Molecular Therapy
Nucleic Acids
Review

Engineered deaminases as a key component of DNA and RNA editing tools

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Over recent years, zinc-dependent deaminases have attracted increasing interest as key components of nucleic acid editing tools that can generate point mutations at specific sites in either DNA or RNA by combining a targeting module (such as a catalytically impaired CRISPR-Cas component) and an effector module (most often a deaminase). Deaminase-based molecular tools are already being utilized in a wide spectrum of therapeutic and research applications; however, their medical and biotechnological potential seems to be much greater. Recent reports indicate that the further development of nucleic acid editing systems depends largely on our ability to engineer the substrate specificity and catalytic activity of the editors themselves. In this review, we summarize the current trends and achievements in deaminase engineering. The presented data indicate that the potential of these enzymes has not yet been fully revealed or understood. Several examples show that even relatively minor changes in the structure of deaminases can give them completely new and unique properties.

INTRODUCTION—DNA/RNA EDITING ENZYMES WITHIN THE FAMILY OF ZINC-DEPENDENT DEAMINASES

Zinc-dependent deaminases form a large superfamily of enzymes that occur across all three domains of life and that catalyze hydrolytic deamination of bases in both free nucleotides and polynucleotide chains.[1,2] These enzymes have been classified into one superfamily because they share a common zinc-chelating structural motif; however, they can play different biological roles.[2,3] The zinc-dependent deaminase superfamily includes enzymes involved in the metabolism of purines and pyrimidines, e.g., (1) deaminases that convert cytidine to uridine in DNA or RNA chains (activation-induced cytidine deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide; AID/APOBECs),[4–10] (2) deaminases that convert adenosine to inosine in tRNA (tRNA-specific adenosine deaminase; TadA/adenosine deaminase tRNA specific; ADAT),[11–13] (3) deaminases that convert adenosine to inosine in mRNA (adenosine deaminases acting on RNA; ADARs),[14–18] and (4) free cytidine deaminases (CDAs)[19,20] and deoxycytidylate deaminases (dCDs) that deaminate cytidine monophosphate.[21–23] In recent years, the enzymes belonging to the first three groups have gained substantial importance in applied practice since they have the unique ability to modify genetic information at the DNA or RNA levels.

The first group includes proteins from the AID/APOBEC family. In the human genome, they are encoded by 11 genes (*APOBEC1*, *APOBEC2*, *APOBEC4*, *AID*, and seven *APOBEC3* genes).[24–26] They alter DNA or RNA sequences by deaminating cytidine (C) to uridine (U).[7] The second group contains a bacterial TadA protein and its eukaryotic homolog, the ADAT2/3 heterodimer, both best known as tRNA anticodon editing enzymes.[27–29] The third group comprises ADARs. In the human genome, three genes encode enzymes of this type: ADAR1, ADAR2, and ADAR3. They deaminate adenine (A) to inosine (I) in double-stranded RNA (dsRNA) substrates.[17,18,30]

All AID/APOBECs catalyze C-to-U deamination in polynucleotide chains, but the biological consequences of their actions are highly diverse (see Table 1). APOBEC1 (A1) was the first identified AID/APOBEC deaminase due to its essential role in lipid metabolism, that is, editing apolipoprotein B (apoB) mRNA.[8,9] Deamination of cytidine 6666 in apoB mRNA leads to the formation of a stop codon, resulting in the production of a truncated apoB48 protein in addition to full-length apoB100.[31] A1 requires protein cofactors to deaminate apoB mRNA but not for single-stranded DNA (ssDNA) deamination. *In vitro,* the activity of A1 is the highest on linear ssDNA within the 5′TC sequence motif.[10] APOBEC2 (A2)[32–35] and APOBEC4 (A4)[36,37] do not exhibit cytidine deaminase activity *in vitro*,[10,38] and their *in vivo* functions are still debated. *A2* has been proposed to play roles in the differentiation of skeletal and cardiac muscle cells, where it is predominantly expressed.[33–35] *APOBEC4* gene expression in humans and mice has been found in testes, which suggests a role of this enzyme in spermatogenesis.[36] Recently, high expression of *APOBEC4* has also been found in cells infected by SARS-CoV-2, which raises questions about the potential role of A4 in the antiviral response.[39] Activation-induced cytidine deaminase (AID) plays an essential role in antibody production.[40–42] It participates in class switch recombination (CSR) and somatic hypermutation (SHM) by deaminating C-to-U in the constant and variable regions of immunoglobulin genes. Therefore, AID function is fundamental for adaptive

