

## Critical Reviews and Perspectives

# Competition for DNA binding between the genome protector replication protein A and the genome modifying APOBEC3 single-stranded DNA deaminases

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### ABSTRACT

The human APOBEC family of eleven cytosine deaminases use RNA and single-stranded DNA (ssDNA) as substrates to deaminate cytosine to uracil. This deamination event has roles in lipid metabolism by altering mRNA coding, adaptive immunity by causing evolution of antibody genes, and innate immunity through inactivation of viral genomes. These benefits come at a cost where some family members, primarily from the APOBEC3 subfamily (APOBEC3A-H, excluding E), can cause off-target deaminations of cytosine to form uracil on transiently single-stranded genomic DNA, which induces mutations that are associated with cancer evolution. Since uracil is only promutagenic, the mutations observed in cancer genomes originate only when uracil is not removed by uracil DNA glycosylase (UNG) or when the UNG-induced abasic site is erroneously repaired. However, when ssDNA is present, replication protein A (RPA) binds and protects the DNA from nucleases or recruits DNA repair proteins, such as UNG. Thus, APOBEC enzymes must compete with RPA to access their substrate. Certain APOBEC enzymes can displace RPA, bind and scan ssDNA efficiently to search for cytosines, and can become highly overexpressed in tumor cells. Depending on the DNA replication conditions and DNA structure, RPA can either be in excess or deficient. Here we discuss the interplay between these factors and how despite RPA, multiple cancer genomes have a mutation bias at cytosines indicative of APOBEC activity.

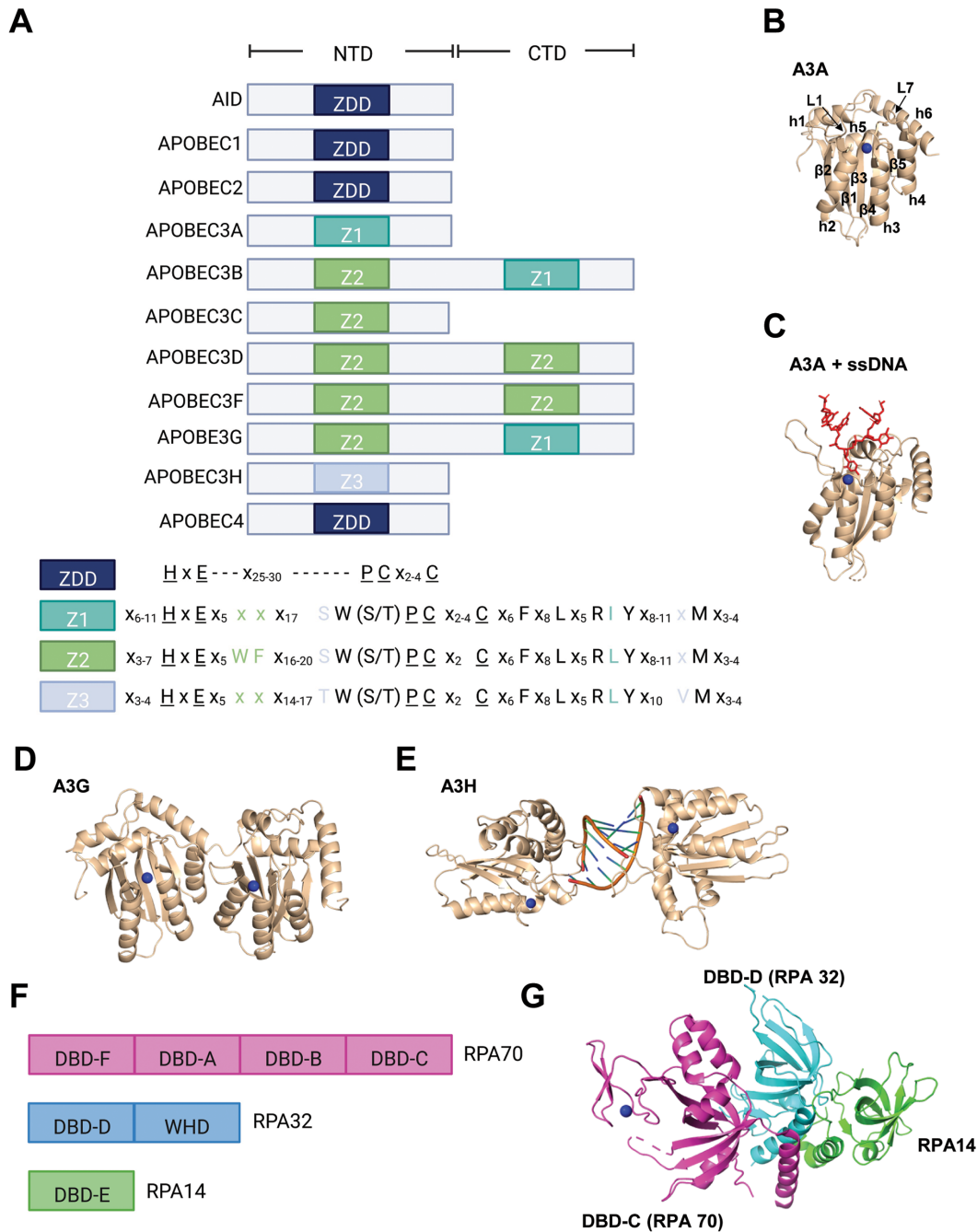
### INTRODUCTION

APOBEC enzymes are cytosine deaminases that deaminate cytosine to form uracil in single-stranded (ss)DNA or RNA. Although these promutagenic enzymes are usually beneficial, having roles in lipid metabolism, antibody affinity maturation, and restriction of viral replication, their unregulated activity can also cause mutations in the human genome that cause cancer evolution. However, in order to access genomic DNA, the APOBEC enzymes need to compete with replication protein A (RPA) that normally binds ssDNA to avoid unwanted degradation or recombination. Here we discuss the biochemistry of RPA–DNA and APOBEC–DNA interactions. These fields have largely stayed separate, despite the growth of the APOBEC/cancer field that has examined APOBEC enzymes and how they access transient genomic single-stranded (ss)DNA substrates, which would also be bound by RPA. The details of the competition of APOBECs and RPA for ssDNA has yet to be fully examined and put into the context of DNA replication and repair and cancer. Here we discuss the biochemistry of each of these DNA binding proteins and based on their biochemistry and the few studies that examined them together we suggest mechanisms and areas of future research.

### Overview of the AID/APOBEC family

Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC) enzymes are a family of evolutionary conserved, zinc-dependent polynucleotide deaminases that deaminate cytosines and consist of Activation Induced Deaminase (AID), APOBEC1 (A1), APOBEC2 (A2), APOBEC3A-H, except E (A3) and APOBEC4 (A4) (Figure 1) (1). A3H is the most variable as it has seven haplotypes (hap), but only A3H hap II,

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**Figure 1.** Protein domains of APOBECs and RPA. (A) The eleven human APOBEC enzymes have either a single or double Zn<sup>2+</sup>-binding domain. In the double domain enzymes, the N-terminal domain (NTD) mediates processivity and the C-terminal domain (CTD) catalyzes cytosine deaminations (8). The amino acid sequences surrounding the catalytic residues (underlined) for the three zinc domains (Z1–Z3) of the A3 subfamily that originated from the ancestral ZDD are illustrated below the NTD and CTD domain schematic. The colored labels for the domains indicate which NTD and CTD contain which amino acid motifs. (B) Crystal structure of A3A demonstrates the conserved APOBEC structure that is comprised of a five stranded β-sheet (labeled β1–5) surrounded by six α-helices (labeled α1–6). Loop 1 (L1) and Loop 7 (L7) control access to the active site and make key interactions with ssDNA that determine deamination motif specificity, respectively. The Zinc molecule is represented by a blue sphere (14). (C) Crystal structure of A3A as in (B), but bound to an ssDNA. The DNA takes a U-shaped conformation, which was also found with A3B and A3G (not shown). A3A structures in (B, C) are from PDB 5SWW (8). (D) Full length crystal structure of Rhesus Macaque A3G is used to represent the structure of double domain enzymes such as A3B, for which a full length structure is not yet available. The NTD and CTD are connected by a flexible linker. PDB 6P40 is shown (175). (E) The human A3H crystal structure revealed that it formed an obligate dimer with dsRNA. There are no protein-protein contacts in the dimer. PDB 6B0B is shown (14). (F) Human RPA is a heterotrimer consisting of RPA70, RPA32 and RPA14 (176). RPA contains six oligonucleotide binding domains called DNA-binding domains (DBD). RPA70 contains DBD-A, DBD-B, DBD-C and DBD-F. RPA32 contains DBD-D and a C-terminal winged-helix (WH) domain. RPA14 contains DBD-E. DBD-D, DBD-C and DBD-E from each subunit form the trimerization core to enable formation of the stable heterotrimer. (G) Structure of the RPA trimerization core demonstrates how the DBD-C of RPA70 (pink) interacts with the DBD-D of RPA32 (teal) and RPA14 (green). The interaction forms through three C-terminal α-helices that arrange in parallel. PDB 1L10 is shown (176). Structural figures were made using PyMol 2.3.1.

