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Addressing the benefits of inhibiting APOBEC3-dependent mutagenesis in cancer

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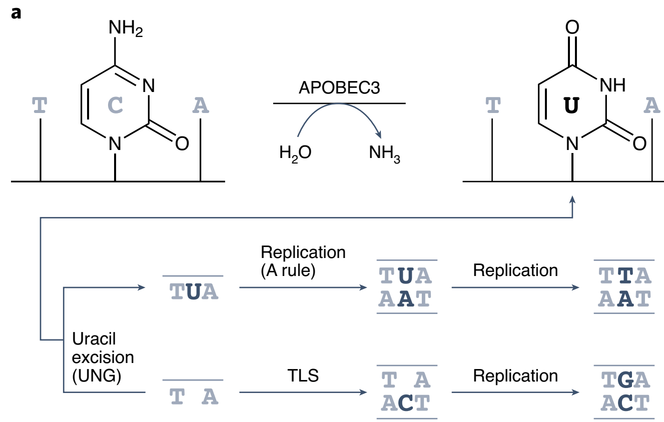
Mia Petljak[1,8] ✉, Abby M. Green[2,3,8], John Maciejowski[4,8] and Matthew D. Weitzman[5,6,7,8]

Mutational signatures associated with apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC)3 cytosine deaminase activity have been found in over half of cancer types, including some therapy-resistant and metastatic tumors. Driver mutations can occur in APOBEC3-favored sequence contexts, suggesting that mutagenesis by APOBEC3 enzymes may drive cancer evolution. The APOBEC3-mediated signatures are often detected in subclonal branches of tumor phylogenies and are acquired in cancer cell lines over long periods of time, indicating that APOBEC3 mutagenesis can be ongoing in cancer. Collectively, these and other observations have led to the proposal that APOBEC3 mutagenesis represents a disease-modifying process that could be inhibited to limit tumor heterogeneity, metastasis and drug resistance. However, critical aspects of APOBEC3 biology in cancer and in healthy tissues have not been clearly defined, limiting well-grounded predictions regarding the benefits of inhibiting APOBEC3 mutagenesis in different settings in cancer. We discuss the relevant mechanistic gaps and strategies to address them to investigate whether inhibiting APOBEC3 mutagenesis may confer clinical benefits in cancer.

The APOBEC family of cytosine deaminases restrict viral pathogenesis through base editing of viral single-stranded (ss) DNA and RNA molecules[1]. The APOBEC enzymes emerged as a source of a frequent mutational pattern in cancer genomes, characterized by mutations at cytosine bases in T<u>C</u>N sequence contexts (mutated base is underlined; N is any base)[2–5]. Mathematical deconvolution of signatures of individual mutational processes in cancer led to identification of single base substitution (SBS) signatures of non-clustered (termed SBS2 and SBS13) and clustered (kataegis, omikli, kyklonas) cytosine mutations in APOBEC-preferred sequence contexts, thus improving the quantification of APOBEC-associated mutations in individual cancers[6–10]. APOBEC-associated signatures have been found in approximately 40–70% of cancer genomes, with a particular prominence in cancers of breast, bladder, lung, cervix and esophagus, suggesting that APOBEC mutagenesis represents one of the most prevalent mutational processes in cancer[7,9,11,12]. Multiple associations indicate that members of the APOBEC3 subfamily may represent the major origins of APOBEC-associated mutations in most cancer types[3–5,13]. Six of seven members of the APOBEC3 subfamily preferentially deaminate cytosine bases in TCN trinucleotides that are commonly mutated in cancer[14,15] (Fig. 1a). In line with these associations, deletion of endogenous *APOBEC3* genes from human cancer cell lines severely decreases the accumulation of APOBEC-associated mutations[2].

Despite progress, the proposal that mutagenesis by APOBEC3 enzymes represents a disease-modifying process that could be inhibited to confer clinical benefit in various settings in cancer[16–28] (Fig. 1b) largely rely on poorly defined roles of APOBEC3 deaminases in cancer and in healthy tissues. First, available surrogate measures of

[1]Broad Institute of MIT and Harvard, Cambridge, MA, USA. [2]Department of Pediatrics, Washington University School of Medicine, St. Louis, MO, USA. [3]Center for Genome Integrity, Siteman Cancer Center, Washington University School of Medicine, St. Louis, MO, USA. [4]Molecular Biology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY, USA. [5]Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia and University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA. [6]Perelman School of Medicine, Epigenetics Institute, University of Pennsylvania, Philadelphia, PA, USA. [7]Center for Childhood Cancer Research, Children's Hospital of Philadelphia, Philadelphia, PA, USA. [8]These authors contributed equally: Mia Petljak, Abby M. Green, John Maciejowski, Matthew D. Weitzman. ✉e-mail: mpetljak@broadinstitute.org