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Table 1. Overview of AID/APOBECs and selected adenosine deaminases and their characteristics

Protein	Deamination activity	Targeted nucleic acid	Preferred motif	Cellular localization	Main biological functions
AID	C-to-U	ssDNA	5′WR**C** (W = A/T, R = A/G)	N/C	antibody diversification
APOBEC1	C-to-U	ssDNA, RNA	5′T**C** in ssDNA 5′A**C**(n_{4-6})UGAUnnGnnnn in RNA	N/C	mRNA editing
APOBEC2	ND	ND	ND	N/C	still debated
APOBEC3A	C-to-U	ssDNA, RNA	5′T**C**	N/C	response against retroviruses and retroelements
APOBEC3B	C-to-U	ssDNA	5′T**C**	N	
APOBEC3C	C-to-U	ssDNA	5′T**C**	N/C	
APOBEC3D	C-to-U	ssDNA	5′T**C**	C	
APOBEC3F	C-to-U	ssDNA	5′T**C**	C	
APOBEC3G	C-to-U	ssDNA, RNA	5′C**C**	C	
APOBEC3H	C-to-U	ssDNA	5′T**C**	N/C depending on haplotype	
APOBEC4	ND	ND	ND	N/C	still debated
ADAR1	A-to-I	dsRNA	5′U**A**G	N/C depending on isoform	sensing of self vs. nonself RNA and preventing autoinflammation
ADAR2	A-to-I	dsRNA	5′U**A**G	N/C	RNA editing
ADAR3	ND	ND	ND	N/C	inhibits functions of ADAR1 and ADAR2
TAdA/ADAT2	A-to-I	RNA, DNA?	5′**A**CG	N	edits the position 34 of the anticodon loop in tRNAs

ND, not determined; N, nuclear; C, cytoplasmic.

immunity.[43,44] AID acts preferentially on ssDNA within the hot spot 5′WR**C** (W = A/T, R = A/G), both *in vitro* and *in vivo,* while avoiding the cold spot 5′SY**C** (S = G/C, Y = C/T), and does not exhibit detectable activity on RNA substrates.[45–47] There are seven *APOBEC3* genes in the human genome. They encode seven proteins (A3A, A3B, A3C, A3D/E, A3F, A3G, and A3H) that are believed to be involved in the innate immune response against retroviruses and retroelements.[24,48–51] APOBEC3 proteins perform their functions using both deamination-dependent and deamination-independent mechanisms.[52,53] *In vitro* and *in vivo,* they act preferentially on ssDNA within the 5′T**C** hotspot motif,[54] with A3A being the most active.[55] The exception is A3G, which favors the 5′C**C** context.[56,57] Additionally, it has been shown that bases flanking deaminated C and located in positions −2 and +1 are also important in some systems, leading to more complex motifs, e.g., 5′CC**C** and 5′TT**C** trinucleotide motifs for A3G and A3F, respectively,[58,59] or 5′CC**C**A and 5′TT**C**A four-nucleotide motifs for A3G and A3F, respectively.[54,60] The sequence motif preferences of AID/APOBECs are summarized in Table 1. Notably, the presence of the nucleotide context alone does not ensure that a site will be deaminated by these enzymes, and there are additional determinants of substrate selectivity. For example, recent data suggest that DNA secondary structure can also significantly influence A3 activity.[54,61,62] It was shown that in some hairpin structures, non-5′TC sites outperform 5′TC sites as A3A mutational hotspots.[63] The secondary structure is also important in the case of RNA substrates. A3A and A3G have been demonstrated to deaminate cytidines in RNA within an optimal structural/sequence context, i.e., the 5′ UC sequence within the four-nucleotide loop of a hairpin structure.[64–66] The overall picture of AID/APOBEC preferences in targeting specific DNA/RNA motifs is further complicated by the fact that at least some of these enzymes use a variety of scanning mechanisms to search genomic DNA for deamination sites.[47,67–69] The proposed processivity involves sliding, jumping, and intersegmental transfers and results in the formation of closely spaced clusters of mutations.[67,70,71] Moreover, some AID/APOBECs (especially A3A and A3H) exhibit *in vitro* deamination activity on cytidines methylated at the 5 position of the pyrimidine ring in DNA (5mC).[55,72–74] However, the activity on 5mC is significantly weaker than that on C, and the functional significance of this phenomenon is still debated.[6,75–78] 5mC typically represses gene transcription and is critical for cell identity.[79–82] Since AID/APOBECs deaminate 5mC to T, generating a mismatch in double-stranded DNA (dsDNA), it has been proposed that they play a role in the active DNA demethylation process.[83–85] However, much more work needs to be done to fully understand the link between DNA deamination and demethylation processes. Regardless of their catalytic capabilities, the subcellular localization of AID/APOBECs (summarized in Table 1) determines their access to specific substrates and is therefore crucial for their biological functions. For example, AID and APOBEC1 are nucleocytoplasmic shuttling proteins whose transport is driven by bipartite nuclear localization signals and nuclear export signals in their N and C termini, respectively.[86,87] Single-domain APOBEC3s (A3A, A3C, and A3H) are small enough to enter or exit the nucleus. A3D, A3F, and A3G lack a nuclear localization signal (NLS); therefore, they are mostly cytoplasmic, whereas