A3H hap V and A3H hap VII are stable in cells (2). All A3 enzymes contain at least one cytosine deaminase domain that is defined by a consensus amino acid (aa) sequence motif (HxEx<sub>25-30</sub>PCx<sub>2-4</sub>C) that is embedded in a conserved fold comprised of five beta sheets and six  $\alpha$ -helices (Figure 1A and B) (3–5). This aa sequence is a zinc-dependent deaminase (ZDD) domain that is crucial for deamination activity on ssDNA and RNA. A3A, A3B and A3G have been shown to bind the ssDNA in a U-shaped conformation through primarily contacts on loops 1 and 7 (Figure 1C) (6–8). Several members of the A3 subfamily, namely, A3B, A3D, A3G and A3F, all contain two zinc coordinating domains, however only the C-terminal domain is catalytically functional (Figure 1A, D). Despite the lack of catalytic activity in the N-terminal domain, this domain still retains RNA- and DNA-binding (9–11). Additionally, it confers processivity enabling these enzymes to deaminate consecutive cytosines in a single enzyme-substrate encounter, rather than deaminating and then dissociating from ssDNA in a non-processive manner (12). The protein sequences within these ZDD domains enable the APOBECs to be grouped further into three sub-families: AID, A1, A3 and the A3s can be further sub-divided into three sub-groups based on their Z1, Z2 and Z3 domains (Figure 1A) (5,13). A3H uniquely forms the Z3 group and dimerizes using a double-stranded (ds) RNA molecule with no protein-protein contacts in the dimer molecule (Figure 1E) (14). These three zinc domains have been predicted to have undergone a minimum of eight duplication events due to positive selection as a protective measure against rapidly evolving pathogenic retroelements and retroviruses (15). This is supported by the discovery of ancestral AID genes that were first identified in jawless fish present over 500 million years ago, which ultimately led to the existence and expansion of today's APOBEC genes in amphibians, birds and mammals (1,16).

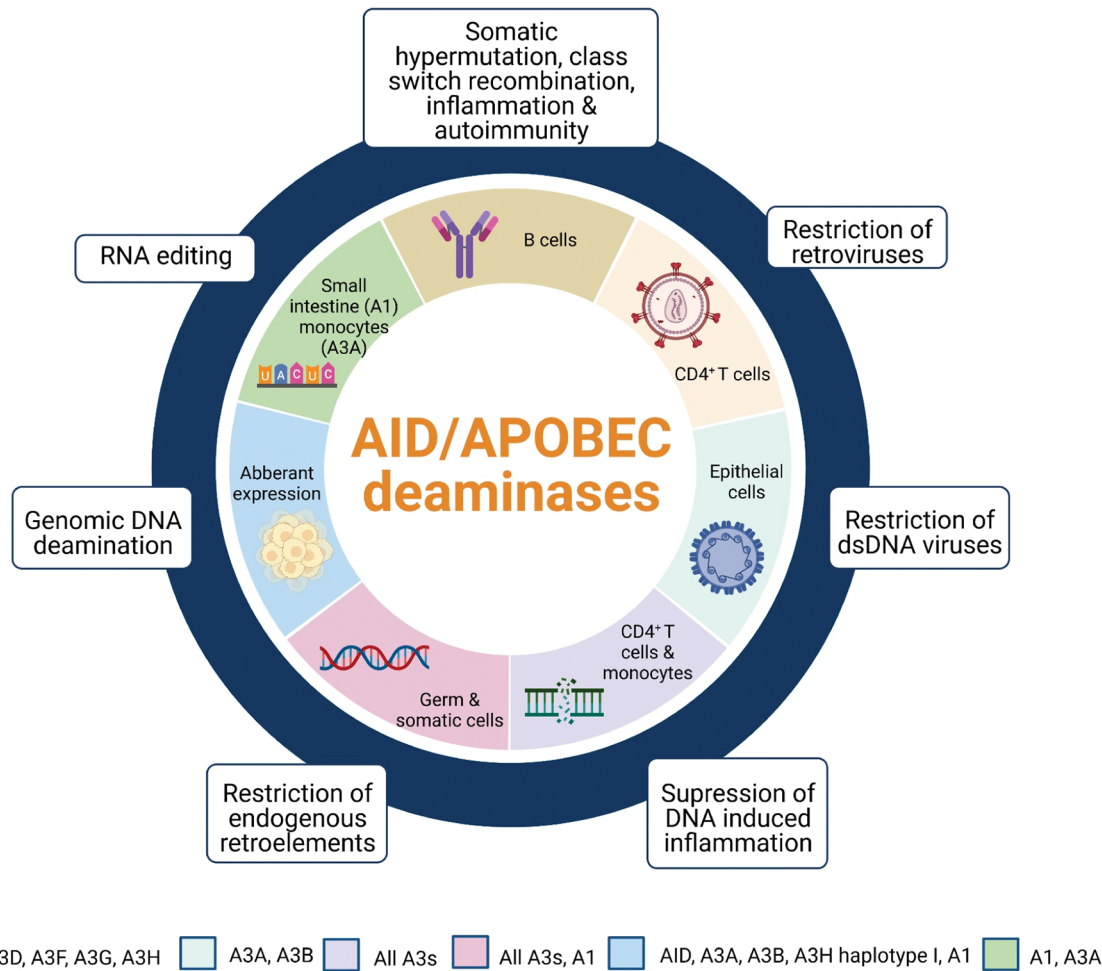
### Diverse functions of AID/APOBECs

APOBEC-mediated cytosine deamination can have different consequences depending on whether it occurs in RNA or DNA. While RNA deaminations are modifications due to the coding functionality of uracils, DNA deaminations are promutagenic lesions that can lead to undesired permanent alterations in the genome if unrepaired or repaired erroneously. Although it may be detrimental to have uracil in DNA under normal circumstances, different cell types use these uracils in a variety of positive ways which emphasizes the importance of regulating these enzymes. In addition, genome engineering technologies have taken advantage of the ability of certain APOBECs to modify genomic DNA by fusing them to Cas9 to allow for programmable CRISPR/Cas9 base editing (17).

The founding member, A1 was originally discovered as an RNA deaminase that plays an important role in lipid metabolism (Figure 2) (18). AID, the ancestral member of the APOBECs is responsible for initiating mutagenesis of immunoglobulin genes to allow production of antibodies with strong affinities (somatic hypermutation; SHM) and different classes, e.g. IgG or IgA (class switch recombination; CSR) (Figure 2) (19,20). When AID is dysregu-

lated it has been found to contribute to inflammation and autoimmunity (Figure 2) (21). The A3 subfamily consists of seven members, A3A-A3H (except E) and are host restriction factors that are constitutively expressed in T cells, macrophages and germ cells and are expressed in epithelial cells in response to viral infection (22–24). The A3 enzymes enable suppression of ssDNA intermediates of retroviruses, retrotransposons, herpesviruses, and other foreign DNA through ssDNA deamination as a means of mutagenesis (Figure 2). A3A and A3G have also been shown to deaminate RNA. A3A RNA editing occurs in response to interferons and hypoxic conditions, but the cell type and function of A3G editing is unknown (Figure 2) (25,26). Retroviral restriction occurs when relevant A3s avoid antagonism from the viral Vif protein and are packaged into viral particles along with the viral genome (27,28). Like other DNA viruses, replication of herpes viruses takes place in the nucleus and Epstein Barr virus encodes the protein BORF2 that depletes A3B from the nucleus to avoid host restriction (Figure 2) (23). A similar antagonism occurs with Herpes Simplex Virus Type 1 ICP6 protein, a BORF2 ortholog and A3A (29,30). The ssDNA of the viral genome is the substrate for deamination and results in hypermutations that lead to inactivation or degradation of the viral DNA (31). Because the expansion of the A3 locus correlates with the expansion of retrotransposons in primate genomes, these genomic elements are thought to be their original target (Figure 2) (32,33).

Biochemical pathways of transcriptional regulation have not been fully elucidated for the AID/APOBEC family, but subcellular localization is an important factor in maintaining proper function. Studies have shown that in cancer cells A3B and A3A transcription correlates with upregulation of other cell cycle and DNA damage response genes (34,35). For other A3s, including A3H hap I and A3A, transcription in cancer cells correlates with immune related genes (35). Overall, further research is needed to fully elucidate the transcriptional regulators of A3 activity. Cytoplasmic A3s (A3D, A3F, A3G) appear to not have access to genomic DNA even during mitosis in normal cells, but there have been reports of A3G in the nucleus in cancer cells (36–38). These A3s have a dominant cytoplasmic retention signal, even with creation of a chimeric NLS, which may be a preventative measure against off-target deaminations (39). The localization of A3H appears to be haplotype dependent where the stable A3H hap II is actively retained in cytoplasm whereas the less stable A3H hap I enters the nucleus (40,41). RNA-binding is required for A3H hap II cytoplasmic localization and HIV-1 restriction. RNA binding mutants showed disrupted cytoplasmic localization and poor encapsidation into viral particles, leading to lower antiviral activity (14). Interestingly, it was also found that A3H hap II is actively retained in the nucleolus in addition to the cytoplasm suggesting A3H may form two different ribonucleoprotein complexes to regulate subcellular localization. Like A1, A3B has N-terminal positively charged residues that mediates nuclear localization through interactions with importin proteins (42,43). A3A that is endogenously expressed in monocytic cells is retained in the cytoplasm whereas expression in other cell types appears cell wide (44,45). The varying localizations of A3s highlight the importance of



**Figure 2.** The diverse functions of the AID/APOBEC family. APOBECs are expressed in different cell types and have many diverse functions. A1 and A3A can undergo RNA editing in the small intestine and in monocytes, respectively. A3G (not shown) can catalyze RNA editing in 293T cells, but the physiological role is not known. In B cells, AID plays an essential role in immunoglobulin diversification through somatic hypermutation and class switch recombination. When dysregulated, AID can cause inflammation and autoimmunity. In CD4<sup>+</sup> T cells, A3s suppress retroviruses through deamination of ssDNA intermediates and inhibition of reverse transcriptase. A3A and A3B can also restrict dsDNA viruses during viral replication in epithelial cells. In CD4<sup>+</sup> T cells and monocytes, restriction of foreign DNA by A3s can suppress the DNA-induced inflammatory response. In germ and somatic cells, restriction of endogenous retroelements occurs through deamination of ssDNA intermediates or RNA binding. Aberrant expression of APOBECs can lead to 'off-target' deaminations in genomic DNA that could result in cellular transformation or cancer.

regulation as nuclear A3s have been implicated in various cancers (46–49).

