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Molecular Origins of APOBEC-Associated Mutations in Cancer

Mia Petljak^{1,*}, John Maciejowski^{2,*}

¹Broad Institute of MIT and Harvard, Cambridge, MA, USA

²Molecular Biology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

Abstract

The APOBEC family of cytidine deaminases has been proposed to represent a major enzymatic source of mutations in cancer. Here, we summarize available evidence that links APOBEC deaminases to cancer mutagenesis. We also highlight newly identified human cell models of APOBEC mutagenesis, including cancer cell lines with suspected endogenous APOBEC activity and a cell system of telomere crisis-associated mutations. Finally, we draw on recent data to propose potential causes of APOBEC misregulation in cancer, including the instigating factors, the relevant mutator(s), and the mechanisms underlying generation of the genome-dispersed and clustered APOBEC-induced mutations.

Keywords

APOBEC mutations; Cancer mutagenesis; Mutational signature; *Kataegis*; Chromothripsis; Cancer cell lines

1. Introduction

Over the past decade the apolipoprotein B mRNA-editing enzyme catalytic polypeptidelike (APOBEC) family of single-stranded DNA (ssDNA) cytidine deaminases has emerged as a prominent putative enzymatic source of mutations in cancer. APOBEC1 was the first member identified for its role in plasma metabolism by apoprotein B pre-mRNA editing [1,2]. It was later recognized that APOBEC1 can also deaminate cytidine in DNA [3,4]. APOBEC2 and APOBEC4 have no confirmed DNA deamination activities [7,8]. The APOBEC3 subfamily, comprised of seven APOBEC members, restricts viral replication through base-editing of viral intermediates during reverse transcription [9]. Importantly, a selection of APOBEC3 enzymes is currently believed to underlie most of the genome-dispersed and clustered APOBEC-associated mutations. Notably, another APOBEC family

*Corresponding authors Mia Petljak, Phone: (617) 714-7049, mpetljak@broadinstitute.org; John Maciejowski, Phone: (212) 639-8581, maciejoj@mskcc.org.

Conflict of interest

The authors have no conflict of interest to declare.

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member, Activation-induced cytidine deaminase (AID) with a role in initiating the hypermutation and recombination of immunoglobulin genes, has been tied to chromosomal translocations and the development of specifically B cell malignancies, reviewed previously [10].

APOBEC-associated mutations represent traces of some of the most common mutational processes in cancer, detected in approximately 75% of cancer types and >50% of all cancers analyzed [11]. Independent of base editing, APOBEC3-mediated cytosine deamination can also elicit a DNA damage response, if DNA double-strand breaks form upon excision of the resulting uracil bases by uracil DNA glycosylase (UNG) [12-14]. This APOBEC3-dependent DNA damage may place unique pressures on the cell and offer opportunities for therapeutic intervention via induction of synthetic lethality [15-18].

The clinical impact of APOBEC-associated mutations appears to be significant [19,20]. APOBEC-associated mutations likely influence tumor evolution through activation of oncogenes and loss of tumor suppressors [21]. Expression levels of some APOBECs also influence the response to therapies: high APOBEC3 expression levels are associated with reduced clinical benefit from tamoxifen in estrogen receptor (ER)-positive breast cancer [22]. These correlative results are corroborated by mouse xenograft models where induced APOBEC3 overexpression abrogates the benefits of tamoxifen [23]. Consistent with activation late in disease progression, APOBEC activity is also associated with tumor subclonal diversification and heterogeneity [22,24]. Finally, overexpression of APOBEC3B can also improve sensitivity to immune checkpoint blockade in a mouse model of melanoma [25]. It is thus possible that APOBEC enzymes may play a role in generating neoepitopes and promote tumor immunogenicity, thus calling for future studies in this direction.

2. Links between APOBEC enzymes and mutations in cancer

Accumulating evidence from yeast and human cell models, transgenic animals, and human cancer genomics point towards a role for APOBEC deaminases in cancer mutagenesis. The first data connecting APOBEC enzymes to cancer was the discovery that expression of *APOBEC1* promotes hepatocellular carcinoma in transgenic animals [26]. The subsequent demonstration that overexpression of APOBECs can induce DNA damage and mutagenesis nominated APOBECs as putative DNA mutators in cancer [3,12], in addition to their known roles as RNA editors in viral restriction. The identification of mutations at motifs characteristic of APOBEC activity in viral restriction and cell models (cytosine bases in 5'-TCN-3' (T = thymine, C = cytosine, N = any base) in cancer genes [27] further suggested that APOBECs are also active in cancer.