A3B is constitutively nuclear due to its N-terminal NLS.[87–90] Regardless of their physiological functions, AID/APOBECs in combination with next-generation sequencing have already been used *in vitro* to identify and map genomic 5mC sites.[91–93] In this technique, enzymatic deamination has been applied as an alternative to other methods, e.g., bisulfite treatment, developed for this purpose.[94,95]

Three ADAR enzymes (ADAR1, ADAR2, and ADAR3) convert adenosine (A) to inosine (I) in dsRNAs (see Table 1).[15–17,96,97] Inosine is interpreted as guanosine (G) by the cellular machinery[98]; therefore, its presence alters the RNA sequence. The expression of *ADAR* genes is ubiquitous among metazoans.[99] Loss of ADAR1 or ADAR2 leads to embryonic lethality in mice.[100–102] In humans, ADAR1 exists as two isoforms: (1) p110 (110 kDa), which is constitutively expressed and specific to the nucleus and whose function is not well understood, and (2) p150 (150 kDa), which is interferon-induced and primarily localized to the cytoplasm.[103] Loss of ADAR1 leads to a dramatic increase in interferon signaling; therefore, ADAR1 is believed to be responsible for detecting and distinguishing self and nonself RNA and for preventing autoinflammation.[30,102,104,105] ADAR2 is most abundantly expressed in the central nervous system and is believed to be responsible for editing many RNAs in their noncoding (mainly) and coding regions.[106–109] ADAR3 is catalytically inactive, although its deaminase domain contains all amino acids necessary for editing activity. In humans, inactive ADAR3 is involved in the regulation of ADAR1 and ADAR2 via competitive binding to target RNAs.[109,110]

It is believed that both AID/APOBECs and ADARs independently originated from an ancestor of tRNA adenosine deaminases (TadA/ADAT), which edit A-to-I at position 34 of the tRNA anticodon loops in eukaryotes and prokaryotes.[13,25,111] Specifically, bacterial tRNA adenosine deaminase (TadA) generates inosine by deaminating the wobble anticodon position of $tRNA^{Arg-227}$. TadA is considered to form a homodimer and shares homology with yeast Tad2.[112] In eukaryotes, a heterodimeric enzyme composed of two sequence-related subunits (ADAT2/ADAT3; Tad2/Tad3 in yeast) is responsible for wobble anticodon $tRNA^{Arg}$ modification, and several others—up to eight cytoplasmic tRNAs from higher eukaryotes—are modified to inosine.[28,29] Additionally, in eukaryotes, the ADAT1 (Tad1) homodimer is solely responsible for A37 deamination during methylinosine formation at position 37 of tRNAAla13. Notably, ADAT1 has greater sequence homology to the ADAR family than to the ADAT2/ADAT3 heterodimer.[13,113] Interestingly, recombinant ADAT2 from trypanosomes catalyzes *in vitro* C-to-U deamination in ssDNA, which seems to support the postulated AID/APOBEC origin.[114] This observation also shows the plasticity of the catalytic pocket toward adopting different nucleotides (A or C) while maintaining the polynucleotide chain binding mechanism.

