. 1B) [4,5], however some

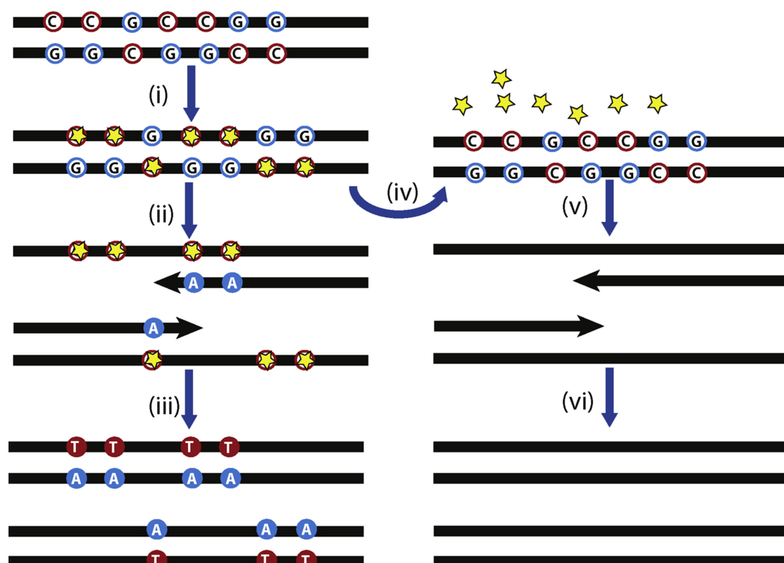

Fig. 3. Strand-coordinated clusters from replication of unrepaired dsDNA. In the presence of a DNA damaging agent, lesions may be formed on both strands of dsDNA molecules. As an example, DNA damage in cytosines is depicted in this figure. (i) Lesions in cytosines are shown as yellow stars in red circles. (ii) Replication of the DNA molecules prior to repair leads to mismatches in the nascent DNA. (iii) Repair of lesions fixes the mutations in the daughter molecules. If the lesions were base specific (as shown here), the mutations will be reciprocally strand-coordinated in the daughter DNA molecules. (iv, v, vi) Alternatively, repair of lesions prior to DNA replication will prevent mutagenesis.

mutations were also observed in purine bases. This increase in strand-coordinated clusters of mutated pyrimidines was in line with the known specificities of UV-damage to DNA. Only very few UV-induced mutations were found in mid-chromosome sections of yeast genome. Mutagenesis was found to completely rely on the bypass of the UV-induced lesions by translesion synthesis (TLS) thus likely reflecting UV-induced lesions specifically formed in ssDNA.

3.2. Chemical and enzymatic deamination of cytosine

One-step enzymatic removal of the amino group from cytosine in ssDNA would result in the formation of a uracil residue. Insertion of an adenine residue opposite this uracil by DNA polymerases, followed by a second round of replication will fix the change as a C→T mutation. Indeed, subtelomeric clusters of strand-coordinated C→T mutations were found in the yeast strain that were prone to the loss of telomere caps and expressing the APOBEC3G cytosine deaminase [31]. In this strain, the gene coding for the Ung1 DNA glycosylase was deleted preventing the creation of an abasic site (AP-site) from excision of uracil in DNA. Copying of uracils resulting in C→T mutations did not require functional TLS. When the same strain was treated with bisulfite, leading to chemically induced cytosine deamination, most of the mutations were also C→T, however up to 90 % of mutagenesis required TLS. Yeast studies on ssDNA mutagenesis by the APOBEC cytidine deaminases revealed that uracils generated in ssDNA are efficiently converted into abasic sites by Ung1 before they are copied by the replicative DNA polymerases. This conclusion was made based on the complete dependence of APOBEC-induced hypermutation in ssDNA of *UNG1* strains on functional TLS, unlike the *Δung1* isolates. In these strains, all base substitutions in cytosines (major substitutions – C→T and C→G and the minor substitution – C→A) were dependent on the activity of TLS (See example in Fig. 1). C→G substitutions completely relied on the catalytic activity of Rev1, a dCTP terminal transferase [31,39,43,44].