2.1 Mutational signature analysis implicates APOBECs in cancer mutagenesis

With the increase in numbers of cancer genomes sequenced and analyzed, a growing number of studies have started to identify mutations resembling APOBEC cytosine deamination in cancer [28,29]. Mathematical deconvolution of somatic mutations from individual cancer genomes identified two separate signatures of genome-dispersed mutations associated with APOBEC deamination, first in breast cancer [29] and later across multiple cancer types [30]. The two signatures, termed 'Signature 2' and 'Signature 13' (also commonly abbreviated as

SBS2 and SBS13 signatures; where SBS stands for ‘single base substitution’), are respectively characterized by C>T transitions, and C>G and C>A transversions, at TCN trinucleotides [11,30] (Fig. 1). These are likely initiated by APOBEC cytosine deamination, as the mutations occur within APOBEC-favored TCN motifs, whereas the distinct mutation types that characterize these signatures may arise from different DNA replication and repair mechanisms [31]. The transversion mutations that characterize Signature 13 are thought to arise via base excision repair (BER), which requires UNG-mediated removal of uracils and insertion of cytosines opposite abasic sites by error-prone translesion polymerases, such as REV1 [31]. The C>T transition mutations that characterize Signature 2 are thought to arise predominantly by replication across the uracil bases [31], but they could also reflect activities of other polymerases with translesion potential, such as POLD, following BER. Further supporting a shared etiology, Signatures 2 and 13 are almost always present together, albeit in different proportions, in individual cancers [11,30].

In addition to the genome-dispersed signatures, mutations in APOBEC-characteristic TCN motifs also occur in patterns of localized hypermutation, where multiple same-strand base substitutions at C:G pairs cluster over kilobase-sized regions [29,32-34]. This regional signature of APOBEC-associated hypermutation has been termed *kataegis* (from Greek for shower or thunderstorm, i.e. mutational showers) and often colocalizes with sites of DNA rearrangements in cancer [29]. The different densities of mutations that form genome-dispersed and clustered APOBEC-associated signatures are thought to be governed by the lengths of the target single-stranded DNA (ssDNA) substrates, required for APOBEC-mediated deamination [35,36].

Since these initial discoveries, researchers have further dissected APOBEC-associated mutations in cancer at extended sequence contexts (i.e. pentanucleotide motifs) [37], at genomic meso-scales (e.g. at longer sequences predicted to form hairpin loops) [38] and at macro-scales (e.g. at regions related to transcription, DNA replication and chromatin organization) [39-42]. Such refinements were often combined with overexpression of individual APOBEC enzymes in model systems to investigate which family member causes mutations exhibiting genomic features that are most similar to the signatures observed in cancer (discussed in the following section) [3,37,43-45]. These studies collectively demonstrated that some APOBEC deaminases, when overexpressed in experimental model systems, can recapitulate mutational patterns seen in cancer.

2.2 APOBEC gene locus polymorphisms associate with mutation burdens

The second collection of links between APOBEC deaminases and mutations in cancer stems from genetic associations between polymorphisms in the APOBEC gene cluster on human chromosome 22 and burdens of the relevant signatures in cancer genomes. A germline polymorphism that results in the loss of the coding sequence of *APOBEC3B*, prevalent in East Asian populations (> 30%) [46], has been associated with an elevated risk of breast cancer in Asian populations [47,48] and the higher overall burden of APOBEC-association mutational signatures in breast cancers [49]. The results on the association between this polymorphism and cancer risk in European populations, where it is less common (~6%) [46], have so far been conflicting [50-52]. Whereas the association was not replicated in

bladder cancers [53], it was found in lung and prostate cancers in individuals under 50 years of age [50]. In addition to the deletion polymorphism, a single nucleotide polymorphism (SNP) [50] rs1014971, located upstream of the *APOBEC3* cluster, was associated with bladder cancer risk, increased *APOBEC3B* expression, and enrichment of APOBEC-signature mutations in bladder tumors [53]. Finally, *APOBEC3H* haplotype I has been implicated as another putative source of mutation in cancers, but its role remains elusive [54]. These studies provide important genetic associations between the APOBEC gene cluster, cancer risk and mutational burdens in human cancer. The impact of individual polymorphisms in different populations and cancer types, as well as their underlying mechanistic bases, require further clarification.