### AID/APOBECs as genomic mutators

The roles of A3s in viral restriction have been the focus of research for many years until it was discovered that there is a cost to this defense mechanism. Current evidence suggests that aberrant A3 expression can lead to the formation of promutagenic uracils on genomic DNA (Figure 2). The fact that normal expression of A3s in their respective cell types does not usually lead to cancer suggests that they have developed their own regulatory mechanisms in addition to the individual intrinsic enzyme properties to prevent aberrant deamination. A3-catalyzed deaminations are one of the most common mutational processes in cancer and were identified in > 50% of cancer genomes (50,51). The APOBEC signature in cancer genomes was

identified as part of 21 single base substitution (SBS). The two APOBEC-associated signatures are SBS2 (C > T) and SBS13 (C > G and C > A) within a TCN (underlined C denotes target cytosine) nucleotide context (50–53). With more extensive studies on the mutation signatures of A3A and A3B since the original identification of the SBS types, it has become apparent that there are specific extended signatures attributable to each A3. A3A prefers deamination at 5' YTCA (Y = C or T) and A3B prefers deamination at 5' RTCA (R = A or G) (54). A3A also can efficiently deaminate cytosine in hairpin loops, unlike A3B and A3H hap I (55). In these hairpins, A3A has been found to be able to deaminate 5'VC (V = A, C, or G) sequences (56).

However, it is unknown as to what stage APOBECs exert their mutagenic potential, namely whether they are involved at the early stages of cellular transformation or the late stages and provide tumor genetic diversity. Additionally, we are lacking an understanding of the effect of these

mutations. It is unclear whether they are deleterious, neutral or advantageous to tumor growth or evolution but it is likely that these effects are cancer type specific. Although this has yet to be tested, it has been suggested that APOBECs provide a 'just right' amount of mutagenesis for tumor diversity, meaning that too little diversity results in the inability of a tumor to adapt to selective pressures but in contrast, too much can be detrimental due to genomic instability (57). For instance, A3 activity has been observed to be a contributing factor in branched evolution as well as the accumulation of subclonal mutations in breast cancer, esophageal squamous cell carcinomas, lung adenocarcinomas, and head and neck squamous cell carcinoma (48,58–60). Conversely, there is also evidence suggesting that A3s can generate 'driver' mutations that are associated with human papillomavirus (HPV)-associated cancers (61). Further studies on the outcomes of A3 deamination activity in tumorigenesis is needed.

Despite the role of A3s in cancer being a relatively new topic of research, the role of AID in cancer had already been discovered years prior. In the early 1980's researchers had already found that genomic rearrangements such as translocations were present in many B-cell lymphomas, specifically between an immunoglobulin (Ig) locus and an oncogene. A number of different oncogenes including *Bcl-2*, *Bcl-6*, *c-myc* have been characterized in B-cell malignancies (62–64). Analysis of breakpoints of *c-myc/IgH* breakpoints revealed that most of the translocations involved the immunoglobulin switch region, a long, repetitive sequence required for CSR. The recombination between two switch regions in the immunoglobulin gene locus allows a B cell to switch from IgM to a more specialized immunoglobulin, e.g. IgG. This is initiated by excision of AID-catalyzed uracils that lead to dsDNA breaks and enable recombination (65). The involvement of AID was confirmed when translocations were not observed in the absence of AID *in vivo* even when artificial breaks were introduced to recapitulate the *c-myc/IgH* translocation (66,67).

Since the discovery of APOBEC-associated mutational signatures, the major focus has been trying to determine which A3s are active during cancer as well as investigate their biological effects. This has been a focus of research because the predominant *in silico* studies have only characterized mutation signatures retroactively or used RNA-seq data from databases like The Cancer Genome Atlas (TCGA) to demonstrate the presence of mRNA, but not catalytically active protein. Additionally, A3 protein levels may differ depending on the type or grade of cancer, which may or may not affect the number and outcome of A3-induced mutations. Determining which A3s are responsible for the SBS2 and SBS13 mutation signatures has been hindered in part because many of the studies use comparisons of normal and tumor tissues and there is evidence of some tumor samples being a mixed population with immune cells that infiltrated the tumor microenvironment (35). Since A3s are also highly expressed in immune cells, the analysis of their mRNA expression in tumor samples needs to be corroborated by other evidence of their involvement in somatic mutagenesis (22,24). Most of the studies revolved around A3B since it was found to be highly expressed in a number of cancers including breast, ovarian, cervical, lung, head and

neck and bladder (47,68). It was suspected to play a major role in A3 mutagenesis as there was evidence demonstrating the correlation between A3B expression and A3 mutation signatures unlike some of the other A3s (47). Conversely, one study found that A3B expression could occur in breast tumors in the absence of an A3 mutational footprint, but A3A expression only occurred in breast tumors that had an A3 mutational footprint suggesting that A3A expression is more indicative of APOBEC deamination (69). As previously described, APOBECs deaminate cytosines within a preferred nucleotide sequence context and in the case of A3B it is 5'A/GTCA (where underlined C is mutated) (47,54,57,70). A3B has also been demonstrated to be a biomarker of poor prognosis for estrogen receptor positive breast cancers, again strongly suggesting that A3B induced mutations contribute to breast cancer progression (71). However, because A3B-null cancers still have a bias of 5'TC cytosine mutations, it meant that there must be at least one other APOBEC family member contributing to the overall mutation load (48,72). Studies have identified mutation footprints of 5'TTCA or 5'CTCA in tumors, which are the preferred context for A3A-catalyzed deaminations, but despite this, a corresponding upregulation of A3A mRNA has not always been detected (73,74).

Nonetheless, A3A can cause genomic damage in cell and tissue experiments. A3A has been suggested to be the most active deaminase as it has been shown to translocate to the nucleus and induce dsDNA breaks that can lead to cell-cycle arrest and death (44,46,70). Due to the inability of some studies to find a correlation between upregulation of A3A mRNA and its mutational footprints, it is difficult to determine if A3A-induced mutagenesis is an ongoing event. It may just be more stable than the other A3s and therefore does not require high amounts of upregulation to see activity (69). Several studies propose that A3A expression is an early event that is later shut off because long exposure to A3A-catalyzed deaminations could be detrimental to cells rather than providing a selective advantage (44,46,54). A study has provided evidence that A3 activity occurs episodically and is difficult to pinpoint specific times since A3s appear to go through periods of inactivity (52). It is unknown what controls the episodic nature of A3s but viral infections and other oncogenic events may result in transient upregulation. Despite the high activity of A3A, there is emerging evidence suggesting a deamination independent role of A3A in tumorigenesis. One study did not find a correlation with mutations and A3A expression in pancreatic cancer and suggested that A3A expression without deamination when in combination with a KRAS mutation or alterations in p53 was sufficient in causing chromosomal instability (CIN), which is a hallmark of cancer (75). Additionally, another study demonstrated that catalytically active A3A can drive tumorigenesis in two murine systems where low expressing A3A enhanced polyp formation in an adenomatous polyposis coli multiple intestinal neoplasia murine model for colon cancer. High expressing A3A resulted in elevated frequencies of hepatocellular carcinoma in a *tp53*-depleted system. Although A3 mutation signatures were detected in both systems, no recurring driver mutations were evident in either system (76). This suggests that A3s may have a possible deamination independent mechanism when

in combination with additional genetic alternations that result in CIN. The role of a deamination independent mechanism remains to be characterized as the mechanistic basis is not yet understood (76). A3A also has RNA editing capabilities and in one study, researchers were able to detect mutation signatures in RNA in the absence of corresponding DNA mutations and this correlated with A3A but not A3B expression (73). Detection of RNA editing by A3A may be a way of measuring ongoing A3A activity as these mutations are not inherited in the genome and are transient (73). However, the impact of RNA editing by A3A in cancer remains to be determined.

It has become apparent that one of the main caveats of past studies was not only relying on mRNA expression, but also studying the A3 enzymes in isolation. Petljak *et al.* used breast cancer cell lines that had the ability to express both A3B and A3A and made knockout cell lines to only express A3B or A3A (74). Interestingly, although *in vitro* deamination assays using cell lysates demonstrated that A3B had more activity than A3A, when the somatic mutations were analyzed, the A3A preferred 5'YTCA signature was more prominent than the A3B preferred 5'RTCA signature and deletion of A3A rather than A3B more significantly reduced SBS2 and SBS13 mutations in the tested cell lines (74). Further, upon A3B deletion, there appeared to be an increase in A3A activity in some cell lines that correlated with increased A3A protein levels/deamination activity, suggesting some type of related transcriptional regulation of A3A and A3B (74). Overall, the authors concluded that A3A is the main driver of SBS2 and SBS13 mutations in breast cancer and B-cell leukemia cell lines.

Despite A3H having seven haplotypes, only A3H hap I has been demonstrated to be able to induce mutations in breast and lung cancer cells in A3B-null cancers despite being far less stable and antiviral compared to the other haplotypes (48,77,78). This is surprising as there is evolutionary evidence suggesting that the unstable A3H hap I was the result of one of two independent polymorphic events that lead to increased ubiquitination and degradation, resulting in reduced antiviral activity (2,77). The majority of the human population have less functional A3H alleles like A3H hap I, whereas the stably expressed proteins, like A3H hap II, are primarily found in African populations perhaps due to ongoing pathogenic pressure to maintain activity (77). This suggests the loss of activity may be an evolutionary protective measure against off-target deaminations. In one study, an A3H hap I K121E single nucleotide polymorphism (SNP) was discovered to correlate with lung cancer (79). Surprisingly, the SNP resulted in active site disruption and destabilization of the RNA-mediated dimer interface (80). This unexpected result suggests that A3H hap I induced mutations in lung cancer may be too mutagenic and lead to death of tumor cells, suggesting that A3H hap I expression in lung cancer is detrimental for the tumor (80). Conversely, there has been another SNP that was identified that had a R18L substitution that had deleterious effects on both the structure and function of the protein and potentially interfered with transcriptional regulation but it was suggested to be associated with lowered risk of lung cancer (81). Further studies are warranted to determine the roles of SNPs of A3H and their association with lung cancer.