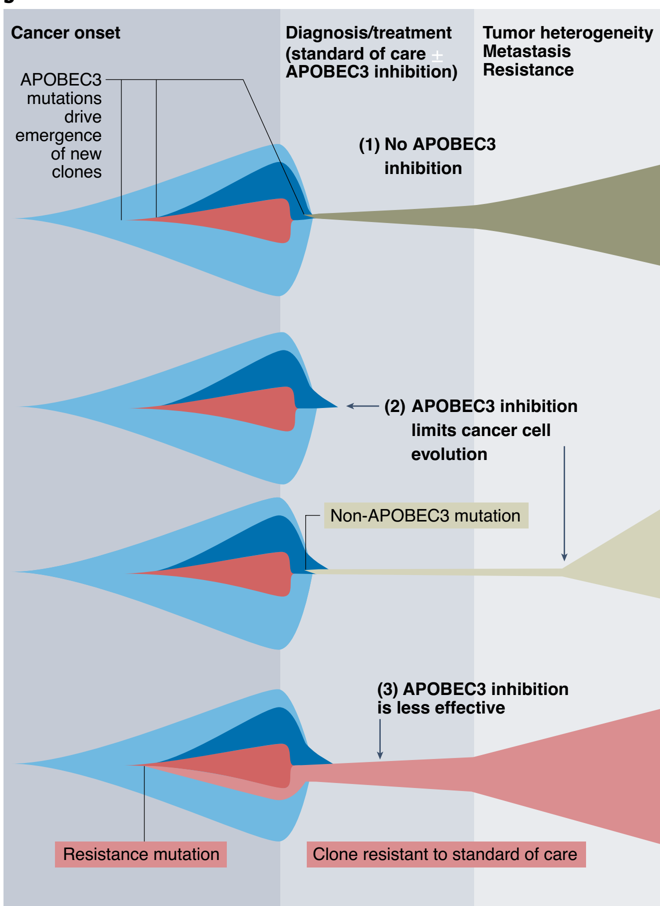


Fig. 1 | APOBEC3 mutagenesis is predicted to be a disease-modifying process in cancer that may be exploited therapeutically. a, APOBEC3 enzymes convert cytosine to uracil by deamination of cytosine bases in ssDNA. Depending on the subsequent uracil processing, different types of mutations can arise. Note that translesion synthesis (TLS) is predicted to give rise to multiple mutation types (C > A, C > G, C > T). **b**, (1) APOBEC3 mutagenesis is speculated to drive cancer cell evolution and associated phenotypes, including tumor heterogeneity, therapeutic resistance and metastases. (2) Inhibition of APOBEC3 mutagenesis alongside the standards of care may limit phenotypes associated with cancer cell evolution. The extent to which such phenotypes would be limited upon APOBEC3 inhibition in individual cancers likely depends in part on the strength of other mutational processes that contribute to cancer evolution. (3) If mutations driving resistance to a standard of care have already been acquired in a cancer before the start of the treatment, APOBEC3 inhibition alongside the standard of care may diminish evolution of tumor heterogeneity but is not predicted to eliminate resistance to the relevant therapy.

APOBEC3-mediated mutagenesis in cancer produce conflicting predictions regarding the relative mutagenic contributions of individual APOBEC3 enzymes[3–5,29,30]. Second, causal links between mutagenesis by individual APOBEC3 enzymes and features of cancer evolution, including tumor heterogeneity, therapy resistance and metastasis, are scarce. Third, the hypothesis that restriction of APOBEC3 mutagenesis may diminish phenotypes associated with cancer evolution awaits thorough investigations across different cancer types and therapy settings where such predictions have been made. Fourth, the therapeutic windows during which APOBEC3 inhibition may confer clinical benefit have not been defined. Fifth, the mechanisms by which APOBEC3 enzymes are regulated in healthy tissues or become dysregulated in cancer are largely unknown, which limits the scope and precision of potential therapeutic opportunities. Finally, the endogenous functions of APOBEC3 enzymes are incompletely understood, which complicates predictions regarding potential toxicities from modulating APOBEC3 activities.

Progress has been compromised by the limited availability of readouts of active mutagenesis by individual APOBEC3 enzymes and limited investigations in preclinical models that closely recapitulate physiological contexts in which APOBEC3 mutagenesis is active. Here, we lay out criteria and strategies to define specific readouts of mutagenesis by individual APOBEC3 enzymes and to investigate the impact of mutagenesis by individual enzymes on cancer evolution. We discuss the need to systematically characterize mechanisms of APOBEC3 misregulation in cancer and APOBEC3 functions in healthy tissues. Addressing these gaps is critical to realize the potential benefits of inhibiting APOBEC3 mutagenesis in cancer.

Addressing the readouts of APOBEC3 mutagenesis

APOBEC3 mutagenesis in cancer genomes is inferred by quantifying mutations at APOBEC3-associated sequence contexts[9,10,31–34], measuring *APOBEC3* transcript and APOBEC3 protein levels[22,23,34,35], in vitro APOBEC3 DNA-deamination assays[22,35,36] and a three-dimensional PCR-based APOBEC3A-associated RNA-editing assay[37] (Fig. 2). Aside from measuring expression of individual APOBEC3 enzymes and APOBEC3A-associated RNA editing, these readouts have not been shown to be specific to individual APOBEC3 enzymes in human cancer cells. Furthermore, mutations detected in cancer genomes represent imprints of historically incurred APOBEC3 mutagenesis, which may not always be ongoing[38], while other readouts have not been directly connected to active genomic mutagenesis by APOBEC3 enzymes. Thus, readouts of active mutagenesis by individual APOBEC3 enzymes are not clearly defined.