2.3 APOBEC expression correlates weakly, but positively, with mutation burden

The third collection of studies linking mutations in cancer and APOBEC deaminases identified correlations between upregulation of expression of individual *APOBEC* genes and burdens of APOBEC-associated signatures [14,28,38,55-57]. Notably, the correlations have been weak in comparison to the often-striking mutational burdens attributed to APOBEC-associated signatures in cancer and have generated opposing results to experimental overexpression systems with respect to the relevant mutator APOBEC enzyme [37,38,56] (discussed later). The major factor limiting comparisons between mutational burdens and RNA abundance are potential differences in APOBEC expression levels at the time of mutagenesis and at the time of RNA sampling. Mutations captured in cancer genomes could have been generated by APOBEC deaminases over the lifetime of a cell lineage, whereas RNA captures expression at the single time point of sample acquisition and not necessarily at the time of active mutagenesis [58]. It is also possible that signals of APOBEC expression in tumors originate from immune cells with naturally higher APOBEC levels [59].

3. APOBEC activity in cancer and non-malignant cells

The following section summarizes the prevalence, timing and activity of APOBEC-associated mutagenesis using mutational readouts from cancer and healthy tissues to provide insights into the features of APOBEC misregulation.

3.1 Prevalence of APOBEC mutagenesis in cancer and non-malignant cells

APOBEC-associated mutational signatures are among the most widespread in cancer and contribute particularly prominent mutational loads (median >0.25 mutations per Mb) in cancers of the cervix, head and neck, bladder, lung, and esophagus [11,28,30]. However, they have not been reported in cancers of the colon, liver, brain, chronic lymphocytic leukemia and acute myeloid leukemias (AML) [11,28,30,60]. Thus, APOBEC-associated mutagenesis is generally prevalent, but absent or rare in some cancer types. Furthermore, APOBEC-associated mutations are rare in childhood cancers [11], suggesting that the underlying misregulation may be age-related.

APOBEC-associated signatures have not been systematically examined in non-malignant cells, due to challenges associated with detection of somatic mutations among non-clonal cell populations. Strategies have recently been developed to circumvent this, including

sequencing of clonal cell colonies [61-65] or clonal microscopic biopsies [66,67] and structures from healthy tissues, such as colonic crypts and endometrial glands [68-71]. Specialized methods [72,73] and single-cell sequencing [74,75] have also been employed to identify somatic mutations. These initial data sets have started to reveal the prevalence and patterns of APOBEC activity across healthy tissues. APOBEC-associated signatures have been detected in a small number of healthy colonic crypts [68] and colonies derived from single bronchial epithelial cells [65], indicating that APOBEC mutagenesis likely occurs in healthy tissues.

APOBEC-associated signatures were not detected in clones from normal esophageal epithelium [66] nor in healthy endometrial glands [71], despite their presence in esophageal and endometrial cancers [11,30], implying that they may be acquired later in cancer evolution, as suggested before [76,77], or that such cancers are more likely to evolve from clones with APOBEC mutagenesis [66]. APOBEC-associated signatures have also not been detected in healthy and cirrhotic liver clones, nor in healthy hematopoietic stem cells and myeloid progenitor cells [63,70]. Over the following years, systematic examination of mutational burdens in healthy tissues will shed further light onto the prevalence and emerging cell-type-specificity of APOBEC activity.

3.2 Episodic APOBEC mutagenesis in cancer cell lines

Generation of mutational signatures was recently assessed during *in vitro* culture of human cancer cell lines [58] (Fig. 2A). Whereas mutations detected in cancer genomes sampled at a single time point reflect readouts of historic mutagenic activities, cell lines are amenable to tracking ongoing mutation acquisition over serial and defined timeframes. Cell lines that carry APOBEC-associated mutational signatures in their genomes, and were therefore exposed to APOBEC mutagenesis in their past, often continued to generate APOBEC-associated signatures over time in culture. This indicates that APOBEC-associated signatures reflect traces of active rather than a historic mutational processes. Given the natural roles of APOBEC deaminases in innate immunity, induction of APOBEC misregulation by immune cells was foreseeable. Identification of cancer cell lines which generate APOBEC-associated signatures over time is the first demonstration that underlying mutagenesis can occur in the absence of the immune system [58].