3.3. Alkylation damage

Yeast strains with long ssDNA regions formed either by DSB induction, break induced replication or uncapping of telomeres and treated with MMS, a model SN_2-type alkylating agent, demonstrated that the predominant mutations in ssDNA regions were in cytosines and adenines. This observation was contrary to the base damage specificities for MMS identified in vitro [45], which identified N7-methylguanine as the predominant lesion created by MMS in both ds and ssDNA. Mutations in guanines made up a very small fraction of the mutations identified in yeast. This observation corroborated previous findings that N7-methylguanines are mostly benign lesions and are copied accurately by DNA polymerases. Moreover, the low prevalence of mutagenesis in guanines in ssDNA in yeast further demonstrated that these lesions are not efficiently excised from ssDNA and no abasic sites are formed for bypass by error-prone TLS.

As mentioned above, base substitutions in cytosines and adenines were the most prevalent changes induced by MMS in ssDNA in yeast [6,38,46,47]. This mutation spectrum was likely due to the formation and mutagenic bypass of N3-methylcytosines and N1-methyladenines [45,48]. The base substitutions in both cytosines and adenines were found to depend upon TLS, with a prominent decrease in A→G and C→G changes in strains with mutations in Rev1. C→G changes were also found to be lowered in strains with the *RAD30* gene deleted, indicating towards a role of Polη in bypassing methylated cytosines in ssDNA [47]. Yeast strains treated with MMS also demonstrated a preference for lesion formation and mutagenesis in adenines on the non-transcribed strand. This was likely due to the formation of single-stranded DNA in the non-transcribed strand during transcription, which would be susceptible to damage by MMS [49].

3.4. Oxidative damage

Oxidative damage was also recently demonstrated to lead to mutagenesis at ssDNA. While 8-oxo guanine is considered to be the major mutagenic product in DNA caused by reactive oxygen species (ROS), the major component of the mutation spectrum in yeast subtelomeric ssDNA was C→T mutations. The potential impact of cytosine deamination on the mutation spectrum was excluded, because these mutations did not depend on the activity of the uracil DNA glycosylase Ung1. Surprisingly, oxidative damage-induced C→T mutations were also TLS independent. The authors proposed that a currently unknown modification of cytosine in ssDNA by ROS was the source of the mutations observed [50,51]. Interestingly, a similar mutation spectrum was found in human mitochondrial DNA in the regions that are expected to be single stranded in the displacement loop formed during mitochondrial DNA replication [51–53]. This observation can be explained by increased ROS formation in mitochondria resulting in ssDNA damage.

4. Multiple sources of hypermutable ssDNA revealed in model studies

Extreme levels of hypermutation that are often visible in ssDNA make it important to understand the mechanism of incidence and genomic contexts prone to formation of vulnerable ssDNA regions. Identifying the mutation spectra and/or signatures in ssDNA that are characteristic of various DNA damaging agents has been instrumental in the identification of such regions in relation to endogenous (e.g. cell cycle, replication) or exogenous stressors. The presence of strand-biased mutations or strand coordinated clustered mutations usually arising due to DNA damage in ssDNA are often indicative of the formation of hypermutable ssDNA in the genome. Below are the mechanisms via which such ssDNA may be formed in cells.

4.1. Lagging strand

Each replication cycle entails the formation of a sliding window of ssDNA that traverses through the genome along the lagging strand template. These ssDNA gaps are protected by RPA and promptly filled in by DNA polymerases Polα and Polδ. Nonetheless, the lagging strand has been reported to be highly susceptible to DNA damage (Fig. 2A). MMS was highly mutagenic to the lagging strand template of a mutation reporter in budding yeast strains wherein the replication fork integrity was compromised by deletions of either *TOF1* or *CSM3* (homologs of the human Timeless and Tipin genes) [6]. The lagging strand template was also preferably mutagenized by the ssDNA-specific cytidine deaminases – APOBEC3A and APOBEC3B even in replication-proficient yeast strains [54]. A similar bias of APOBEC3-induced mutagenesis was observed in the genomes of replicating *E. coli* strains [55]. Downregulation of lagging strand polymerases Polδ or Polα in yeast strains expressing APOBEC3B resulted in hypermutation strongly biased to the lagging strand template, with much smaller effect and no strand-specificity when the leading strand polymerase Polε was downregulated [56]

4.2. 5′→3′ resection and break-induced replication (BIR)