The phylogenetic analyses suggest that the ancestor of AID/APOBEC proteins originated from TadA/ADAT2 enzymes at the beginning of vertebrate evolutionary radiation.[4,25] The earliest members of the AID/APOBEC family to evolve (AID, A2, and A4) have been found in jawed vertebrates.[111] For example, the lamprey AID ortholog PmCDA1 is believed to be engaged in the somatic diversification of variable lymphocyte receptor repertoires.[115,116] *APOBEC1*-like genes have been found in the anole lizard and zebra finch genomes and most likely arose as a duplication of the *AID* locus.[117] *APOBEC3* genes are restricted to mammals and evolved through a complex history of gene duplications and fusions.[118] The APOBEC3 subfamily most likely arose from two ancestral domains (called Z1 and Z2) that constitute either double-domain or single-domain APOBEC3s (see the next section).[119,120] Therefore, the *APOBEC3* gene family varies widely among species. The human genome contains seven *A3* genes. Mice have only one *A3* gene, whereas in pteroid bats, 18 putative *A3* coding regions have been identified.[121,122] It is believed that the rapid evolution of *A3* genes in mammals is driven by strong selection pressure exerted by retroviruses and retroelements.[118] ADARs are present in the earliest branching metazoan lineages, such as those of the sponges and ctenophores. Therefore, it is postulated that the ADAR family evolved from ADAT2 ancestors after the split of *Protozoa* and *Metazoa*.[123] The *ADAR2*-like gene emerged first when a region encoding the dsRNA-binding domain (dsRBD) was incorporated into the duplicated *ADAT* gene via domain shuffling.[124] The *ADAR1*-like gene appeared later since it required the incorporation of an additional domain(s) (one or two Z-DNA-binding domains; see the following section). *ADAR3* appeared much later, after the Urochordata–Vertebrata divergence.[123] The role of ancestral ADARs is currently unclear. The structure of the family has undergone several changes, such as gene loss and duplications, which have been reported for certain animal lineages; however, expansion and diversification have not occurred in the evolution of the *ADAR* gene family, in contrast to the *AID/APOBEC* gene family.[123–125]

In recent years, the potential applications of both AID/APOBECs and ADARs have been recognized. Their ability to modify genetic information at the DNA and RNA levels has opened up the possibility of correcting disease-causing point mutations, creating desired genetic variants, and modulating gene expression.[126,127] Among deaminases, those in humans are best characterized and are therefore the deaminases that are most often engineered and used as DNA/RNA editing tools. However, the activity of their homologs from different species is beginning to be recognized, which may expand the potential applications of deaminases in the future.

Based on the latest advances in the research of zinc-dependent deaminases, in this review, we describe the structural features affecting the substrate specificity and catalytic activity of these enzymes. Next, we discuss the approaches taken thus far in deaminase engineering that ultimately led to the development of DNA/RNA editing technologies. Finally, we present the current challenges of nucleic acid editing systems and new perspectives for their implementation and improvement.

MODULAR STRUCTURE OF DEAMINASES—AN OPPORTUNITY FOR ENGINEERING

While zinc-dependent deaminases are diverse in substrate selection and functions, they share similar core structural features