Active APOBEC-associated mutagenesis was estimated to persist in at least 75% of cancer cell lines, with no ongoing APOBEC-associated mutagenesis detectable across only a smaller proportion of cell lines [58]. However, cancer cell models of APOBEC mutagenesis should be selected with caution, because the mutagenesis may not remain strongly or at all active in all cell lines that were previously exposed to it and carry the relevant signatures. The amenability of cell lines to serial single-cell cloning, followed by short propagation timeframes and whole-genome sequencing, revealed that unlike other examined mutational signatures, APOBEC-associated signatures exhibit substantial fluctuations in mutation rates over time [58]. In some cases, acquisition of signatures virtually completely ceased before reactivating and then discontinuing again. This phenomenon was termed ‘episodic mutagenesis’ [58]. The variability of APOBEC-associated mutational burdens was also detected between individual branches of phylogenetic trees from primary tumors [78,79] and

healthy bronchial epithelial cells [65]. Collectively, these data indicate that some or all aberrant APOBEC activities *in vivo* may be unleashed onto the genome in mutagenic bursts. Genetically amenable cancer cell lines that exhibit active accumulation of APOBEC-associated mutations during propagation *in vitro* represent useful tools to probe the mechanisms of APOBEC mutagenesis (Fig. 2A).

Whereas the origins of APOBEC dysfunction remain unknown, these data imply that the instigating factors can be episodic in nature and possibly endogenous to affected cells. In addition, APOBEC-associated mutations seem to present rarely in healthy cells and/or may be more damaging to certain cell types. Collectively, these are some of the fundamental features to be considered in future investigations on the mechanisms underlying APOBEC misregulation in cancer.

4. Prime candidate mutators underlying APOBEC-associated mutagenesis

The identity of the APOBEC deaminase that is primarily responsible for mutagenesis in cancer is a persistent question in the field. Recent efforts have employed correlative analyses of APOBEC3 expression with mutational burden and developed transgenic mouse and cell overexpression models to assess the mutagenic potential of specific APOBEC3 paralogs. These studies nominated two APOBEC3 family members, APOBEC3A and APOBEC3B, which preferentially operate in a TCN context which brands the associated signatures in cancer, as the most likely candidates. Current focus has shifted away from the other nine AID/APOBEC members as potential mutators, due to lack of known DNA deaminase activity, distinct sequence preferences and/or apparent limited access to genomic DNA (reviewed in [80,81]), although their contributions cannot be in entirety dismissed.

4.1 Studies supporting a role for APOBEC3B in carcinogenesis

APOBEC3B mRNA is upregulated in at least 6 distinct cancers (bladder, cervix, lung adenocarcinoma, lung squamous cell carcinoma, head and neck, and breast) relative to healthy tissue [14,55]. The other APOBEC3 paralogs are generally expressed at significantly lower levels than *APOBEC3B*, aside from a few limited examples (e.g. *APOBEC3A* overexpression in AML [15]). Several lines of evidence implicate the observed *APOBEC3B* upregulation with carcinogenesis.

First, *APOBEC3B* expression correlates with specific clinical outcomes indicating a role in disease progression. *APOBEC3B* expression is positively correlated with adverse outcomes in estrogen receptor-positive breast cancers [22]. High *APOBEC3B* expression predicts worse outcomes in lung cancer and recurrence of clear cell renal cell carcinomas [82,83]. However, in a subset of clear cell ovarian carcinomas, high *APOBEC3B* expression is associated with improved progression-free survival [84]. Improved progression-free survival in this subset of patients may be explained by enhanced sensitivity to cisplatin from increased DNA damage resulting from APOBEC3B-mediated deamination [84].

Second, *APOBEC3B* expression levels were reported to weakly, albeit positively correlate with overall mutation burden [14]. Breast cancers with high levels of *APOBEC3B* expression exhibit nearly twice as many C-to-T mutations [14]. This correlation extends to

distinct cancer types including cervical, bladder, and other cancers [55]. In line with APOBEC3B being a putative mutator in cancer, it is the only APOBEC3 that is constitutively nuclear as it exhibits a nuclear localization in all cell types examined thus far [14,85]. However, another widely considered mutator, APOBEC3A, also appears to access the nucleus and displays a pan-cellular localization in some cell types, but restriction to the cytosol in others [12,85].

Third, APOBEC3B expression levels correlate with the course of disease. Consistent with clinical data showing that high *APOBEC3B* levels inversely correlate with response to tamoxifen in estrogen receptor-positive disease, *APOBEC3B* overexpression promotes tamoxifen resistance in murine xenografts [23] and increases sensitivity to immune checkpoint blockade in murine models of melanoma [25].