Initiation of DSB repair by gene conversion necessitates the generation of ssDNA upon resection of the break. Mutation clusters can often arise in such regions where the ssDNA accumulated DNA damage. In the case of mutagens which have a defined mutation spectrum in ssDNA, these mutation clusters may be strand coordinated. If the hypermutable ssDNA was a product of bidirectional 5′→3′ resection, mutation clusters would form on both sides of the break, and the bias in base substitutions would reflect mutagenesis in the top (Watson) strand to the left of the break followed by a switch to mutagenesis in the bottom (Crick) ssDNA strand to the right of the break (Fig. 1B). In the case of asymmetrical resection, a cluster would span only one side of the site-specific DSB with mutation strand bias reflecting 5′→3′ resection. Formation of large strand-biased mutation clusters in the vicinity of a site-specific DSB repaired by homologous recombination (HR) was first detected in a reporter gene placed in the DSB vicinity on either the left or right side of the DSB. Mutation specificity in each case agreed with long 5′→3′ resection [4,39]. Whole genome sequencing of yeast strains exposed to chronic alkylation damage (MMS) or yeast strains repairing multiple simultaneous radiation-induced DSBs in the presence of the APOBEC3A cytosine deaminase allowed the detection of single-switch clusters presumably near DSBs [6,57]. In both studies, single-switch clusters displayed mutation bias agreeing with bidirectional resection from a DSB.

Damage-induced hypermutation in ssDNA formed during BIR has been documented in yeast strains. During BIR, 5′→3′ resection of the break is followed by the invasion of one 3′-end into a homologous sequence and synthesis via a migrating bubble which generates long ssDNA in its wake. Importantly, ssDNA region on both sides of the position of the break would be in the same strand. ssDNA-specific damage during BIR would result in the formation of a strand-coordinated mutation cluster with the same base substitutions seen on both sides of the break (Fig. 1C). Based on the length and location of strand-coordinated clusters of mutations induced by either alkylation agent (MMS) or by ssDNA-specific cytosine deaminase APOBEC3A in yeast strains undergoing BIR, it was ascertained that hypermutable ssDNA stretches in yeast can exceed 100 kb [38,44].

Yeast cells repairing multiple radiation-induced breaks could contain more than 30 mutation clusters, some of them spanning over 100 kb. Analysis of strains defective in either bi-directional or unidirectional resection and strains with defects in BIR have defined the spectrum of mutation clusters in yeast repairing bursts of DSBs by HR [57].

4.3. Unprotected telomeres

4.3.1. 5′→3′ resection at uncapped telomeres in yeast

Unprotected (uncapped) telomere ends in yeast are recognized as the end of a DSB, triggering multi-kilobase 5′→3′ end-resection. In the temperature-sensitive *cdc13-1* yeast mutants, telomere uncapping followed by resection and G2-arrest is efficiently triggered by shifting cells to non-permissive (37 °C) temperature [58,59], which renders the subtelomeric chromosomal ends single-stranded, and highly susceptible to DNA damaging agents. When yeast cells are shifted back to permissive temperature (23 °C), capping is restored and ssDNA subtelomeric gaps revert to a dsDNA state. Treatment of yeast strains expressing the *cdc13-ts* allele with genotoxic agents, and subsequent sequencing of subtelomeric reporters or the entire genome has been shown to produce strand-coordinated mutation clusters marking stretches of ssDNA up to 50 kb (Fig. 1A) [4,5,31,43,47,60].

4.3.2. Telomere crisis in human cells

Mammalian telomeres are protected from being recognized as DSB ends ensuing resection and the triggering of checkpoints by a large shelterin protein complex including the TRF2 protein [61]. Expression of a dominant-negative *TRF2* allele results in a telomere crisis entailing telomere-telomere fusion followed by chromosome breakage in anaphase [62]. Interestingly, whole-genome sequencing of transient telomere crisis survivors revealed multiple chromosomal rearrangements and clusters of strand-coordinated mutations carrying a clear mutation signature of the endogenous activity of the APOBEC3B cytidine deaminase, which was expressed in the cell line used in these experiments. Resolution of anaphase bridges and formation of mutation clusters depended on the TREX1 single-strand exonuclease [63,64]. These clusters of APOBEC-induced mutations indicated formation of large stretches of ssDNA due to the telomere crisis.