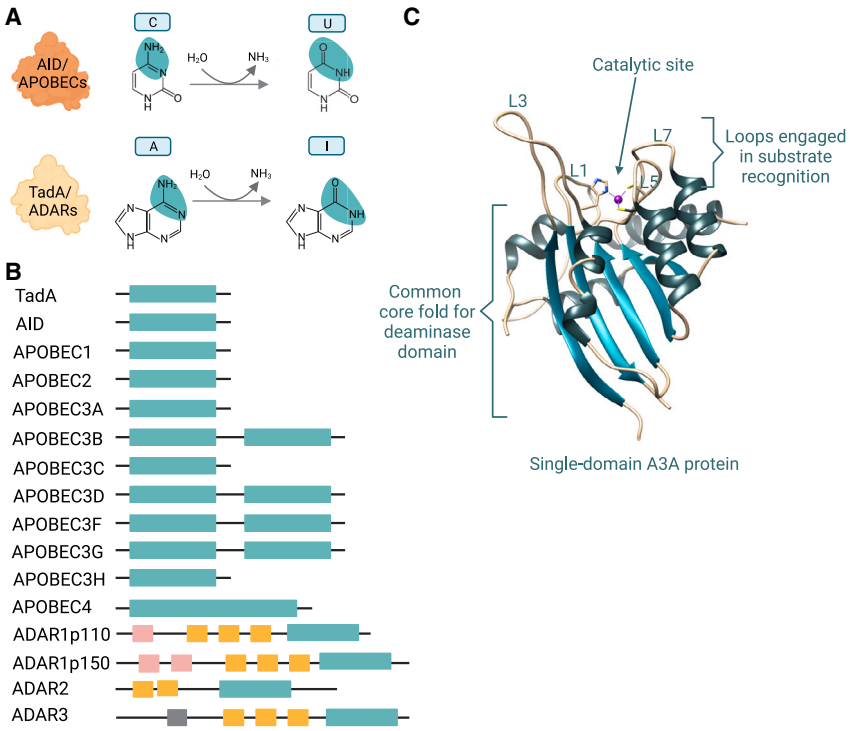

Figure 1. Specific characteristics of the deaminase family

(A) The reaction of deamination of cytidine to uridine and adenine to inosine catalyzed by AID/APOBECs and TadA/ADARs, respectively. (B) The specific composition of domains in AID/APOBECs and ADARs (green rectangle: deaminase domain; pink square: Z-RNA-binding domain; orange square: dsRNA-binding domain; gray square: arginine-rich domain). (C) Characteristic structural features of deaminases on the example three-dimensional structure of A3A (PDB ID: 5SWW). Created with BioRender.com.

(Figure 1).[2,3,113] The superfamily is characterized by the canonical structural motif $HxEx_{25-30}PCx_{2-4}C$, in which conserved Cys and His residues and a water molecule coordinate a zinc ion. The motif is located within one or two deaminase domains (DDs), whose typical fold comprises five β strands that form the backbone of the enzyme, which is surrounded by six α helices.[2,128–131] The mechanism of enzymatic deamination (Figure 1A) is conserved within the superfamily and presumes a nucleophilic attack at position C4 of the cytidine ring (or at position C6 of the adenine ring) by the activated water molecule coordinated by the zinc ion and the conserved glutamate (which acts as a proton donor).[20,132–134] The mutation of glutamate or any of the zinc-coordinating residues results in a loss of enzymatic activity.[135,136] AID/APOBEC structures consist of one (AID, A1, A2, A3A, A3C, A3H, A4) or two (A3B, A3D, A3F, and A3G) independently folded DDs, whereas Tad/ADATs and ADARs possess only one DD (Figure 1B). Interestingly, in the dual-deaminase domain AID/APOBECs, only the C-terminal domain is catalytically active. The N-terminal domain does not exhibit enzymatic activity, although it retains the typical fold.[128,130,137,138]

Despite the common core fold, deaminases exhibit functional specialization. In the AID/APOBEC structure, the conserved catalytic pocket is surrounded by loops L1, L3, L5, and L7, which are the least conserved regions (Figure 1C). The variability of their lengths, amino acid composition, plasticity, and dynamics are believed to be critical for substrate sequence specificity.[61,128] Additionally, subtle sequence differences in the structural core affect the surface charge, catalytic rate, and oligomeric propensities. The latter strongly influences the enzymatic activity of AID/APOBECs and can be driven by both protein-protein and protein-nucleic acid interactions.[139–143] Importantly, nucleic acids (both RNA and DNA) are often bound outside the catalytic center of AID/APOBECs, and a regulatory role of these interactions has been proposed.[46,140,144–146] Structural features that could be responsible for AID/APOBEC enzyme selectivity toward RNA or DNA substrates have not been identified thus far. The recognition of different nucleic acids and sequence contexts by AID/APOBECs seems to be a multifactorial phenomenon driven by structural determinants of both the enzyme and the substrate.[54,61,128]

ADAR structures consist of multiple independently folded and functionally distinct domains (Figure 1B).[130] The single deaminase domain is located on the C terminus and shares a common core fold with other zinc-dependent deaminases.[147,148] RNA binding is mediated by dsRNA-binding domains present in two (ADAR2/3) or three (ADAR1) copies. In addition, ADAR1 possesses one or two N-terminal Z-DNA-binding domains (Zα and Zβ in the interferon-inducible isoform or only Zβ in the constitutively expressed isoform).[103,149] Zα binds left-handed Z-RNA and Z-DNA with high affinity and directs ADAR1 to Z-forming sequences within dsRNA substrates and actively transcribed genes. Zβ does not interact with Z-DNA/Z-RNA, and its function is still debated.[150,151] The presence of dsRNA-binding domains in ADAR structures restricts the activity of these enzymes to double-stranded RNAs of sufficient length. Therefore, for deamination to occur, the reactive base must be flipped out from the helix into the catalytic center, which significantly differentiates ADARs from AID/APOBECs.[147]