Computational analysis identified the potential role of A1 to be a genomic mutator. Evidence of a mutational signature correlating with the expression of A1 was found in esophageal adenocarcinomas (82). These data suggested that aberrant A1 expression outside of intestinal epithelial cells could lead to undesired somatic mutations and potentially cellular transformation (82). Mutator assays in both bacterial and human cells have also demonstrated its ability to induce mutations in DNA as well as produce a mutator phenotype (82,83). A1 mRNA editing targets the 3'UTRs of transcripts (84). This indicates that both RNA and DNA can be deaminated by A1 and suggests that A1 can both deregulate mRNAs as well as hypermutate cancer genomes. However, measurement of  $\gamma$ H2AX foci as a marker for dsDNA breaks or slowed replication in the presence of A1 showed that it can only cause a low-level of DNA damage (85–87). Although A1 has similar specific activity to A3H hap I and A3A on naked ssDNA, biochemical studies showed that A1, in contrast to A3H hap I and A3A, was unable to compete with replication protein A (RPA) for ssDNA (85). Thus, these data suggested how the ssDNA conditions in cells can modulate APOBEC-induced damage.

### Replication protein A

RPA is a ssDNA binding protein that is essential for all aspects of DNA metabolism including DNA replication, transcription, recombination, DNA damage checkpoints and repair pathways such as base excision, nucleotide excision, and double-stranded break (DSB) repair (88,89). Its main role is to protect it from unwanted nuclease activity, secondary structure formation and to promote DNA processing (88,89). RPA interacts with numerous proteins involved in these pathways and ensures the proper proteins are able to access the DNA while still protecting and maintaining the integrity of the DNA (90–93).

Human RPA is a hetero-trimer consisting of RPA70, RPA32 and RPA14 (70, 32 and 14 kDa, respectively) (94) (Figure 1F, G). All three subunits contain at least one DNA-binding domain (DBD) designated from A-F and have oligonucleotide/oligosaccharide binding (OB) folds (Figure 1F, G). RPA70 contains four OB-folds where the DBD-A and DBD-B central folds are the DNA-binding core and are necessary for high affinity binding (89). DBD-F is at the N-terminus and is required for binding partially duplex DNA and checkpoint activation through phosphorylation. DBD-C is located at the C-terminus and has roles in DNA interaction, trimer formation and DNA damage recognition (Figure 1F, G). RPA32 contains a DBD-D fold, an N-terminal phosphorylation domain to signal DNA damage and modulate activity, and a C-terminal winged-helix (WH) domain that is primarily involved in protein-protein interactions (Figure 1F, G). RPA14 only has a DBD-E domain and interacts weakly with DNA but is required for complex formation (Figure 1F-G) (95). RPA is very flexible due to its modular structure and can adopt multiple conformations with varying modes of interaction with DNA nucleotides (nt): a low affinity mode (binds ~8 nt,  $K_d \sim 100$  nM), a moderate mode (binds 12–23 nt,  $K_d \sim 5$  nM) and high affinity mode (binds 30 nt,  $K_d \sim 0.05$  nM) (96).

## DISCUSSION

### INTERSECTIONS OF RPA AND A3 ENZYMES

#### DNA replication

Since APOBECs catalyze deaminations on ssDNA, these ‘off-target’ deamination events only occur when ssDNA intermediates are present such as during transcription, replication, or DSB repair (34,97–99). During DNA replication initiation, RPA is recruited by the helicase to bind on emerging ssDNA to stabilize ssDNA regions formed by origin unwinding and to help recruit proteins such as DNA polymerase alpha/primase to the nascent replication fork (100–102). A3-mediated mutations frequently occur during DNA replication and are most enriched in early-replicating, gene-dense and regions with active chromatin (98). Despite the protection provided by RPA during leading and lagging strand synthesis, there is a lagging-strand bias of clustered and sporadic A3-induced mutations (Figure 3A). Additionally, A3A-induced DNA damage has been demonstrated to lead to cell-cycle arrest at early S-phase further indicating that replication forks are susceptible to A3 activity and that A3-induced DNA damage has the potential to lead to stalled replication forks (44). During stalled replication, it is known that the helicase can become uncoupled from the polymerase where it can continue unwinding DNA without synthesis, generating long stretches of DNA and creating an abundance of substrate for A3s to deaminate (Figure 3B) (103). Altogether, this strongly suggests that there is a correlation between the number of mutations and the amount of time DNA remains single-stranded (97,104,105). Furthermore, A3A, A3B, and A3H can deaminate both methylated and non-methylated cytosines and study found that there were 2-fold higher number of mutations on unmethylated compared to methylated DNA, further illustrating that these off-target deaminations are occurring on newly synthesized DNA when DNA methyltransferases have not had the opportunity to methylate the DNA (104,106–108).

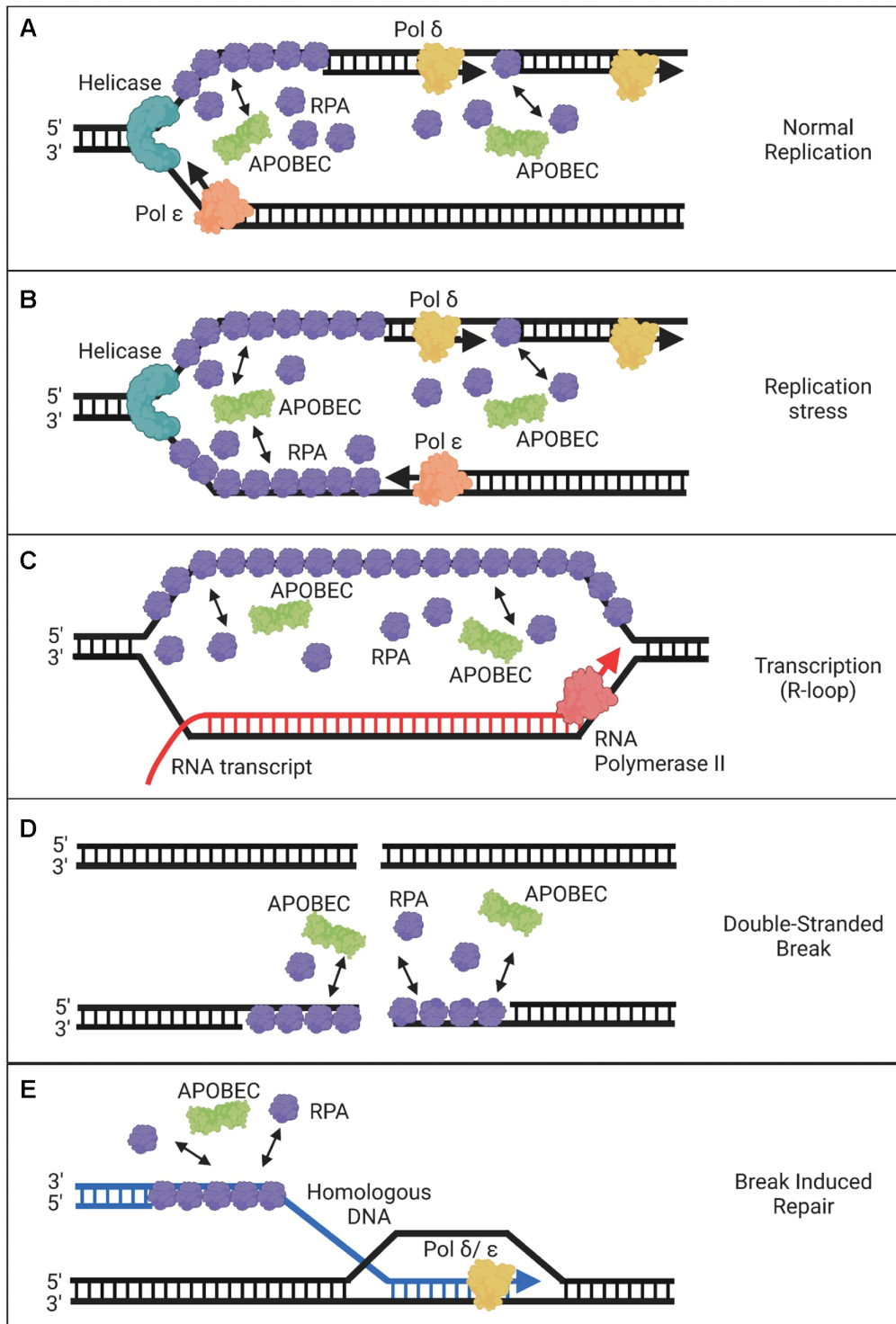
In addition to recruiting proteins necessary for DNA replication, RPA also functions as a sensor for ssDNA at stalled replication forks and DNA damage. Transformed cells with pre-existing genomic instability have been shown to experience higher levels of replication stress and fork stalling leading to more ssDNA exposure and opportunities for A3-mediated mutagenesis (97,99,103,104,109). There are several factors that can cause replication fork stalling and collapsing to prevent erroneous replication. Exposure to chemicals such as hydroxyurea (HU), which causes dNTP pool depletion, has been shown to enhance A3 activity (34). Reduced or depleted dNTPs can lead to reduced DNA synthesis due to a lack of substrate for DNA polymerases. DNA synthesis arrest may occur before nucleotide pools are exhausted to preserve dNTPs for DNA repair or prevent synthesis under these suboptimal conditions. Low levels of DNA polymerases have also been shown to affect the formation of mutations. It has been demonstrated in yeast that low levels of DNA polymerase causes replication stress, which can result in stalled replication forks that are prone to breakage or uncoupling of leading- and lagging-strand synthesis (110). This leads to high levels of ssDNA, suggesting that the high mutation rates observed arise from

the instability of replication forks that have long stretches of ssDNA. Both DNA breaks and uncoupling of strand synthesis results in large ssDNA regions at the fork, which are susceptible to A3-induced mutagenesis.