The limitations inherent to the surrogate readouts of active mutagenesis by APOBEC3 enzymes are illustrated in often inconclusive or discordant results regarding the relative contributions of individual APOBEC3 enzymes to mutations in cancer. Based on multiple associations, APOBEC3A and APOBEC3B have emerged as the most likely causes of APOBEC3-mediated mutations in cancer[3,5,22,23,31–35,37]. Among APOBEC3 family members, expression levels of *APOBEC3A* and *APOBEC3B* correlate most strongly with mutational burdens in cancer genomes[22,23,34,35]. However, such correlations are generally weak, complicating assignment of the enzyme underlying the majority of the APOBEC3-associated mutations in cancer[22,23,34,35]. More prominent expression of *APOBEC3B* relative to that of *APOBEC3A*, strong deaminase activity in cell extracts and association between *APOBEC3B* expression and mutational burdens in cancer have been used to define APOBEC3B as a major mutator in breast and other cancers[22,23]. Despite these associations, cancers more frequently present with mutations enriched in extended sequence contexts otherwise preferred by APOBEC3A (YTC̲N contexts, where Y is a pyrimidine base) rather than contexts preferred by APOBEC3B (RTC̲N, in which R is purine)[31]. Such 'APOBEC3A-associated' cancers also harbor over tenfold more

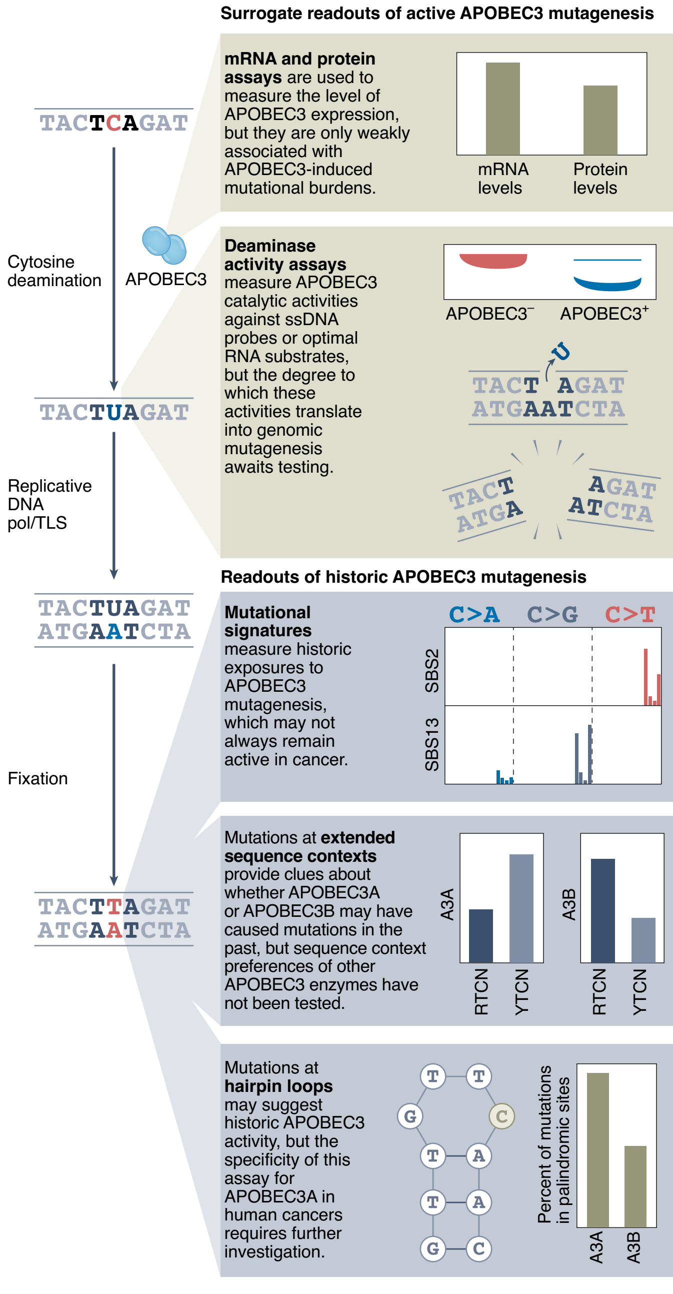


Fig. 2 | Surrogate readouts of active mutagenesis by individual APOBEC3 enzymes. The degree to which assays of APOBEC3 expression (mRNA and protein) and deaminase activity upon DNA probes (for example, probe-cleavage assays) or upon endogenous RNA (for example, three-dimensional PCR of commonly targeted transcripts) reflect active mutagenesis upon a genome is not clear. Mutations in cancer (including mutational signatures, mutations at extended sequence contexts and mutations at hairpin loops) indicate the historic exposure of a genome to APOBEC3 mutagenesis, but such readouts do not inform on whether APOBEC3 mutagenesis is active in a given tumor at the time of sampling. Pol, polymerase; A3A, APOBEC3A; A3B, APOBEC3B; RTCN/YTCN: R, purine base; Y, pyrimidine; N, any base.

mutations than 'APOBEC3B-associated' cancers, supporting the notion that APOBEC3A may be the more potent mutagen[31]. Refinement of the correlation analysis between APOBEC3 expression levels and change of conditions in deaminase activity assays further indicate that APOBEC3A may be a more potent mutator[35].

In a direct test of the mutagenic activities of endogenous APOBEC3A and APOBEC3B, individual *APOBEC3A* and *APOBEC3B* genes were deleted from human cancer cell lines that generate APOBEC3-associated signature over time[2,38]. Deletion of *APOBEC3A* severely diminished mutation acquisition, especially in YTCN contexts, demonstrating that endogenous APOBEC3A can mediate prevalently found cytosine mutations enriched at YTCN contexts in cancer[2]. Deletion of both *APOBEC3A* and *APOBEC3B* caused the most severe reduction in APOBEC3-associated mutations without completely eliminating them, indicating that APOBEC3B, and perhaps an additional APOBEC enzyme, contributes smaller mutation burdens[2]. In cell lines in which APOBEC3A contributed the majority of mutations, its relatively weak activity could be detected against model probes in extracts and against model RNA substrates[2], in agreement with prior work[35,37]. However, APOBEC3B protein levels and deaminase activity were substantially elevated compared to those of APOBEC3A in these cell lines, indicating that these assays may have limited utility as readouts of relative APOBEC3 mutagenic activities[2].