4.4. Non-transcribed strand and APOBECs

Transient ssDNA can be created during transcription by complementary pairing of the transcript with the transcribed strand (R-loops). Lesions in such transiently single-stranded non-transcribed DNA strands can be repaired by nucleotide- or base-excision repair (NER or BER) upon RNA removal and DNA rewinding. However, if lesions in the non-transcribed strand persist until DNA replication, they can be fixed into mutations by TLS. This would result in a mutation bias favoring the non-transcribed strands (Fig. 2B). Such a bias was seen in BER-deficient yeast strains expressing the APOBEC/AID cytidine deaminases. Indeed several studies reported increased mutagenesis in the non-transcribed strand caused by various cytidine deaminases expressed in *Δung1* yeast strains incapable of excising the uracil formed in DNA and initiating BER [40,65,66].

5. Hypermutation of ssDNA in cancer genomes

Hypermutation of ssDNA has also been detected in a large number

of cancer genomes that have been sequenced. The best characterized source of hypermutation in ssDNA currently are the single-strand-specific cytidine deaminases (AID and APOBEC3 enzymes). Nonetheless, various studies have now demonstrated strand-biased mutagenesis in cancer genomes due to other mutagenic factors including DNA alkylation and oxidative damage. Such mutagenesis may also be indicative of DNA damage in ssDNA.

5.1. Somatic hypermutation by AID

Somatic hypermutation (SHM) in immunoglobulin genes by the ssDNA-specific activation-induced deaminase (AID) was the first example of hypermutation detected in ssDNA. The AID cytosine deaminase belongs to the protein family often referred as AID/APOBEC cytosine deaminases because of the structural similarity of the group members. Further, hypermutation by AID is important in antibody diversification, and AID can cause mutations in secondary genomic targets including potential oncogenes [67]. Clustered mutagenesis with canonical AID mutation signature was detected near active transcription start sites in chronic lymphocytic leukemias, with an additional non-canonical AID signature also identified in non-clustered mutations in chronic lymphocytic leukemias [68]. Both the canonical and non-canonical AID signatures were also detected in multiple myelomas [69,70].

5.2. Hypermutation in cancers by the APOBEC cytidine deaminases

There are 11 APOBEC/AID ORFs encoded in human genome. Seven proteins encoded by APOBEC3 gene cluster and the APOBEC1 encoded by a gene located on a different chromosome have demonstrated ssDNA-specific cytidine deaminase activity [71,72]. In agreement with various model system studies on mutagenesis induced by the APOBEC enzymes, in cancers with a high enrichment of the APOBEC-specific tCw→T or tCw→G changes, C- or G-coordinated clustered mutations were also found to carry tCw→T or tCw→G changes (w = A or T, mutated base capitalized). Unlike most members of the APOBEC3 family, APOBEC3G preferably mutagenizes the cytosines in a cCn motif. Interestingly, cCn→T or cCn→G changes were depleted in C- or G-coordinated mutation clusters in multiple types of cancers, indicating that APOBEC3G is likely not responsible for the hypermutation in cancers [6,73].

Soon after discovery of the APOBEC3-induced mutation signature in clustered hypermutation within cancer genomes [6,74] the activity of the APOBEC3B enzyme was postulated to be the major source of this hypermutation in cancers [75,76]. This assumption was made based on the high expression level of APOBEC3B mRNA in solid tumors carrying APOBEC hypermutated genomes and supported by positive, albeit weak, correlation between mRNA levels and mutation load. Detailed statistical analysis in yeast subtelomeric ssDNA revealed the specific motifs preferred by either APOBEC3A or by APOBEC3B. APOBEC3A preferred pyrimidine (ytCa motif), while APOBEC3B preferred purine (rtCa) in the -2 position. This allowed a distinction between the APOBEC3A-like and APOBEC3B-like hypermutation in cancers [60] and to classify mutation catalogues of whole-genome sequenced human tumors as APOBEC3A-like or APOBEC3B-like. Analysis of hundreds of cancer samples revealed that APOBEC3A-like tumors contained about 10-fold more APOBEC-signature mutations as compared to the APOBEC3B-like tumors [60]. On the other hand, the APOBEC3B-like signature was clearly present in tumors with lower levels of APOBEC mutagenesis [60,77]. Interestingly, APOBEC3B-like signature was present in mutation clusters of prostate tumors even if genome-wide APOBEC mutagenesis was barely detectable. The levels of clustered APOBEC3B-like mutagenesis was found to increase with the age of diagnosis, thus leading to the identification of APOBEC3B-induced mutagenesis as a "clock-like" mutation process in human cancers [78]. A recent study demonstrated that in the presence of cellular RNA, the APOBEC3A enzyme has about a 100-fold greater activity than APOBEC3B, which may in part explain higher level of APOBEC3A-like mutagenesis in tumors with abundant APOBEC3B mRNA [79].