#### Transcription

Unlike DNA replication and repair, there is little evidence suggesting the involvement of RPA during normal transcription. However, one study suggested that it may be involved in elongation and stabilizing the non-transcribed strand because Yeast Rfa1 (equivalent to RPA70 in humans) was found to associate with the RNA polymerase II (RNAPII) complex, interact with elongation factors Spt4 and Bur2, and in a proteomic screen in budding yeast be present at active genes transcribed by RNAPII and RNAPIII (111). Further studies are required to elucidate the role of RPA during normal transcription. A3 enzymes have not been found to significantly target ssDNA in transcription bubbles, in contrast to AID (97,104,112). Due to the known and specific role of AID deaminating cytosines in the immunoglobulin gene locus during transcription, the role of A3 deaminase activity during transcription was investigated when they were discovered to induce somatic mutagenesis (Figure 3C). In yeast cells, A3A can deaminate during transcription, but to such a lesser amount than during DNA replication that the authors of the study concluded this was not a source of A3-induced mutations (97). The reasons for this are not known, however *in vitro* studies with T7 transcription systems have raised some possibilities, an obvious caveat being that a phage RNA polymerase is not similar to a human transcription complex. A3s have been demonstrated to have the ability to deaminate the non-transcribed strand during an *in vitro* phage T7 transcription system (113). A3A and A3H were both able to deaminate during T7 RNA polymerase mediated transcription whereas A3B was unable (113). If we take into consideration that A3H hap I and A3B deamination activity decreases in the presence of RPA-saturated ssDNA, it is highly probable that in the tight confines of the transcription bubble their activities would be further reduced (113). In contrast, A3A would be the most probable candidate to deaminate during transcription, however it is unknown why this is not observed to be significant in human cells (97). Data from Brown *et al.* found that even A3A cannot deaminate ssDNA in a transcription bubble generated by T7 RNA polymerase if the polymerase is stalled (114). Rather their data suggest that A3s can only deaminate in R-loops behind the RNA polymerase and not actually during active transcription (114).

Stalled transcription can trigger the stress response signal that is driven by RPA and Ataxia-telangiectasia mutated and Rad3-related serine/threonine kinase (ATR) as they monitor elongation during transcription. This leads to the phosphorylation of Ser-15 of p53 in a replication-independent matter (115). In contrast to DNA polymerases, RNAPII is unaffected by DNA lesions but will stall at bulky DNA adducts such as thymidine dimers and cisplatin-induced crosslinks and trigger transcription-coupled repair (116). Stalling of RNAPII by blocking agents can trigger



**Figure 3.** Models of the RPA/APOBEC interface during DNA replication, transcription, and repair. **(A)** During normal replication, APOBEC enzymes compete with RPA primarily for access to the lagging strand where there is more ssDNA. **(B)** Replication stress can cause uncoupling of the DNA polymerase with helicase and can result in larger regions of ssDNA that are bound by RPA on both the leading and lagging strands. APOBECs can compete with the RPA that accumulates at these sites for access to ssDNA. **(C)** RPA can bind the non-template DNA strand of a stalled transcription bubble that has formed an R-loop. The R-loop is intentionally depicted larger than what would occur in a cell to illustrate potential protein–DNA interactions. Although there does not appear to be a major role of RPA during normal transcription, it does accumulate when transcription stalls or on R-loops. The APOBEC enzyme AID is more likely to deaminate during transcription than A3 enzymes, although some A3 activity during transcription has been demonstrated. **(D)** DNA repair resulting from DSB can lead to end-resection of DNA exposing ssDNA that is bound by RPA. APOBECs compete with RPA for access to this DNA, which may be facilitated if there is an RPA deficiency in the cell due to excessive firing of replication forks or high amounts of DNA damage. **(E)** BIR gives extended access to ssDNA. The migrating bubble replication during BIR creates ssDNA that persists and is thought to be a major source of A3-induced kataegis.



the stress response signal. Since ATM serine/threonine kinase (ATM) that is activated by DNA DSB is not involved in Ser-15 phosphorylation, it is unlikely that transcription stalling results in this type of damage (115). Transcription can also be impaired if the RNA transcript re-anneals to the template strand leading to formation of an R-loop. These RNA-DNA hybrids called R-loops are formed when the newly synthesized RNA transcript is transiently paired to the coding strand. The non-coding strand is looped out and remains single-stranded (Figure 3C). This can trigger a DNA damage response since this can impair transcription and DNA replication leading to replication stress and DSB formation (117). RNase H is required to degrade the RNA of the RNA-DNA hybrid in a sequence independent manner. Absence of RNase H during transcription-associated DSB results in stabilization of the DNA-RNA hybrids around the DSBs and prevents RPA recruitment whereas overexpression of RNase H results in the opposite where enhanced resection and recruitment of RPA is observed (118). RNase H1 activity on R-loops is enhanced by RPA. The loss of interaction between RPA and RNase H1 results in the inability to suppress R-loops leading to genomic instability (119). Therefore RPA is also important for sensing R-loops and regulating RNase H1 activity, further building on its role for sensing DNA damage and replication stress. A3A, A3B and A3H hap I can all deaminate an *in vitro* R-loop suggesting that they are able to access the small segment of ~8–20 nt ssDNA available albeit with lower activity than fully ssDNA (113). Further analysis suggested that the larger oligomeric state of A3B may hinder its ability to stabilize itself on an active transcription bubble (113). Despite this, R-loops can span 0.1–2 kb and so depending on the length of the R-loop, A3B may be able to compete with RPA for binding on bigger R-loops (120).

### DNA repair

DNA repair processes are more likely to leave ssDNA exposed for longer periods than DNA replication enabling higher levels of deamination by A3 enzymes (Figure 3D-E). These higher levels of deamination are seen as clustered mutations called kataegis (meaning thundershowers in Greek), but the mechanism enabling this extended access to ssDNA was not initially known (121). Kataegis are clustered C-to-T and/or C-to-G mutations that are enriched at TCN motifs (N denotes any nucleotide) located on the same DNA strand (51). In addition, several studies found that A3 expression can cause  $\gamma$ H2AX foci (80,122,123). These foci represent dsDNA breaks and/or slowed replication (86,87). Most studies have focused on the DSB formation, but the distribution of  $\gamma$ H2AX foci due to DNA breakage or simply just slowed replication is not known. Nonetheless, when DSB are induced after uracil removal, usually by overexpressing A3A, several repair pathway options ensue (Figure 3D) (123). However, one type of repair that gives extended access to ssDNA is DSB repair break-induced replication (BIR) (Figure 3E) (124). BIR is used to repair DSB with only one repairable end, such as would occur at collapsed replication forks. The DNA synthesis during BIR is a migrating bubble where lagging strand synthesis is significantly delayed in comparison to leading strand synthesis

(125). This accumulation of ssDNA behind the replication bubble is thought to be a major substrate for A3 enzymes, leading to kataegis (Figure 3E) (126,127).

In response to DNA damage, DNA checkpoints are activated to arrest cell cycle progression and enable repair. RPA is essential for a number of DNA repair pathways including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and DSB repair because it is needed for signalling damage and recruiting and positioning various repair factors (94). The N-terminal domain of RPA32 is phosphorylated in response to DNA damage by ATM, ATR and DNA-dependent protein kinase (DNA-PK). The amount of phosphorylation of RPA depends on the type of DNA damage where RPA32 S33 is primarily phosphorylated by ATR and S4/S8 and Thr21 by ATM and DNA-PK (128). This phosphorylation happens sequentially by first ATR at S33 followed by DNA-PK at S4/S8. During replication catastrophe caused by the absence of ATR and accumulation of ssDNA by HU, T21 is phosphorylated as well (129). DNA damaging agents that result in DSB only result in modest phosphorylation whereas UV, HU and camptothecin that result in generation of large amounts of ssDNA from fork stalling can induce hyperphosphorylation where the N-terminal domain of RPA32 is phosphorylated at five or more distinct sites, which serves as a marker for DNA damage (130). It has been suggested that the addition of negative charges through phosphorylation will generate electrostatic repulsive forces between hyperphosphorylated RPA32 and negatively charged ssDNA that will encourage dissociation to enable DNA repair (89,131). If this was the case, then it would mean that A3s would be able to gain easier access to ssDNA when RPA is phosphorylated because of its weakened interaction with ssDNA. However, this decreased ssDNA binding affinity of phosphorylated RPA for ssDNA was only observed for purine-rich substrates (132). In general, phosphorylated RPA has a decreased ability to bind and destabilize dsDNA (132–134).