Collectively these studies suggest an important role for APOBEC3B in cancer. However, most experiments have used correlations to implicate APOBEC3B as the relevant mutator. In fact, recent refinement of the expression-based studies, suggests that *APOBEC3A* expression correlates more strongly with APOBEC-associated mutation load in breast cancer than *APOBEC3B* expression [56]. It is thus conceivable that high *APOBEC3B* expression influences disease progression through independent means, such as the generation of DNA breaks and induction of chromosome rearrangements. In the future it will be important to experimentally connect endogenous APOBEC3B activity to mutations in cancer to assess its role as a mutator. Thus far, endogenous APOBEC3B has also been implicated in generation of *kataegis* in a cell model of telomere crisis (more below) [34]. This type of mechanistic clarity is especially important because several studies have suggested that APOBEC3B plays a limited role in cancer mutagenesis [37,56].

4.2 Studies supporting a role for APOBEC3A in cancer mutagenesis

Independent lines of evidence point towards the APOBEC3A paralog as a more prominent source of mutations in cancer. The first piece of evidence to suggest the presence of APOBEC3B-independent sources of mutagenesis comes from the *APOBEC3B* deletion polymorphism discussed above, associated with increased APOBEC-associated mutational burdens in some populations [46,49]. This deletion effectively removes *APOBEC3B* and fuses the *APOBEC3A* coding sequence with the 3'UTR of *APOBEC3B*. This alteration is proposed to stabilize the *APOBEC3A* mRNA thus resulting in higher mutation burdens [49,86]. An alternative proposal is that APOBEC3H may be a putative mutator in cancers with the *APOBEC3A/B* deletion polymorphism [54], but conclusive experimental evidence is lacking.

Data from yeast suggest that human APOBEC3A and APOBEC3B preferentially operate at distinct motifs and that the APOBEC3A-preferred motif is more common in cancer [37,43]. This study used overexpression of human *APOBEC3A* and *APOBEC3B* in yeast models engineered to accumulate long, ssDNA substrates to generate sufficient mutations to distinguish sequence motifs preferred by each paralog [37]. This analysis revealed that APOBEC3A favors YTCA sites, while APOBEC3B favors RTCA sites (Y = pyrimidine, R = purine) [37]. The structural basis for this sequence preference is not clear as APOBEC3A and a chimeric version of APOBEC3B have limited contact with ssDNA aside from the

central 5'-TC motif [87]. The APOBEC3A-preferred YTCA site is a more prominent source of mutations in cancer genomes and in cell lines with ongoing mutagenesis [37,58]. This correlation suggests that APOBEC3A may be a prime mutator in human cancer, but the substrate preferences of other APOBEC3s expressed in yeast and human systems have not been rigorously assessed. More recently, APOBEC3A was found to prefer DNA substrates predicted to form stem-loops and multiple recurring APOBEC-resembling mutations were found at such APOBEC3A-favored sequences in cancer genomes [38]. It thus seems likely substrate preferences of APOBEC3A, rather than selection, drive recurring occurrence of mutations at such hotspot sequences in cancer, highlighting how understanding the substrate preferences at which mutational processes operate may be helpful in distinguishing 'driver' mutations with functional consequences from 'passenger' mutations in cancer genomes that not affected by selection [38]. Importantly, although APOBEC3A may prefer targeting sequences predicted to form stem-loops, the majority of the APOBEC-associated mutations is expected to present outside of such sequences, that are otherwise rare across the human genome.

Unlike APOBEC3B, APOBEC3A, is expressed at a low level in most cancers [28] posing a significant challenge to accurately detect its activities. A lack of sensitive, APOBEC3A-specific antibodies has further compounded the challenge of detecting APOBEC3A protein in cancer. Recently, RNA stem-loops were found to be commonly edited in cancer, within the sequence motifs characteristic of APOBEC mutagenesis on DNA [88]. Although the specific RNA loci were not mutated in their DNA templates of analyzed tumors, these data indicated that structural preferences of the APOBEC3A activities on RNA, mimic its ability to also mutate DNA within the similar hairpin-like structures [38] and as such differ from the preferences of APOBEC3B [88]. A selection of the RNA hotspots was subsequently leveraged in a digital droplet PCR assay to quantify the RNA-editing activities associated with APOBEC3A in individual tumors. In the future, it will be critical to determine whether detection of APOBEC3A-associated RNA editing also translates to active APOBEC3A mutagenesis in cancer genomes. APOBEC3A activity, as inferred from the active RNA editing, was frequently observed in the panel of AML and myeloproliferative neoplasms (MPN), in keeping with such activities previously reported in monocytes and macrophages during M1 polarization [89]. However, APOBEC-associated mutational signatures have not been detected in AML and are rare in MPN [11]. It is thus possible that APOBEC3A activities on RNA may not represent an optimal proxy for an active mutagenesis in cancer.