The use of surrogate assays of APOBEC3-mediated mutagenesis continues to nominate either APOBEC3A or APOBEC3B as a more prominent mutator in different settings in cancer[3,5,29,30]. Expression-based readouts in cancer can be complicated by tumor-infiltrating immune cells, which can have high levels of APOBEC3 expression[39]. Thus, bulk tumor mRNA sampling may not accurately reflect APOBEC3 expression in tumor cells[3]. APOBEC3-associated mutational signatures can be acquired episodically over time in cancer cell lines, and some human cancer cell lines with signatures of historic APOBEC3 mutagenesis exhibit no evidence of continued or episodic APOBEC3-associated mutation acquisition[38]. Therefore, expression of individual APOBEC3 enzymes detected at the time of cancer cell sampling may not accurately reflect origins of detected mutations. Furthermore, protein-based assays for APOBEC3 detection remain limited due to the lack of reagents, while more commonly measured transcript abundance may be a poor predictor of corresponding protein levels. These challenges likely underlie the weak correlations reported between expression of APOBEC3 enzymes and APOBEC3-associated mutations in cancer[22,23,34,35].

In vitro cytosine deamination assays, in which a labeled ssDNA oligonucleotide is incubated with extracts from cell lines, have been widely used to measure APOBEC3 activities[22,35] (Fig. 2). However, non-uniformity of experimental conditions confounds interpretation of these assays. Important parameters of these assays include RNA-mediated inhibition of deaminase activity[35,40] and variability in sequence context preference of APOBEC3 deaminases upon different probes[33]. Distinct predictions regarding mutagenic activities of APOBEC3A or APOBEC3B may emerge upon altering these parameters across experimental conditions[22,33,35]. Recent work suggests that quantitative measures of RNA editing at hotspot hairpin loops targeted by APOBEC3A may serve as a marker of ongoing APOBEC3A deamination activity[37]. However, an APOBEC3A-induced mutation within a cancer genome reflects a combination of APOBEC3A-mediated deamination and subsequent errors introduced or enabled by DNA-repair and DNA-replication processes. Indeed, DNA-repair processes can modulate the spectra and burdens of APOBEC3-associated mutations[2,41,42]. While both RNA editing and in vitro DNA-deamination assays are important tools to study deamination activities of APOBEC3 enzymes, they do not measure the extent to which deamination events are converted into genomic mutations. Thus, these assays may not always reflect accurate readouts of APOBEC3 mutagenesis upon a genome, and the extent to which they predict APOBEC3-mediated genomic mutagenesis has yet to be tested.

Analysis of cytosine mutations at extended motifs has gained traction as a readout of activities of the major candidate mutators APOBEC3A and APOBEC3B (Fig. 2). These include enrichment of mutations at, respectively, APOBEC3A- and APOBEC3B-preferred YTCN and RTCN tetranucleotides[31] and quantification of 'hotspot' cytosine mutations in sequences predicted to form hairpin loops, which

otherwise make up a minor proportion of total APOBEC3-associated mutations in cancer genomes, to infer mutagenesis by APOBEC3A[32,33]. Extended sequence analysis of mutations accumulating in human cancer cell lines[2] showed that endogenous APOBEC3 enzymes exhibit sequence preferences similar to those of APOBEC3A and APOBEC3B overexpressed in yeast[31]. However, preferences of many other APOBEC3 family members, which can otherwise cause mutations at TCN trinucleotides and thus possibly contribute signatures in cancer, have not been investigated at these sequence contexts, and their activities cannot be excluded from these assays alone. Moreover, experiments in human cancer cell lines show that APOBEC3B can contribute smaller mutational burdens in the cell lines that display enrichment of mutations at YTCN contexts mediated by APOBEC3A[2]. Therefore, extended sequence analyses cannot exclude minor contributions of some APOBEC3 enzymes. Furthermore, while hairpin loops represent preferred substrates for APOBEC3A and mutations at hairpin loops are less frequent in 'APOBEC3B-associated' cancers, APOBEC3B can be active at hairpin loops[31,33,43]. Thus, the specificity of this assay for activity of APOBEC3A in human cancers requires further investigation[43]. Importantly, mutations at tetranucleotide sequence motifs and sequences predicted to form hairpin loops measure historically acquired APOBEC3-associated mutations and do not inform on whether mutagenesis remains active in samples under analysis. In addition, while these assays can point toward historic APOBEC3 activities in major clonal lineages captured in cancers under analysis, relative contributions of individual APOBEC3 enzymes may vary between individual cancer cell lineages and change during cancer evolution[2].

Defined readouts of active mutagenesis that distinguish individual APOBEC3 enzymes are necessary to detect mutagenic activities of the relevant APOBEC3 deaminases in tumors and to enable experimental investigations into causes and functional consequences of APOBEC3 misregulation during cancer evolution. Human cancer cell lines with ongoing acquisition of APOBEC3-associated mutational signatures represent a suitable set of models to establish readouts that translate into active acquisition of the relevant mutations in cancer cells[38]. Deletion of individual APOBEC3 enzymes in such cell lines can be combined with overexpression of APOBEC3 enzymes across additional experimental models to further refine abilities of relevant assays to measure mutagenesis by individual APOBEC3 enzymes upon a genome[2,13,32]. It is possible that a combination of assays will be required to adequately identify active APOBEC3 mutagenesis. Once the optimal readouts of APOBEC3-mediated mutation acquisition have been determined in controlled experimental systems, the next challenge will be to assess their potential as clinical biomarkers of active mutagenesis by individual enzymes in human cancers.