### Mechanisms of competitive binding of ssDNA

Although RPA binds very tightly to ssDNA, it must be displaced from ssDNA to allow access by downstream proteins during replication or repair processes. The rate and extent of RPA dissociation are dependent upon the concentration of free RPA (135,136). RPA can remain bound to ssDNA for hours at an infinite dilution limit but when there is an excess of free RPA present in solution, the protein exchanges between free and bound states (137). This mechanism ensures that RPA remains bound and protects ssDNA while still maintaining the flexibility to be displaced when necessary. Interestingly, one study observed that a small subpopulation of RPA remained resistant to facilitated exchange, which could suggest that different binding modes exist when RPA binds DNA and that it may be dependent on the number of DBDs interacting with ssDNA (135,138). Another study proposed a different model suggesting that all DBDs transiently bind ssDNA with high affinities but differ in their dynamic interactions on ssDNA therefore enabling other proteins to compete with DBDs for binding (138). Adjacent RPAs can also contact each other via the DBD-A of one RPA and DBD-E of the other suggesting

that this interaction could stabilize a conformation that linearizes ssDNA (139). However, this interaction is weak and occurs after ssDNA binding since no cooperative binding was observed. Based on this tight interaction on ssDNA and protein-protein interaction between adjacent RPAs, the likelihood that facilitated exchange is sufficient to provide enough access to ssDNA for repair proteins is low. A mechanism through phosphorylation was proposed as a means to facilitate more exchange. The phosphomimetic mutant, Rfa1 S178D (equivalent to RPA70 S180 in humans), was observed to have a weakened interaction with ssDNA (mediated by DBD-A and DBD-B) but enhanced affinity with adjacent RPA (mediated by DBD-A and DBD-E). This shift in binding association between ssDNA and adjacent RPA molecules suggests that it may be a switch mechanism that RPA allows for displacement and exchange with other ssDNA-binding proteins. (139).

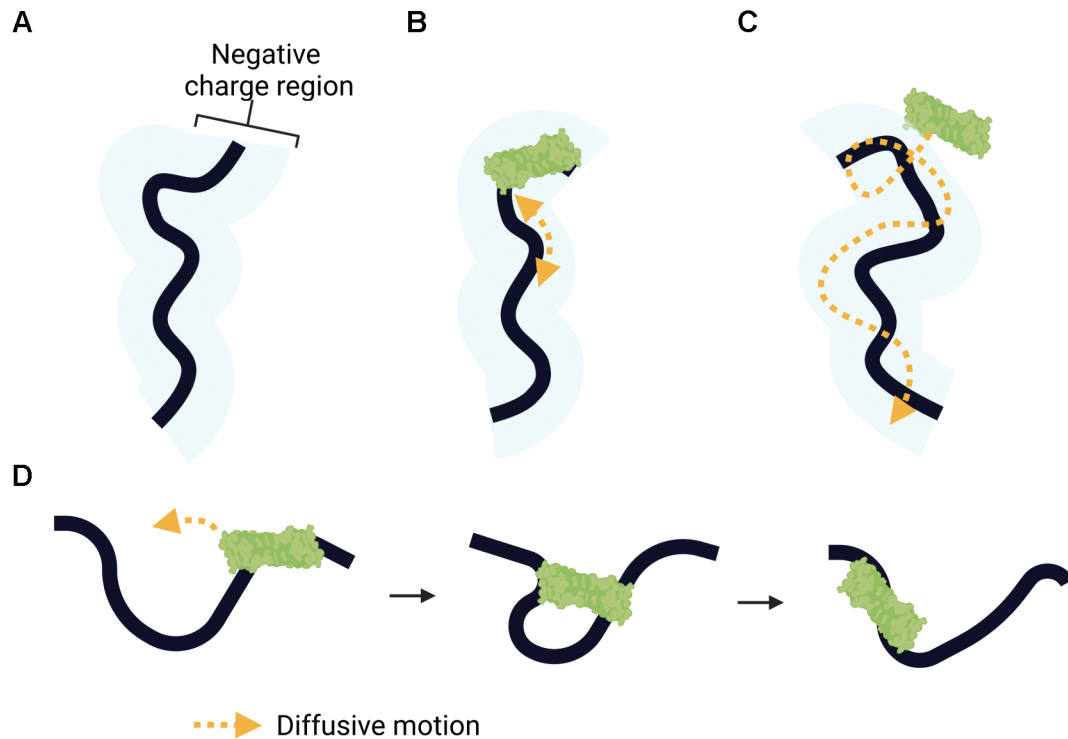
In addition to facilitated exchange between RPA proteins, RPA has been demonstrated to diffuse rapidly on ssDNA while remaining bound to invade and melt hairpin structures as well as coordinate the assembly and disassembly of other proteins (140). This mechanism of diffusion may involve sequential local dissociation and rebinding of two or more of its OB-folds since bound RPA can undergo a concentration dependent exchange with free RPA. This ability to diffuse on ssDNA enables RPA to melt DNA hairpins (140). However, another study has proposed that at the replication fork RPA is unable to efficiently bind ssDNA that forms strong hairpins, suggesting that in some situations RPA cannot melt DNA hairpins (56). This has implications for A3 deamination of structured ssDNA at replication forks.

Similar to RPA, A3s use facilitated diffusion as a means of moving on DNA to search for a specific target motif since they do not use an energy source (12,141,142) (Figure 4). Electrostatic interactions with the DNA enables enzymes to undergo Brownian motion driven diffusion which facilitates the enzyme binding longer to the DNA rather than being released into the bulk solution (Figure 4A) (12,141,142). Diffusion can be further classified depending on whether the A3s diffuse along the negatively charged phosphate backbone; termed sliding, diffuse along the DNA while intermittently making contact with the phosphate backbone; termed jumping or hopping, or move through a doubly bound state; termed intersegmental transfer (12,141–143) (Figure 4B–D). The ability for the enzyme to undergo these types of motions increases efficiency as they are able to processively catalyze deaminations in a single enzyme-substrate encounter. Sliding movements encompass <20 nucleotides (nt) of DNA and are essential for locating the deamination motif. Each A3 studied to date is processive except A3A (144). The other A3 enzymes each have a unique processive mechanism with some relying primarily on sliding and jumping (A3G, A3H), jumping (A3F), or are very efficient at intersegmental transfer in addition to sliding and jumping (A3B) (145–147). Intersegmental transfer is movement of enzymes on DNA by an intermediate doubly bound state to a distal segment of the same or a different DNA (141–143). With intersegmental transfer enzymes are more likely to bind a different DNA than the same DNA as the local DNA concentration increases (145). As a result, this mech-

anism can be processive or non-processive depending on the conditions.

Since it was initially thought that A3s could not displace RPA, the original theory was that A3s only had limited access to ssDNA and could only deaminate the transient ssDNA gaps not bound by RPA that may be present normally or during RPA depletion (148,149). While several studies have demonstrated that A3 processivity and staying bound to viral DNA is important to increase deamination efficiency, it is not the main driving factor for A3 activity on genomic ssDNA (144). Rather, it was found that the ability to undergo rapid cycling between ssDNA substrates, either by non-processive cycling or intersegmental transfer, increased the deamination activity of A3s on RPA coated ssDNA and ability to induce  $\gamma$ H2AX foci (85,113) (Figure 5). A1, A3B, and A3H have been shown to be able to cycle between ssDNA substrates and maintain processivity whereas A3A can only cycle on and off rapidly (113). In contrast, A3G, which is the most processive A3 enzyme, is unable to cycle as frequently as the others (113). A3G is primarily a cytoplasmic enzyme, although one study has observed A3G in the nucleus and others have correlated genomic instability to A3G expression (36,150). Nonetheless, A3G is still a useful control for studying the role of processivity and access to ssDNA in the presence of RPA. Biochemical assays performed in the presence of RPA showed that while processivity decreased approximately 2-fold for A3B and A3H hap I, the specific activities of A3A, A3B and A3H hap I were fairly similar. In contrast, A1 and A3G exhibited 7- and 10- fold decreases in specific activity (85,113). The reasoning for this is their inability to cycle as effectively prevents them from displacing RPA (Figure 5). For example, A3G binds ssDNA as a monomer and then oligomerizes, which stabilizes its binding and it can remain bound for 5–10 min (151,152). Without oligomerization, A3G cannot form a stable enough association to remain on the DNA and presumably RPA blocks that oligomerization, even if A3G can initially compete with RPA to bind the DNA. A1, which is a multimer off or on ssDNA, may not have enough room to stay bound (85). This suggests that the combination of the constant on and off of A3A, A3B and A3H hap I and ability to bind without further oligomerization can actively displace RPA and enable ssDNA binding, with this cycle repeating itself. A3s must be able to bind to the ssDNA quickly before a free RPA can bind as RPA has a very fast  $k_{on} = 1.1 \text{ M}^{-1} \text{ s}^{-1}$  (138). Ultimately, the data suggests that the faster the A3 on/off cycling, the better the competition for ssDNA with RPA during facilitated exchange (Figure 5). It is likely due to rapid cycling that A3A is not found to colocalize with RPA (153). A3s other than A3A have not been directly studied for colocalization with RPA, but it is likely that they also do not colocalize with RPA. Even though A3B and A3H hap I are processive enzymes, they use intersegmental transfer to different degrees, which means that the enzymes can easily move from one DNA segment to another distal DNA segment and that high local concentrations of DNA promote enzyme dissociation and rebinding at another location (145). As a result, the A3 enzyme dissociation rate on ssDNA is faster than RPA (138,152).