Finally, transgenic expression of *APOBEC3A* also supports a role for this paralog in carcinogenesis. Transgenic *APOBEC3A* expression in cell models results in more cellular DNA damage than overexpression of other APOBEC3s [12,13]. This is consistent with observations that APOBEC3A exhibits the highest catalytic activity among the human APOBEC3 enzymes [90]. The structural basis of this high catalytic activity may reflect the relatively open conformation of APOBEC3A that is capable of binding to DNA in the absence of modification [87]. In contrast, APOBEC3B exhibits a closed active site that must be converted in order to bind DNA substrate [87]. Transgenic expression of *APOBEC3A* in mice has also recently been shown to induce APOBEC signature mutations and increase tumorigenesis in murine colon and liver tissues [45].

Overall, the discussed studies suggest that APOBEC3A likely generates APOBEC-associated mutations in cancer genomes. However, the experimental evidence and investigation into the extent of its mutagenic activities relative to APOBEC3B and possibly other APOBECs that may contribute additional mutations, are outstanding.

4.3 APOBEC3B generates clustered mutations during telomere crisis

In addition to mutations throughout the genome, APOBEC-induced mutations can also appear in *kataegis*, a pattern of localized hypermutation often associated with chromosome rearrangement breakpoints [29]. Recent work has identified *kataegis* in >60% of cancers and at high frequency in lung squamous cell carcinoma, bladder cancer, acral melanoma, and sarcomas [91]. APOBEC signatures account for the majority of *kataegis* events, but some may be driven by independent mechanisms including error-prone DNA synthesis [91,92]. APOBEC3B appears to be a prime driver because, along with structural variant burden and age of diagnosis, *kataegis* positively correlate with *APOBEC3B* expression levels [91].

Recent work implicates endogenous APOBEC3B in the generation of clustered mutations during telomere crisis [34] (Fig. 2B). Telomere crisis is a period of genome instability that occurs during tumorigenesis when depletion of the telomere reserve generates unstable dicentric chromosomes [93]. Dicentric chromosomes persist through mitosis to form DNA bridges that are generally broken hours after mitotic exit [33]. Rearranged clonal cell lines isolated after transit through telomere crisis exhibit frequent chromothripsis often associated with *kataegis* [33]. Reduction of *kataegis* in *APOBEC3B* knockout cells recovered after telomere crisis indicates that APOBEC3B is the major enzymatic source of *kataegis* in this telomere crisis model [34]. *Kataegis*-like clusters have also been generated in yeast by expression of AID/APOBEC family deaminases [32,43,94].

APOBEC3B base editing during telomere crisis occurs independently of *APOBEC3B* upregulation and instead depends on ssDNA formation at DNA bridges [34] (Fig. 2B). The ability of APOBEC3B to generate clustered mutations may derive from its ability to execute processive deamination [95]. However, it is not clear if APOBEC3B is uniquely capable of catalyzing *kataegis* during telomere crisis. RPE1-hTERT cells are non-tumorigenic and do not express APOBEC3A [34]. Therefore, a potential role for APOBEC3A in generating *kataegis* during telomere crisis is not clear from these experiments.

The identity of the prime mutator(s) may depend on unknown contexts specific a particular cell or tissue. Clustered and genome-dispersed mutations may also reflect the activities of distinct APOBEC3 mutators. The identity of the prime mutator is a fundamentally important problem because answers can guide the development of targeted therapies and studies on factors that instigate APOBEC mutagenesis. Cell line models that recapitulate APOBEC3 mutagenesis during propagation *in vitro* [33,34,58] will be useful tools to dissect the mutagenic potential of specific APOBEC3 paralogs.

5. Speculative mechanisms of episodic APOBEC misregulation in cancer

Mechanisms which drive APOBEC-mediated mutagenesis remain a major open question. We specifically focus on putative intrinsic sources of APOBEC misregulation in cancer

cells, in the light of recent observations of endogenous and episodic APOBEC-associated mutagenesis in cancer cell lines and in a model of telomere crisis [34,58].