APOBEC3A-like tumors from the PCAWG dataset [80] revealed a large number of scattered APOBEC-signature mutations as well as many mutation clusters with only C- or(and) G-mutations highly enriched with the APOBEC3A-like mutation signature [57]. Only a small fraction of these clusters was categorized as single-switch (only C- mutations, followed by only G-mutations) and in half of these clusters the order of C- and G-stretches did not conform with canonical bi-directional 5′→3′ resection. Completely C- or G-coordinated clusters represented the largest category. By comparing distribution of cluster types in APOBEC3A mutated cancers with clusters occurring in yeast repairing radiation-induced DSBs in the presence of heterologous expression of APOBEC3A, Gordenin and co-authors proposed that the major mechanism generating large stretches of hypermutable ssDNA in human tumors is BIR (Fig. 1C) or another unusual form of DNA replication resulting in long range uncoupling of synthesis between two DNA strands [57]. They also estimated the total length of hypermutable ssDNA formed in a single yeast cell repairing a burst of DSBs by summing up the lengths of clusters in a genome as approximately a 100 kb. Surprisingly, cluster size-based estimate of hypermutable ssDNA in tumor genomes demonstrated the same (~100 kb) ssDNA lengths as yeast, despite the fact that the human genome is ~300-fold larger than the yeast genome. They proposed that hypermutable ssDNA in tumors is formed as rare bursts of events, rather than in small amounts spread across many cell generations. Additionally, the upper limits on the total length of ssDNA tolerable by a cell is set by the probability of forming an unrepairable DSB. It has been suggested that a single unrepaired DSB would be lethal regardless of the genome size [81]. As such, if a single catastrophic event in a tumor genome yields a large amount of ssDNA in the form of DNA repair intermediates, the proportion of the ssDNA formed would directly correlate with the probability of creation of an unrepairable DSB.

5.3. Strand-coordinated clusters by Polη

A- or T-coordinated clusters have also been found in APOBEC3-mutated cancers [6,77,80,82]. The mutation signature in these A- or T-coordinated clusters was found to resemble the mutations induced by the TLS-polymerase, Polη, during filling in of gaps formed during repair of mismatches in the course of somatic hypermutation in the immunoglobin genes [83].

5.4. Other strand biased mutagens in cancers

Experiments in model systems have demonstrated a variety of other environmental mutagens that preferably mutagenize ssDNA. Extrapolation of the observations in such model systems to cancers has provided insights into the roles of such carcinogens in ssDNA mutagenesis in human tumors. Recently, it was shown that oxidative damage preferably mutagenizes ssDNA formed at uncapped telomeres in yeast and in the displaced D-loop strand in the human mitochondrial genome during replication. The ssDNA-specific oxidative damage signature in mitochondria was C→T changes in gCg and ggCg motifs. High enrichment for C→T at gCg and ggCg motifs was also detected in human cancers across the genome and in mutation clusters which were indicative of DNA damage in ssDNA [51].

A similar approach was utilized to identify the mutation signatures of alkylating agents in yeast and in human tumors. SN_2-type alkylating agents were found to preferentially mutagenize cytosines and adenines in ssDNA. Mutations in adenines led to an equal amount of A→T and A→G changes, likely due to the formation and mutagenic bypass of N1-methyladenines in ssDNA. Such A→T and A→G changes were also found to be biased towards the non-transcribed strand in lung and head and neck tumors. The authors hypothesized that these changes were

likely induced by the activities of SN_2-type alkylating agents present in tobacco smoke. The authors also identified a separate mutation signature in thymines (nTg →nGg and hTg→hGg, h is a, c or t) that was highly specific for SN_2-type alkylation-induced DNA damage. This signature along with the strand-biased A→T and A→G changes was indeed found to be enriched in the genomes from smokers, indicating that tobacco smoke-specific alkylating agents induce A→T and A–G changes in ssDNA in lung and head and neck tumors [47].

6. Genome profiling of APOBEC mutagenesis reflects propensity for hypermutable ssDNA formation

The trinucleotide-specific APOBEC3-induced mutation signature revealed the preferences for APOBEC3-induced mutagenesis in various parts of cancer genomes. Since the APOBEC3-induced cytidine deamination requires an ssDNA substrate, these preferences would indicate increased propensity to form hypermutable ssDNA. Similar to yeast and bacterial studies (see above), a clear increase in APOBEC-signature mutagenesis was detected in the lagging strand template of cancer genomes which carry a high mutation load attributable to APOBEC3 activity [84–86], however unlike the yeast model no transcription bias was detected in tumors.