Although the ability of A3s to compete with RPA for linear ssDNA has been established, it has also become ap-

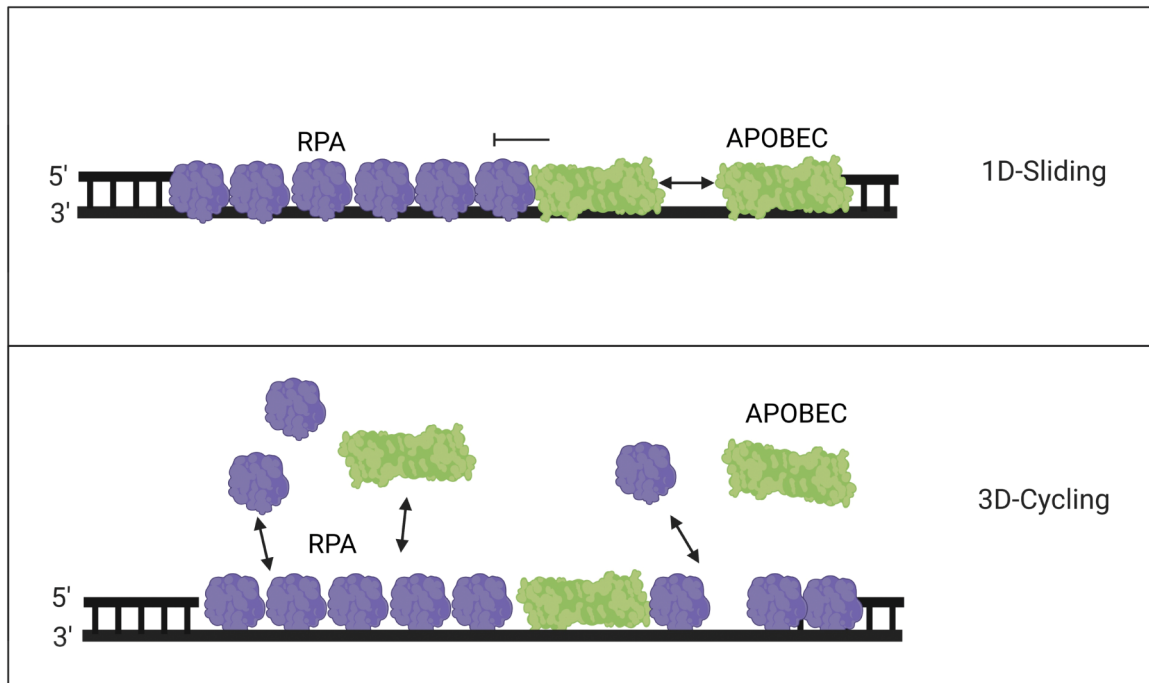


**Figure 4.** Mechanisms of ssDNA scanning by facilitated diffusion. (A) Electrostatic interactions between APOBECs and the negatively charged region (blue shaded region) of ssDNA are essential for facilitated diffusion. (B) One-dimensional sliding of the enzyme enables short-range localized searching ( $\leq 20$  nt). (C) Three-dimensional scanning of the enzyme by jumping enables larger translocations through micro-dissociations and reassociations on ssDNA without diffusion into the bulk solution, but lacks a local search process. (D) Three-dimensional scanning by intersegmental transfer enables even larger translocations mediated through a doubly bound state. Enzymes with two binding domains can simultaneously bind two different regions of ssDNA before dissociating from one region to move onto the other.

parent that A3A-induced mutations frequently occur in the loops of hairpin structures that can form from ssDNA during replication (55). Although A3A has a higher deamination rate *in vitro* on hairpin loops than A1, A3B, or A3H hap I, when comparing A3A deamination on hairpin loops and linear ssDNA in the absence of RPA, the difference in specific activity is marginal to none, depending on the study, and cannot account for observations in cells (55,85,114). Since RPA is proposed to be unable to bind ssDNA that can form strong hairpins during replication, different groups have proposed that A3A has a higher rate of deamination of hairpin loops simply because there is no competition with RPA (56,114). Brown *et al.*, found that RPA bound hairpin loops  $\sim 7.5$ - (0.5 RPA:1 DNA) to 3.6- (2 RPA:1DNA) fold less than linear ssDNA (114). Often these loops have small ssDNA regions, e.g. 4–6 nt, and a single RPA trimer binds 8–30 nt of ssDNA, suggesting that RPA is excluded or is less able to bind these regions (55,56,96,114). However, Brown *et al.* did find a 3.5-fold decrease of A3A activity on hairpin loops in the presence of RPA, compared to 6.3-fold on linear ssDNA (114). In contrast, Wong *et al.* found that there was no *in vitro* inhibition of A3A specific activity on linear ssDNA in the presence of RPA (85). Wong *et al.* used a high salt buffer which makes the *in vitro* DNA binding ability of proteins more competitive than Brown *et al.*'s reaction conditions which had no salt. However, Wong *et al.* did not test inhibition of A3A by RPA on hairpin loops, precluding any direct comparisons (85,114). In addition to

the different reaction conditions on linear ssDNA between these two studies, the different sequences of the DNA substrates are potential reasons for the different results. More studies using the same DNA substrates and reaction conditions are needed to resolve these different *in vitro* observations. Overall, these studies highlight that more direct experiments need to be carried out to determine the effect of RPA on A3 deamination activity in cells, and the extent to which RPA localizes to hairpin regions as cellular conditions with multiple proteins can be difficult to mimic *in vitro*.

A similar protective mechanism by an ssDNA binding protein is observed in HIV-1 replication. In HIV-1, nucleocapsid, a nucleic acid chaperone protein, rapidly binds and unbinds the RNA or ssDNA to remodel secondary structure and protect ssDNA. A3s must also compete with nucleocapsid to access viral (-) ssDNA. However, unlike RPA, nucleocapsid binds non-specifically at every 5–7 nt on RNA or DNA only at high concentrations (154). Unlike genomic DNA deamination, cycling is not a required characteristic to deaminate HIV-1 (-) ssDNA and instead, studies have shown that processivity directly correlates to the number of mutations (146,155). The restrictive potential of the enzyme is determined by a balance of sliding mechanisms to perform local searches and jumping mechanisms to scan larger distances. In the case of A3F which lacks the sliding mechanism, but can jump, it is unable to perform localized searches and therefore induces less mutations than A3G which is capable of performing both scanning mechanisms



**Figure 5.** Model of competitive binding of APOBECs and RPA. The tight binding of RPA on ssDNA acts as a roadblock, limiting APOBECs from catalyzing processive deaminations. (*Top panel*) This effectively prevents competitive exchange of RPA with APOBECs that can only slide along the DNA backbone, leading to lowered APOBEC specific activity. (*Bottom panel*) Bound RPA can undergo exchange with free RPA through facilitated dissociation allowing an opportunity for APOBECs to bind. Three-dimensional rapid cycling on/off ssDNA enables APOBECs to compete with RPA for ssDNA as both proteins exchange between bound and free states. The displacement of RPA enables deamination activity from A3s that do not require processive sliding, but instead locate cytosines by cycling on/off the ssDNA.

(146). These data demonstrate how A3 activity is highly specialized. While A3G does not restrict any viruses that replicate in the nucleus or have dsDNA intermediates, A3B and A3A do restrict dsDNA viruses during DNA replication in the nucleus, highlighting how although the somatic mutagenesis during cancer is considered an off-target event, the involved enzymes are specialized to access this type of substrate (23,29,156,157).

### The significance of APOBECs and involvement in somatic mutagenesis

RPA is essential for ssDNA protection during replication and is estimated to be at a 6- to 10-fold excess to deal with perturbations that result in fork stalling (129). The fact that cells will accumulate such high amounts of a specific protein highlights how crucial is RPA for maintaining genomic integrity. During replication fork stalling, RPA-coated ssDNA activates ATR which in turn will phosphorylate RPA to facilitate recruitment of repair factors. Additionally, ATR activates checkpoint signalling to suppress dormant origin firing to limit the number of active forks and reduce potential sources of ssDNA (129). This is meant to keep RPA in sufficient supply to ensure the stalled forks remain stable. Cells with supra-physiological RPA levels have been demonstrated to sustain longer durations of checkpoint inhibition, replication stress and tolerated high quantities of ssDNA without fork breakage, suggesting that as long as there is a surplus of RPA the active or stalled replication forks remain stable (129). These data make RPA critical

for managing the replication stress and recruiting the necessary proteins to protect the surrounding area, facilitating repair, and preventing catastrophic collapse of the replication fork. Additionally, fork breakage was also observed to be a sudden occurrence rather than a gradual accumulation of isolated events at active replicons (129).

Another competition for A3 enzymes, perhaps not for ssDNA *per se*, but for activity is with DNA repair enzymes. This is because the WH domain of RPA is known to bind multiple proteins involved in DNA repair and genome maintenance, such as SMARCAL1, TIPIN, ETAA1, RAD52, XPA and UNG (90–93). The binding of RPA to UNG enables UNG to efficiently excise uracils from RPA-bound ssDNA (158). The normal function of this interaction is to remove any dUMPs incorporated by the DNA polymerases (159). Thus, each uracil the A3s catalyze may be easily and subsequently removed. This could mean that cancers would be biased with SBS13 (C→G mutations), which results from insertion of a C opposite abasic sites by Rev1 during replication (74,121). However, there are still C→T mutations (SBS2) present in cancer genomes, which result from using uracil as a template during replication. It appears that somehow A3 enzymes induce enough uracils to overwhelm DNA repair. A similar conundrum was recently solved for AID where it was found that a protein of previously unknown function, Fam72A, induces degradation of UNG in order to promote AID-induced mutations during SHM (160,161). Although Fam72A is expressed in cancer cells, it has no associated function as of yet (161). This overwhelming of DNA repair with excessive