Thus far, recurrent mutations in APOBEC genes that are associated with the relevant mutational signatures in cancer have not been identified [49], implying a perturbation in activity or abundance. Expression of APOBEC genes in healthy tissues is considered low, with notable exceptions of *APOBEC1* expression in small intestine, where it has a critical role in plasma metabolism by apoprotein B pre-mRNA editing [1,2], and expression of *APOBEC3* members in immune cells [9]. Among the most prominent basal levels of APOBECs in healthy tissues is that of *APOBEC3A* in myeloid cells [96-98]. Upregulation of several APOBEC3 family members in cancer compared to healthy tissues has been reported [14,28,37,57]. However, correlations between individual APOBEC gene expressions and mutational burdens in cancer are weak [14,28,37,55-57], possibly due to aforementioned limitations or because other factors are involved. Prolonged APOBEC3 expression is cytotoxic in cell culture models [14]. Stable hyperexpression in cancer thus may be cytotoxic, but short and intermittent periods of APOBEC3 expression may limit genotoxicity in individual cancer cells.

5.1 Viruses and retrotransposons

APOBEC3 family members are believed to have evolved primarily as editors of viral and retrotransposon nucleic acid intermediates [80]. It was proposed that APOBEC-associated signatures in cancer may represent collateral damage on human DNA during APOBEC-mediated restriction of retrotransposons or exogenous viruses [30]. APOBEC-associated signatures are among the most prevalent in human papilloma virus (HPV)-associated cervix and head and neck cancers [11]. However, APOBEC-associated signatures are also found in many HPV-negative head and neck cancers [99], and are prevalent in many cancers types otherwise not linked to obvious infections (e.g. breast, lung, esophageal cancers) [11,30]. Furthermore, APOBEC-associated signatures have not been found in liver cancers [11,60], commonly infected with hepatitis viruses restricted by APOBEC deaminases [100] and active APOBEC-associated mutagenesis does not persist in cervical cancer cell lines where HPV sequences and related transcripts were detected [58]. Similarly, the burden of APOBEC-associated signatures did not correlate with somatic retrotransposon insertions in analysis across >2,300 primary cancers and the association was only weak in cell lines [58]. Whereas these data do not exclude the roles of viruses and retrotransposons in modulating APOBEC-associated mutagenesis, it indicates that there are cases in which they are neither required nor sufficient to drive it.

Although viruses and retrotransposons may not be the major activators of aberrant APOBEC activities, the same underlying modulators of APOBEC upregulation in the context of an anti-viral response may be involved in the context of cancer. APOBEC3 genes are interferon (IFN)-stimulated during the innate immune response, with *APOBEC3A* showing exceptional sensitivity to IFN stimulation [44]. Both IFN-dependent and IFN-independent mediators of APOBEC3 upregulation may play a role and have been extensively reviewed elsewhere [9], but are not likely to be thoroughly captured by correlations between mutation

burdens and signals from RNA-sequencing [101] due to the potential for temporal disconnect between APOBEC mutation burden and sample acquisition discussed above.

Expression of some APOBEC3 enzymes can be upregulated by the cytosolic nucleic acid sensors, which detect single- and double-stranded RNA and DNA [98]. In myeloid cells, STING (stimulator of IFN genes) pathway can modulate *APOBEC3A* expression via IFN- β induction [102]. Multiple sources of cytosolic nucleic acid in cancer cells have been documented, including micronuclei [103-105] and expression of endogenous retroviruses [106]. Whereas *APOBEC3A* is induced by IFN to mediate clearance of foreign DNA from human cells, nuclear DNA remains intact and the restriction mechanisms are possibly compartmentalized to mitigate the risk to the genomic DNA [44]. Nuclear envelope rupture is one event that may break such barriers and that is common in cancer [107-111]. Transient upregulation of APOBEC expression and intermittent access to nuclear DNA are compatible with the episodic nature of underlying mutagenesis.

Multiple exogenous stimuli can activate APOBEC expression in experimental cell systems and have been reviewed elsewhere [9]. It is not clear whether these exogenous stimuli also induce mutagenesis and account for mutations observed in cancer as APOBEC upregulation may not always translate to active mutagenesis. However, it is likely that multiple environmental agents, including viruses, may contribute to misregulation. For example, lung adenocarcinomas from smokers carry more APOBEC-associated signatures compared to non-smokers [112], possibly because inflammatory responses associated with tobacco smoking also trigger APOBEC activity. Cancer cell lines with ongoing APOBEC-associated mutagenesis represent experimental models to investigate endogenous sources of episodic APOBEC misregulation [58]. Epidemiological associations may shed light further on the environmental modulators of APOBEC-associated mutagenesis.