Addressing the impact of APOBEC3 enzymes on cancer evolution

Endogenous APOBEC3 enzymes can drive acquisition of APOBEC-associated signatures prevalent in cancer genomes[2]. Germline polymorphisms in the *APOBEC3* locus are associated with increased mutational burdens and elevated cancer risk[44–47]. Expression of the human *APOBEC3A* transgene can promote tumorigenesis in mice predisposed to colon and liver cancers[9,48,49]. APOBEC3-associated mutations have been found in metastatic cancers and in subclonal branches of tumor phylogenies[50–55]. Driver mutations have been detected in APOBEC3-associated sequence contexts at different stages of cancer evolution, including in therapy resistance[29,30,50–53,55–58]. APOBEC3-associated signatures can continue to be acquired in human cancer cell lines over long periods of time[38]. These studies collectively provide support for the concept that APOBEC3 mutagenesis drives cancer evolution and contributes associated phenotypes, such as tumor heterogeneity, metastasis and resistance, and that its inhibition may confer therapeutic benefit (Fig. 1b). However, therapeutic pursuit depends on establishing causal relationships between mutagenesis by endogenous APOBEC3 deaminases and cancer evolution and on demonstrating that perturbing mutagenesis by individual enzymes can alleviate associated phenotypes in preclinical models that closely recapitulate physiological settings where APOBEC3 inhibition is predicted to confer therapeutic benefit.

First, validation of associations between individual APOBEC3 enzymes and mutational signatures detected in various contexts, such as in different types of primary or therapy-resistant cancers, requires experimental demonstration that nominated endogenous enzymes generate the relevant signatures in such contexts. Deletion of *APOBEC3A* from a panel of breast cancer and lymphoma cell lines diminished acquisition of the majority of APOBEC3-associated mutational signatures[2]. Genes encoding APOBEC3 enzymes can be systematically deleted across cell lines from additional cancer types and upon treatment with relevant therapies, such as standard of care or therapies associated with APOBEC3-mediated resistance[5,29,30], to ascertain the relevant mutator enzymes across a broader spectrum of contexts in which APOBEC3-associated signatures have been detected (Fig. 3). Second, once the relevant mutators have been experimentally validated, their impact on cancer evolution should be investigated across preclinical models that closely recapitulate settings where APOBEC3 mutagenesis has been linked to cancer evolution (Fig. 3). Critically, the hypothesis that APOBEC3 inhibition may confer therapeutic benefit and diminish the phenotypes associated with cancer cell evolution, such as sensitivity to therapy, metastatic potential and tumor heterogeneity, should be tested by assessing the relevant phenotypes upon perturbing mutagenesis by the relevant enzymes. Unlike humans, mice encode only a single APOBEC3 enzyme[59]. Therefore, mimicking human APOBEC3 mutagenesis in murine models relies on expression of the human *APOBEC3* genes. Generation of transgenic models should be guided by human data. The choice of transgenic systems should be directed toward modeling mutagenesis in tissues where APOBEC3-associated mutational signatures have been observed in cancer. Reconstructions of the evolutionary trajectories of tumor and normal tissues[60–62] will likely unveil genetic backgrounds upon which APOBEC3-associated mutations commonly occur in different tissues. Such backgrounds can be engineered into transgenic mouse models to recapitulate the settings in which APOBEC3 mutagenesis is hypothesized to drive cancer evolution and associated phenotypes. APOBEC3 mutagenesis in mouse models should be recapitulated by ectopic expression of human APOBEC3 enzymes that were a priori established as responsible mutators in human cell models of the cancer type and/or therapy setting under analysis. Complementary studies using depletion of the relevant enzymes in patient-derived xenografts and human cancer cell lines with active APOBEC3 mutagenesis are important to mitigate the potential neomorphic effects of APOBEC3 overexpression in mice. These experiments can be combined with treatments with relevant therapeutics to further mimic clinical settings in which APOBEC3 inhibition is speculated to confer therapeutic benefit.

Third, therapeutic windows during which inhibition of APOBEC3 mutagenesis may confer the most effective clinical benefit should be investigated by conducting experiments in preclinical models representing various stages of the disease. Additionally, the driver mutations arising in APOBEC3-associated sequence contexts in preclinical models should be compared to drivers identified in matching types of human cancers and therapy settings to establish the relevance of such mutations in human cancer cell evolution. APOBEC3 enzymes are likely sources of driver mutations occurring at TCN trinucleotides[53]. In classifying driver mutations as APOBEC3 induced in human cancers and experimental models, it will be relevant to statistically account for signatures of other mutational processes that can contribute mutations at TCN contexts[58,63,64], such as signatures associated with ultraviolet light exposure and mutations in polymerase ε, particularly in cancers in which such processes contribute high mutational burdens. Furthermore, identification of APOBEC3-associated driver mutations should take into