Additionally, it was noted that the overall density of mutations on the evolution time scale and in cancer genomes is greater in late replicating regions as compared to early replicating regions [87–90]. This tendency is usually explained by lower access of DNA repair enzymes to late replicating regions enriched with heterochromatin. Surprisingly, Sunyaev and colleagues noticed that in several whole-genome sequenced human cancers clustered as well as scattered APOBEC mutagenesis was stronger in early replicating regions [91]. A similar tendency for clustered APOBEC mutagenesis was reported in a recent pancancer study [80], while, another study reported a flat profile of APOBEC3-induced mutagenesis across late and early replicating regions of breast cancer genomes [86]. One possible explanation for the preference of APOBEC mutagenesis in early replicating regions could be a higher propensity of ssDNA formation in these genomic areas due to higher frequency of breakage as indicated by increase in rearrangements seen in early replicating sections [92,93]. This suggestion is supported by the frequent colocalization of APOBEC mutation clusters with breakpoints of gross chromosomal rearrangements as was noticed in the initial studies [6,73,74] and later confirmed by analyses of large cohorts [78,93,94]. On the other hand, breast cancer genomes are predisposed to DSBs and the high incidence of DSBs may cause the formation of large amounts of ssDNA even in late replicating regions, causing a flatter mutation landscape of APOBEC3-induced mutations across the genome.

7. Meiosis and the germline

The phenomenon of increased rates of auxotrophic reversion in meiosis (meiotic effect) was discovered nearly 60 years ago in budding yeast suggesting the mutagenic effect of DNA recombination [95]. Decades later Strathern and colleagues confirmed the phenomenon using defined direct and reverse mutation reporters and demonstrated that increased mutation rate depends on the proximity of the tested locus to the meiotic break hotspot as well as on the function of Spo11 generating meiotic breaks [96]. Increased frequency of de novo mutations in the vicinity of meiotic breaks was also demonstrated by pull-down and accurate single-molecule sequencing of DNA next to sites of meiotic DSBs in human males [97].

Human germline de novo mutations detected by sequencing genomes of trios made up of two parents and their child, also led to the detection of mutation clusters [98–101]. Accounting for parent of origin led to the identification of the source of the majority of germline mutations as an age-dependent mutational process occurring in oocytes [99]. One study reported clustering next to germline rearrangement breakpoints [102]. C→T (or G→C) mutations were prevalent in clusters across the different studies. It is worth noting that hypermutation in ssDNA or DNA damage in dsDNA followed by replication prior to resolution of the damage can lead to clustered mutations along with strand coordination (Fig. 3). As such, lesions accumulated in oocytes and then fixed as mutation in the first cell division of a zygote would manifest as strand-coordinated clustered mutations seen in the human germline. More studies are needed to assess the sources and mechanisms of clustered hypermutation during meiotic recombination.

8. RNA

Unlike DNA, damage in RNA cannot be repaired and can result in altered protein upon translation. Mutant protein molecules resulting from such mutagenesis in transcripts may even have pathological consequences [103]. In addition to RNA lesions there are two types of RNA editing enzymes, ADAR adenine deaminases (changing adenines to inosines) and AID/APOBEC cytidine deaminases. The latter can work on DNA as well as on RNA. RNA editing of a small number of specific sites is a well-known phenomenon with physiological function, however widespread editing of random sites in mRNAs is occurring at a low frequency in each transcript (reviewed in [104,105]. RNA editing by ADARs [106,107] and APOBECs [108] was suggested to play a role in viral evolution and restriction in cells. Recently, it was also found that RNA viruses may be hypermutated as well [109]. A heavily mutated propagated Rubella virus was isolated from cutaneous granulomas of children with primary immunodeficiency. The virus isolated was a mutated isoform of the propagation-incapable rubella vaccine administered to all children after birth. Hundreds of mutations in the 10 kb viral genome mostly conformed to tCn APOBEC signature and were strongly biased to the viral plus strand. ADAR signature was also detectable but was less strand biased and represented a minor fraction of the mutations. Since replication cycle of Rubella virus is the same as in many other + strand ssRNA viruses, including Coronaviridae, hypermutation by APOBECs may be detected in other viral RNA genomes. Interestingly, the presence of APOBEC mutation signature was recently reported in Coronavirus from two COVID-19 patients [110]. This finding leads to more questions regarding the abundance of RNA hypermutation in model systems and human cells, and if in addition to viral RNA, mRNA may also be hypermutated.