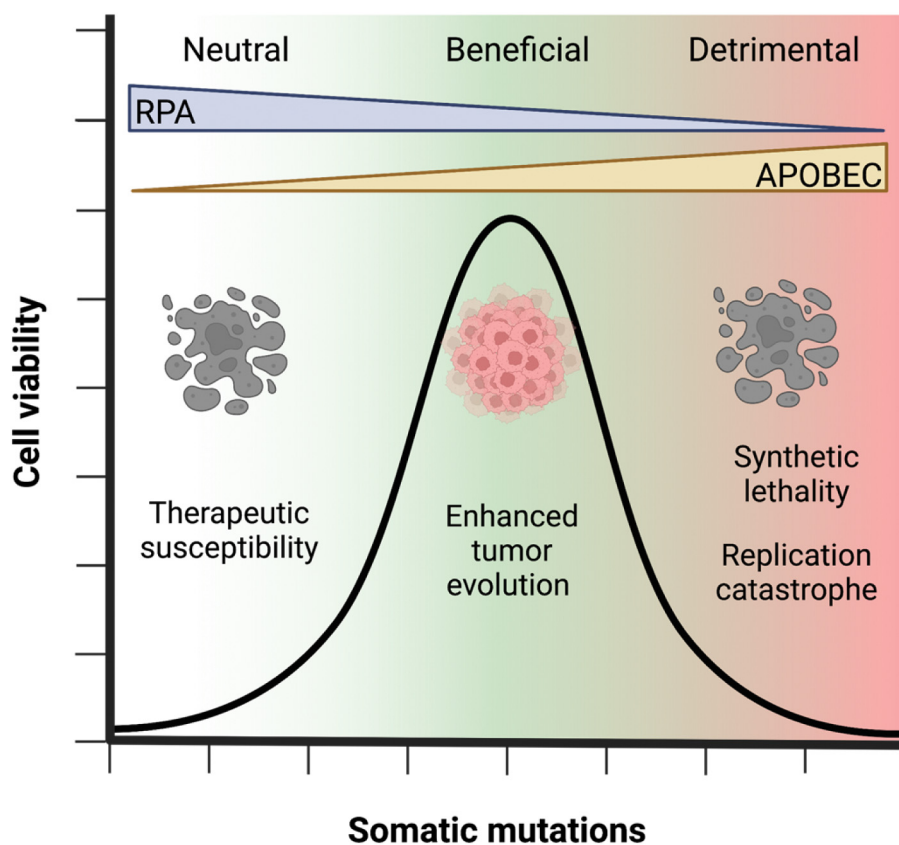
uracils could occur during BIR, which is thought to enable kataegis (126,127). Although RPA would still be involved in binding these ssDNA intermediates, the WH domain of RPA may be bound by other proteins such as RAD52, excluding UNG. Although A3 induced mutations can occur during lagging strand synthesis, considering these factors, it is likely that the original number of deaminations on the ssDNA far exceed the mutation frequency. Thus, the timing of A3 catalyzed deaminations and the type of ssDNA substrate appear to be very important in determining the mutation frequency and these types of analyses need to be an area of future research.

Another condition that needs to be considered is the level of free RPA in the cells. If excessive ssDNA accumulates due to replication fork stalling or excessive replication fork firing, the free RPA may become depleted if it is all bound to the ssDNA. Depletion of RPA can also leave significant amounts of ssDNA exposed and if combined with A3s, the normally less active enzymes can have the perfect opportunity to deaminate ssDNA that is usually not easily accessible. Assuming A3s are present during this RPA depleted state, the exposed ssDNA would be subjected to high amounts of deamination and would allow for A3s to undergo all possible processive scanning mechanisms as it would no longer be restricted to rapid cycling on/off ssDNA. This may tip the balance of the effect of A3-induced mutations to catastrophe, killing the cancer cell. This scenario also depends on the availability of the nuclease Mre11 since in the absence of RPA, the ssDNA is sensitive to Mre11-dependent degradation, which would remove any A3 substrate, but also likely lead to elevated levels of cell death in itself (162). However, the likelihood of this happening is very low since RPA depletion only occurs when ATR signalling is inhibited and would likely cause cell death due to the high number of mutations from unrepaired deaminations, if the cell was not forced prematurely to enter into mitosis (129,163). Accordingly, ATR defective cancers are rare (164). ATM is a similar checkpoint kinase that plays a pivotal role in the detection of DSB, signalling, and regulating DSB resection to activate homologous recombination (HR) (165). ATM is also activated when there is an excess of single-stranded breaks (SSB) generated during BER since ligation of SSB is slower than DNA incision by APE. By delaying S-phase entry, ATM ensures SSB intermediates generated by BER have sufficient time for repair, thereby preventing DSB formation (166). In contrast to ATR, ATM defective cancers are more common as they are resistant to apoptosis and despite having defective homologous recombination, can rely on the redundant DSB repair pathway and non-homologous end-joining (NHEJ) for repair (167). Unlike HR, NHEJ can be activated anytime to repair DSB and therefore does not appear to pose the same risk since RPA depletion occurs only during replication stress. Under these conditions, ssDNA is limited and RPA concentration is high and therefore A3s are reliant on their ability to compete with RPA. There are effects of other proteins on RPA, such as RECQ1, a helicase that can suppress firing of dormant origins of replication to ensure a normal replication rate in cells (168). In the presence of RECQ1 defects, which have been found in tumors, excess replication fork firing results in sequestration of RPA on the nascent ssDNA

(168). The sequestration of RPA also inhibits NER, further contributing to genomic instability (162). Altogether the genetic background of a cancer cell also determines the effect of A3s and this has not been analyzed in previous studies. Some cancers, such as bladder or breast cancer, have a slight bias for more SBS13 than SBS2 mutations and it would be interesting to determine if this bias correlates with the availability of RPA when compared to other cancers that have equal amounts of SBS2 and SBS13 mutations (50).

One caveat to consider in the RPA depletion theory and how it relates to A3 deamination activity is that the scarcity of RPA also means that there is a higher propensity for secondary structure formation. RPA has strand melting abilities that resolve DNA secondary structure, perhaps not during replication, but during processes such as BIR or stalled replication that create tracts of ssDNA that persist for longer times (Figure 3E) (103,124,169). Biochemical studies have demonstrated that RPA acts as a physical roadblock for certain A3s and prevents A3s from 1D sliding thereby reducing processivity and specific activity (85,113,114). Although RPA depletion would appear to circumvent this issue and allow enzymes such as A1 that are less able to compete with RPA to induce similar amounts of damage as A3A, A3B or A3H hap I, only A3A has the ability deaminate hairpin loops efficiently, although A1, A3B, and A3H hap I can to a lesser degree (55,85,113). If there is enough secondary structure to block A3s other than A3A remains to be determined. What is also not yet known is if the cell can recover from RPA depletion in the presence APOBECs and what effect this has on the cell post recovery. Depending on the amount of APOBEC-induced mutagenesis, there can be different results. Fewer deaminations by less active A3s may have a lesser effect if the cell is able to recover. Alternatively, a more active enzyme such as A3A which has a rapid on/off rate and can deaminate large stretches of DNA may lead to replication catastrophe or induce synthetically lethal mutations. Due to the uncertainty of APOBEC induced mutagenesis and dependence on the cellular state, the effect of their mutations result in a spectrum of beneficial or detrimental outcomes for the host (Figure 6). The amount of APOBEC-induced DNA damage strongly correlates with their ability to displace RPA as this is a determinant for deamination activity. Further biochemical characterization can provide insights as to how A3 enzymes would manage an RPA depletion scenario. Overall, RPA depletion appears to be a rare event, seemingly more often encountered in laboratory experiments than *in vivo*, suggesting that for the most part, A3s will need to compete for ssDNA with RPA (149,170).

In the context of chemotherapy, RPA depletion and DNA repair can be beneficial. ATR inhibitors (ATRi) are in development but there are concerns of possible severe side effects in highly proliferative normal tissues since it is an essential gene. This issue could be circumvented by using ATRi only in cancers expressing high levels of A3s since they exhibit a synthetic lethal relationship with ATRi (171). A3s induce a unique type of replication stress where the resulting abasic sites from UNG are susceptible to ATRi which leads to greater accumulation of ssDNA substrate for A3s, ultimately leading to RPA depletion and cell death (Figure 6). Another example of where A3-induced replica-



**Figure 6.** Spectrum of APOBEC induced mutagenesis. The effects of APOBEC induced mutagenesis is dependent on the cellular state and can be either neutral, beneficial or detrimental to the host. APOBEC-induced DNA damage strongly correlates with their ability to displace RPA and the amount of RPA available to protect ssDNA. Low levels of APOBEC-induced mutagenesis may not have an effect in the development of drug resistance in cancer cells and as a result, cancer cells are susceptible to therapies. An optimal amount of APOBEC-induced mutagenesis may provide genomic diversity to cancer cells that can enhance tumor evolution and growth. High levels of APOBEC-induced mutagenesis can lead to cell death as a result of synthetic lethality and replication catastrophe due to RPA exhaustion.

tion stress is beneficial is during the use of platinum based chemotherapeutic drugs such as cisplatin that induces inter-strand crosslinks (ICL). Although overexpression of A3s in several cancer types have been associated with poor clinical outcomes and drug resistance, studies have investigated the combinatorial effect of A3s and DNA repair pathways and observed that A3 deamination activity can confer sensitivity to ICL agents (172,173). BER and MMR repair of A3-catalyzed uracils physically inhibits NER and HR from repairing ICLs and leads to cell death (174). This approach of using cancers that are overexpressing A3s to exploit synthetic lethal interactions is a way to ensure that A3-induced mutations do not enhance tumor genetic diversity and growth.

## CONCLUSIONS

Since the discovery that A3 enzymes are involved in somatic mutagenesis during cancer, significant knowledge has been acquired on the A3-induced mutation types, their distribution across the genome, and the different cancers affected. However, since most of the studies in the A3 cancer field are *in silico* analysis of cancer genomes or animal studies, the mechanistic details on how they access DNA are lack-

ing. The sources of ssDNA are known, but the details of how A3s intersect with RPA and DNA repair enzymes are not as well studied (Figure 6). The DNA repair field has several methodologies to study the efficiency of specific DNA repair pathways. Using these methods without or with the addition of A3s would enable A3 researchers to test directly how A3s effect different DNA repair outcomes and the effect of the DNA structure, e.g. overhangs, on A3 deamination activity. Merging existing DNA repair biochemical assays with purified A3 enzymes and A3 biochemical knowledge would be useful to answer many of the questions on how uracils become heritable mutations. Although there has been more of a clinical focus that has resulted in more broad-based genomic analyses, the mechanistic details of how mutations arise can be highly useful for designing potential therapies. The synthetic lethality of A3 activity with ATRi is one example, and there may be many more if we understood their dynamics on ssDNA with the multiple other proteins that gather at replication forks and sites of repair.

## DATA AVAILABILITY

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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