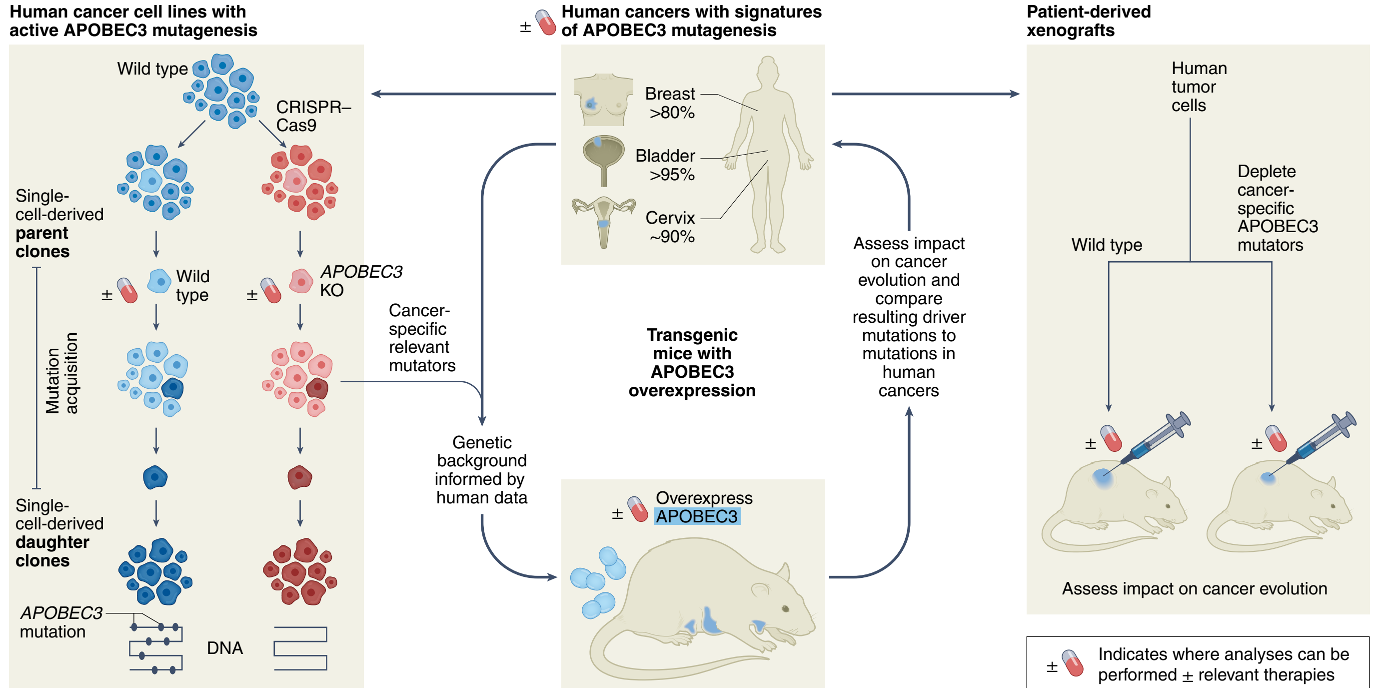


Fig. 3 | Addressing impact of APOBEC3 enzymes on cancer evolution. APOBEC3 mutators can be identified by knockout (KO) of individual enzyme-coding genes in human cell lines, which actively acquire APOBEC3-associated mutations, from cancer types commonly presenting with APOBEC3-associated signatures (percentages of cancers presenting with APOBEC3-associated signatures in exemplar cancer types are indicated). To detect the impact of *APOBEC3* deletions on mutation acquisition, genomes of single-cell-derived parent clones can be compared to genomes of their respective daughters obtained after defined in vitro periods. To investigate the roles of APOBEC3 mutagenesis in cancer cell evolution, identified APOBEC3 mutators can be expressed in murine models, which lack most human *APOBEC3* gene orthologs. APOBEC3 expression can be performed upon genetic backgrounds and in tissues in which APOBEC3-associated mutations present in human cancers. Tumors that may result from APOBEC3 activities can be profiled for driver mutations and these can be compared to driver mutations in human cancers. To control for neomorphic effects, experiments in transgenic models can be accompanied by experiments in patient-derived xenografts and human cancer cell lines with active APOBEC3 mutagenesis. Analyses can be performed in cancers and models of various stages of the disease and/or upon therapy treatment to investigate the ability of APOBEC3 mutagenesis to contribute to different stages of cancer cell evolution, such as tumor heterogeneity, metastasis and resistance.

account sequence features such as the potential to form DNA hairpins, which are preferred substrates for APOBEC3A[32,33]; and thus recurrent mutations at such sequences may not reflect positive selection.

Additionally, APOBEC3A and APOBEC3B have been implicated in induction of chromosomal instability[65,66]. Expression of APOBEC3A in avian cells was recently shown to induce genomic deletions in addition to SBS signatures[13]. Therefore, putative non-SBS alterations should be taken into account when investigating APOBEC3-mediated driver mutations. In addition, APOBEC3 activities may contribute to tumor progression by other means. For example, APOBEC3B activity in estrogen receptor (ER)-positive breast cancer cell lines can be directed to ER binding regions and mediate chromatin remodeling that promotes expression of ER target genes[67], providing a possible mechanistic basis for association between APOBEC3B expression and poor survival of patients with ER$^+$ breast cancer[19,67,68].

APOBEC3 enzymes have been experimentally linked to acquired resistance to tyrosine kinase inhibitors in lung cancer[29,30]. Such efforts should be expanded across a broader range of cancer types and therapy settings to investigate existing predictions about the impact of APOBEC3 mutagenesis on cancer cell evolution phenotypes, which await experimental validation[16–28]. Overall, investigations across models that closely recapitulate physiological settings in which APOBEC3 mutagenesis is active in cancer will be critical to assess the impact of individual APOBEC3 mutators on cancer evolution and associated phenotypes such as tumor heterogeneity, resistance and metastasis. Identification of the specific readouts of active APOBEC3 mutagenesis discussed in the previous section will accelerate such efforts.