9. Open questions

The ubiquitous presence of ssDNA intermediates in various DNA transactions and the increased propensity of accumulation of DNA damage in these intermediates justify further efforts to understand mechanisms of genome instability associated with hypermutable ssDNA. We envision several questions in this field that promise important progress in understanding the biological contexts and mechanisms that impact the hypermutability of ssDNA.

9.1. The impact of direct reversal into prevention of ssDNA hypermutation

Various proteins involved in direct reversal of DNA damage have been shown to process lesions in ds- as well as in ssDNA substrates in vitro [3,111,112]. However, whether these enzymes also reverse DNA damage in ssDNA in vivo is still unknown.

9.2. Resection size in mammalian cells

In yeast 5′→3′ resection from some DSB ends or from an uncapped telomere may proceed through dozens of kilobases if not channeled into a pathway restoring dsDNA. Unlike yeast, resection in mammalian cells is constrained by 53BP1 and the shieldin protein complex [113]. Nussenzweig and colleagues showed that in cells proficient in DSB repair, resection can extend to 3 − 7 kb [114] and in p53BP1 mutant mouse

embryonic fibroblasts resection could go up to 15 kb [115]. It not still clear how far rare individual resection tracts can go in cells with wild-type or mutated 53BP1. Interestingly, a novel method relying on the binding of an ssDNA specific antibody to stretched DNA fibrils demonstrated hundreds of kilobases stretches of ssDNA in U2OS human cells after gamma irradiation [116]. It is not known how the size of resection tracts varies between different cell and tissue types and physiological conditions.

9.3. Does BIR generate long ssDNA in mammalian cells?

Although BIR has been shown to generate long ssDNA stretches in yeast strains, direct evidence demonstrating the ability of this repair pathway to generate long and persistent ssDNA in mammalian cells is still lacking (Also reviewed in [32]).

9.4. Do long persistent stretches of ssDNA occur in meiosis

Multiple DSBs are induced and then successfully repaired in meiosis. In this sense it makes cells undergoing meiosis similar to mitotic cells repairing multiple simultaneously induced DSBs. The bulk of resection measured from yeast meiotic DSBs is relatively short, up to 2 kb [117], and the comparable size was reported for resection yeast cells repairing multiple gamma-induced DSBs [118]. Nevertheless, the small, but well detectable fraction of such breaks in yeast mitotic cells expressing the ssDNA specific cytidine deaminase APOBEC3A resulted in formation of up to a 100 kb stretches of hypermutable ssDNA, as reveled by APOBEC mutation clusters found in whole-genome sequenced post-irradiation isolates [57]. Similar experiments in yeast meiotic cells can assess the chance of long hypermutable DNA forming in meiosis.

9.5. How much of spontaneous mutagenesis is occurring via hypermutable ssDNA?

All genomic loci are single stranded at some point during the cell cycle and long persistent ssDNA is formed in several kinds of DNA transactions aside from normal replication fork. Since such ssDNA is prone to accumulating DNA damage, it is conceivable that mutagenesis at ssDNA plays a significant role in spontaneous mutagenesis across the genome. There are several observations that could be put together in support of this speculation. In *E. coli,* stress-associated mutagenesis, presumably associated with ssDNA formed during DSB-repair, was estimated to be responsible for up to 50 % of the spontaneous mutagenesis detected upon starvation of the cultures [21]. We speculate that the phenomenon of the constant rate of spontaneous mutation per genome in DNA-based microbes with either small or large genomes [119–121] can be also explained by major contribution of ssDNA hypermutation in spontaneous mutagenesis. Based on the study by Sakofsky *et al.* it was suggested that the tolerated amount of hypermutable ssDNA in genomes of yeast and cancer cells is comparable irrespective of the genome size [57]. Generalizing this observation, we speculate that the amount of ssDNA that can be tolerated by a cell could dictate cell survival. Thus, in biological contexts where hypermutable ssDNA would be the major source of spontaneous mutations, mutation rate would be proportional to the absolute size of hypermutable ssDNA which would be limited to a similar extent in small and in large genomes

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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