Conclusions

Extensive data implicates APOBEC deaminases as some of the most prominent endogenous sources of mutations in human cancer. However, current links between APOBEC enzymes and mutations in cancer are based on associations, not causation. The critical evidence that APOBEC deaminases generate mutations observed in cancer cells is outstanding and the responsible mutator remains only speculative and is under debate [14,28,37,55-57]. These fundamental gaps remain because genetically amenable models that accurately recapitulate mutagenesis were not available until recently. Transgenic murine models of APOBEC mutagenesis should be cautiously interpreted. APOBEC3 sequences are rapidly evolving and exhibit significant divergence between rodent and primate species [113-115]. Given the mutagenic potential of aberrant APOBEC3 activity, it is possible that primates may have evolved mechanisms to protect against the risks associated with the expression of endogenous enzymatic mutators. Newly discovered negative regulators of APOBEC3 mutagenesis provide some experimental support for this view [116,117]. The absence of these potential regulatory mechanisms in rodents may compromise the utility of transgenic mouse models to fully understand APOBEC mutagenesis.

Recently described human cancer cell lines which generate APOBEC-associated mutational signatures endogenously over time were reported [58]. These cell lines also carry signatures of the historic exposure to mutagenesis, which is predicted to have at least in part occurred in the corresponding primary cancers. The *in vitro* mechanisms of APOBEC misregulation in cell lines thus likely operated *in vivo* and were inherited from their originating cancers [58]. Such cell lines with intrinsic APOBEC-associated mutagenesis represent suitable experimental systems for future investigations on its origins in cancer, to identify the relevant mutator member(s) and identify mechanisms which instigate the mutagenic behavior.

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Abbreviations:

AID	activation induced cytidine deaminase
AML	acute myeloid leukemia
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide
BER	base excision repair
ER	estrogen receptor
HPV	human papilloma virus
MPN	myeloproliferative neoplasms
SBS	single base substitution
ssDNA	single-stranded DNA
UNG	uracil DNA glycosylase

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Figure 1. APOBEC-associated genome wide signatures SBS2 and SBS13.

SBS2 and SBS13 identified from whole-genome sequences of 2,780 primary cancer from various types, as part of the Pan Cancer Analysis of Whole Genomes (PCAWG) effort [9]. Each signature is displayed according to the alphabetical 96-substitution classification on horizontal axes, defined by the six color-coded substitution types (C>A, C>G, C>T, T>A, T>C, and T>G; whereby all substitutions are referred to by the pyrimidine of the mutated Watson—Crick base pair) and sequence context immediately 5' and 3' to each mutated base. Vertical axes indicate the percentage of mutations attributed to specific mutation types, adjusted to the actual frequencies of each trinucleotide in the reference human genome version GRCh37. Images acquired from COSMIC (Mutational Signatures v3, May 2019).

Figure 2. Cell models of APOBEC mutagenesis.

A. Human cancer cell lines which generate APOBEC-associated mutations episodically *in vitro* represent genetically amenable models to experimentally explore origins of such mutations in cancer cells. CRISPR-Cas9 modifications of the candidate genes, such as individual APOBEC enzymes or other putative modulators of mutagenesis, can be generated in such cell lines to examine changes in mutation acquisition on various genetic backgrounds and during the defined *in vitro* timeframes (*t*). Genetically modified single-cell derived clones ('parent clones') are cultured for specified periods of time, e.g. depending on the mutation rates previously determined in cell lines [58]. Multiple 'daughter clones' are derived subsequently from single cells of individual parent clones. DNA isolated from parent clones and their corresponding daughters are subjected to WGS. Clonal mutations in daughter clones absent from their respective parent clones represent *de novo* mutations acquired during the specified *in vitro* timeframes spanning the two single cell bottlenecks. APOBEC-associated mutational burdens can be examined among *de novo* mutations acquired in different genetic backgrounds. **B.** Dicentric chromosomes formed by telomere fusion persist through mitosis intact and develop into cell-connecting DNA bridges [33]. Nuclear membrane rupture at DNA bridges enables the cytoplasmic exonuclease TREX1 to access and resect DNA. The resulting single-stranded DNA is targeted by APOBEC3B when broken DNA bridges are re-integrated into the nucleus. Repair of the fragmented DNA generates chromothripsis chromosomes frequently associated with *kataegis*. Uracil excision by UNG2 and abasic site cleavage by APE1 at single-stranded DNA may further enhance chromothriptic fragmentation, but APOBEC3B is not required for chromosome fragmentation.