Addressing (mis)regulation of APOBEC3 mutagenesis

The processes that regulate and misregulate APOBEC3 mutagenesis are key knowledge gaps that present barriers to therapeutic opportunities. Available data suggest that APOBEC3 mutagenesis may be instigated by both endogenous and environmental factors. APOBEC3-associated mutational signatures accumulate in some human cancer cell lines in the absence of obvious environmental stressors, indicating that APOBEC3 mutagenesis can arise from endogenous misregulation in cancer cells[38]. APOBEC3-associated signatures can be acquired episodically, rather than continuously, over time in individual cancer cell line lineages, suggesting that endogenous instigators of mutagenic APOBEC3 behavior may be intermittent[38]. Episodic APOBEC3 misregulation may account for highly variable APOBEC3-associated mutational burdens among phylogenetic branches of primary tumors[54,69] and healthy bronchial cells[70]. On the other hand, multiple environmental factors, such as viral infections, genotoxic stress, some targeted therapies and cytotoxic drugs, can induce APOBEC3 expression and/or deamination activities in experimental models[5,27–30,47,71–73]. Although inductions of APOBEC3 expression and/or activity may not always translate into active genomic mutagenesis, some of the exogenous stressors, such as tobacco smoking, cytotoxic agents and some targeted therapies, have been associated with increased APOBEC3 mutational burdens[28–30,74,75]. Furthermore, APOBEC3-associated signatures are prevalent in cancers associated with human papillomavirus (HPV) otherwise restricted by APOBEC3 enzymes, as well as enriched in HPV-positive compared to HPV-negative head and neck cancers[1,76].

Thus, APOBEC3-associated signatures in cancer genomes may in part represent collateral damage from a response originally directed toward virus restriction[76]. However, APOBEC3-associated signatures have been detected in HPV-negative cancers and in many cancer types in which viral infections are not apparent, while they have not been commonly observed in cancer types associated with viruses that can be restricted by APOBEC3 enzymes, such as in hepatitis-associated hepatocellular carcinoma[1,56,76]. Therefore, factors other than viral infections likely contribute to APOBEC3 misregulation in many cancers, and viral infections may not always instigate APOBEC3 misregulation. In conclusion, environmental factors likely trigger APOBEC3 mutagenesis, but understanding the relative contributions of environmental and endogenous factors to APOBEC3 mutagenesis will require further investigation.

The molecular details of how APOBEC3 dysfunction translates into genomic mutations are largely unknown but likely include a combination of APOBEC3 expression, subcellular localization, availability of the genomic substrate, interacting proteins and DNA repair. The mechanisms may vary depending on the exogenous or endogenous instigator of aberrant APOBEC3 behavior. Expression of *APOBEC3* genes in malignant and healthy tissues varies among family members[34,77–79]. While many prior studies have evaluated factors that activate the innate immune function of APOBEC3 enzymes, the signaling pathways that control physiological APOBEC3 expression are not completely understood[39]. Furthermore, multiple signaling pathways, such as interferon and protein kinase C (PKC)–nuclear factor (NF)-κB signaling, can induce expression of major mutator candidates APOBEC3A, APOBEC3B or both in normal and malignant cells depending on cell type or context[5,27,29,30,39,71]. It is possible that contexts common in cancer cells promote misregulated *APOBEC3* expression. For example, *APOBEC3* induction in cancer could result from activation of DNA-damage signaling pathways in response to replication stress, DNA breaks or chemotherapeutic agents[28,74]. Furthermore, the *APOBEC3* genes may be regulated by p53 in response to DNA damage. While some APOBEC3 enzymes may be responsive to p53 activation, others such as APOBEC3B appear to be repressed by p53 and may therefore be misregulated by tumor-associated p53 mutants[80–82]. Overall, the signaling pathways that elevate APOBEC3 expression in cancer remain incompletely understood, and it is not clear to what extent APOBEC3 upregulation translates into mutagenesis of a cancer genome. Furthermore, post-transcriptional and post-translational modifications of *APOBEC3* transcripts and proteins may play contributing roles but have not been explored thoroughly.

APOBEC3 activity on the cellular genome may be further regulated through access to genomic ssDNA, for example, by cellular localization of APOBEC3 enzymes. Some family members are exclusively nuclear (for example, APOBEC3B), and some are predominantly cytoplasmic (for example, APOBEC3G), while others are found in both nuclear and cytoplasmic compartments (for example, APOBEC3A)[83,84]. The episodic nature of APOBEC3 mutagenesis[38] calls for further investigation into the possibility that aberrant localization of these enzymes occurs transiently, which may not be captured by standard assays. Furthermore, availability of ssDNA substrate may represent a relevant barrier or facilitator for mutagenesis. Deamination substrates may be impacted by factors such as replication fork progression, DNA resection, break-induced replication and chromatin conformation[8,28,41,74,85–88]. For example, associations from human cancer data and experiments in model systems implicate replication stress in increased APOBEC3 mutagenesis[28,74,89]. ssDNA generated during telomere crisis, a period of genomic instability that occurs following depletion of the telomere reserve, enables APOBEC3B-mediated generation of kataegis[90]. In this system, APOBEC3B-dependent mutagenesis occurred independently of increased *APOBEC3B* mRNA or APOBEC3B protein levels. Instead, APOBEC3B was shown to target available ssDNA that accumulated in aberrant nuclear compartments that formed as a result of chromosomal instability during telomere crisis. Additionally, ssDNA-binding proteins such as replication protein A (RPA) or RNA polymerase may limit APOBEC3 access to genomic substrates[91].

APOBEC3 activities may also be regulated via protein interactions. Interaction between APOBEC3A and the cellular CCT chaperonin complex was recently found to minimize deaminase-induced DNA damage and cytotoxicity[92]. Viral oncoproteins provide further insights into how APOBEC3 activities may be regulated via protein interactions, for example, by redirecting ubiquitination[93] or cellular localization[94]. Finally, the DNA-repair and DNA-replication processes involved in processing APOBEC3-induced DNA edits can modulate the final burden and types of APOBEC3-associated mutations incurred on a cancer genome[2,41,42]. For example, deletion of the gene encoding translesion polymerase REV1 diminishes large amounts of APOBEC3-associated signatures, while deletion of the gene encoding uracil DNA glycosylase (UNG) decreases APOBEC3-associated transversion mutations[2].

Overall, combinations of endogenous and environmental processes at different molecular levels likely determine the dynamics of regulation and misregulation of APOBEC3 mutagenesis upon a genome. Molecular, proteomic and epidemiologic characterization is required to define the processes that ultimately regulate and misregulate APOBEC3-associated mutational burdens observed in cancer genomes. Improving the readouts and models of APOBEC3 mutagenesis discussed above will facilitate these efforts. Initially, the instigators of the APOBEC3 misregulation and molecular levels at which misregulation translates into mutagenesis of the human genome must be defined. Subsequent characterization of the factors involved in APOBEC3 misregulation at the relevant molecular levels may identify additional targets to halt APOBEC3-mediated mutation acquisition in cancer. Understanding the ways in which misregulation in cancer may differ from regulatory mechanisms in healthy tissues will help prioritize strategies to limit adverse effects.

Addressing physiological APOBEC3 functions

The *APOBEC3* gene locus has undergone a rapid evolutionary expansion, resulting in seven genes in primates[59]. The expansion is thought to be underscored by the selective pressure on the *APOBEC3* genes from their retroviral and retrotransposon targets[95–97]. An evolutionary advantage would predict deleterious effects on APOBEC3 inhibition. However, current knowledge of the physiological roles of APOBEC3 enzymes across human tissues is incomplete, limiting predictions regarding consequences of targeting APOBEC3 deaminase activities.

The best-defined function of the APOBEC3 family is in innate immune restriction of retroviruses and retroelements. The first APOBEC3 enzyme identified, APOBEC3G, was characterized by its ability to restrict Vif-deficient human immunodeficiency virus through deamination of the viral genome in its cDNA intermediate phase[98,99]. Activities of APOBEC3G and other APOBEC3 members have now been linked to additional targets, including ssDNA viruses, double-stranded DNA viruses and RNA viruses[1,100]. The major candidate mutators in cancer, APOBEC3A and APOBEC3B, have been reported as viral restriction factors against a narrow range of pathogens[101]. Despite recent progress, in vivo data regarding APOBEC3-mediated viral restriction are lacking. Furthermore, the functions of APOBEC3 enzymes beyond the immune system have not been thoroughly investigated.

Limited available evidence suggests that APOBEC3 inhibition may not severely compromise overall health. *APOBEC3*-knockout mice exhibit increased susceptibility to mouse retrovirus infection but normal viability[102,103]. Furthermore, the germline *APOBEC3B* polymorphism, which effectively deletes *APOBEC3B* and fuses *APOBEC3A* to the 3′ untranslated region of *APOBEC3B*, is common in some ancestries[44]. It is thus possible that there is redundancy among cellular pathways that affect virus and retroelement restriction. However, the *APOBEC3B*-deletion polymorphism has been associated with increased mutational burdens and susceptibility to some cancer types within specific ancestries[45,47,104–107]. The increased mutational

burdens in carriers of the *APOBEC3B* polymorphism may result from production of a stabilized version of the *APOBEC3A* transcript[108]. APOBEC3B loss can increase APOBEC3A protein levels and activity and APOBEC3A-associated mutational burdens in some cancer cell lines that do not form the *APOBEC3A–APOBEC3B* 3′ untranslated region hybrid transcript[2]. These results raise the counterintuitive possibility that the absence of some APOBEC3 members may result in higher overall mutational burdens, further highlighting the need to investigate interactions between APOBEC3 enzymes.

More thorough examination of physiological APOBEC3 functions will determine the consequences of perturbing APOBEC3 activities. Future investigations should include population-based studies of the risk factors associated with APOBEC3 polymorphisms and systematic assessments of phenotypic and genotypic consequences of APOBEC3 perturbation in healthy and malignant tissues of model organisms and human cell systems.

Conclusion

Therapeutic approaches based on inhibiting APOBEC3 activities in cancer are commonly discussed, and inhibitor development is underway[16–28]. However, critical aspects of APOBEC3 biology remain unknown or rely on associations that have not been validated in preclinical models that closely resemble human cancer physiology. This lack of understanding thus limits predictions pertaining to efficacy and safety of proposed inhibitor approaches. We have highlighted these gaps and outlined experimental strategies to address them. Ultimately, effective investigations into the potential to inhibit APOBEC3 mutagenesis as a therapeutic strategy in cancer depend on reprioritization of experimental approaches and experimental validation of associations made between APOBEC3 activities and cancer evolution. Additional proposals aimed at therapeutic exploitation of APOBEC3 activities in cancer have emerged. These include induction of APOBEC3 mutagenesis to increase the efficacy of immune checkpoint blockade[17,51,109,110] and exploiting synthetic lethality opportunities arising from APOBEC3-mediated DNA damage[111–114]. Future studies into APOBEC3 biology will facilitate understanding the potential of these alternative therapeutic avenues.

Data availability

No new data were generated for this text.

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Author contributions

All authors contributed to the writing of the paper.

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Competing interests

M.P. is a shareholder in Vertex Pharmaceuticals and a compensated consultant through GLG Network. J.M. has received consulting fees from Ono Pharmaceutical. J.M.'s spouse is an employee of and has equity in Bristol Myers Squibb. M.P. and J.M. are inventors of the patent application entitled 'Tracking APOBEC mutational signatures in tumor cells' (patent pending). The remaining authors declare no competing interests.

Additional information

Correspondence should be addressed to Mia Petljak.

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