TadAs from prokaryotic organisms are relatively small proteins that display the α/β/α three-layered fold typical of deaminases.[27,112,152] The eukaryotic enzymes ADAT2/ADAT3 are composed of catalytically active (ADAT2) and inactive (ADAT3) subunits.[153] The N-terminal fragment of ADAT3 functions as a tRNA-binding domain, and the C-terminal fragment forms an inactive pseudocatalytic domain. It has been proposed that the deamination activity of ADAT3 is abolished by a dual mechanism, in which the potential catalytic pocket

is blocked and the key glutamate (involved in proton transfer during catalysis) is replaced with a catalytically inactive residue.[28]

Recent reports indicate that relatively minor structural changes can result in significant modifications of deaminase enzymatic activity.[154–157] The plasticity of deaminases can be utilized in their engineering to modify their functions or to obtain enzymes not known to exist in nature. With the catalytic core remaining unchanged, the part responsible for substrate specificity can be modified by, for example, grafting it from another deaminase[158,159] or redesigning it using rational or random mutagenesis.[154,160] It is also possible to exchange the subcellular localization signals and modify residues responsible for oligomeric propensities or the surface charge.[161,162] A single-domain deaminase can also be changed into a double-domain enzyme and vice versa.[163,164] In the following sections, we present the approaches taken thus far to obtain enzymes with altered characteristics. Specifically, we discuss four strategies of deaminase engineering: (1) single amino acid substitutions; (2) region shuffling and extensive alterations in the deaminase architecture; (3) the construction of fusion systems for precise base editing; and (4) split technology application (see Figure 2). Notably, each of these strategies can be used in combination with the others.

DEAMINASE ENGINEERING BY SINGLE AMINO ACID SUBSTITUTIONS

Single amino acid substitutions are powerful tools for studying protein function because one or several amino acid changes are often sufficient to significantly modulate enzymatic activity, binding capacities, or intermolecular interactions while maintaining the overall protein fold (see Figure 3). This approach has also been successfully applied in deaminase engineering, either as random or structure-guided mutagenesis. Five main types of functional effects of such substitutions on deaminase function can be distinguished: (1) improved or modulated canonical activity, (2) alterations of the recognized sequence context, (3) alterations of the recognized nucleic acid type, (4) modulation of deaminase selectivity for modified or nonmodified nucleobases, and (5) changes in intermolecular interactions of deaminases. We discuss these effects in the following subsections.

Improved or modulated canonical activity

The first type of effect caused by single amino acid substitutions is a change (increase or decrease) in the deaminase canonical activity. As mentioned in the previous section, despite very similar structures, each AID/APOBEC protein has unique deaminase activity. In a family-wide comparative analysis, A3A, A3H, and A3C exhibited higher *in vitro* deaminase activity than other members of the family.[55] Consequently, it can be concluded that the catalytic center of these A3 proteins is more effective than that of others in the family, and it might be possible to utilize this knowledge (in combination with structural data) to modulate the activity of the other members. The correctness of this supposition is evidenced by the studies of Wang et al., who, by applying random mutagenesis (error-prone PCR) coupled with a selection process, identified a panel of AID variants (so-called upmutants) that exhibit higher activity than the wild type (WT) *in vitro*.[154] Notably, many of the upmutations brought the sequence of AID closer to that of A3s, and many of the mutations included residues likely implicated in protein:substrate interactions (regions conserved within the family). From the identified panel, the triple AID mutant K10E/E156G/T82I was further tested in CSR *in vivo*. Despite being expressed at a lower level, the triple mutant was 20% more effective than the WT analog at promoting CSR. This result suggested a possible application of this mutant in the development of an efficient technology for the production of monoclonal antibodies in transgenic mouse lines. However, because upmutations of AID also led to an increased frequency of chromosomal translocations, the risk of genomic instability emerged as an important factor limiting the practical use of the engineered enzyme.[154]

Maintaining enzyme specificity while increasing catalytic efficiency is also a significant challenge faced by researchers investigating ADARs. For example, Kuttan and Bass applied the random mutagenesis approach to generate ADAR variants with improved activity.[155] They obtained 24 active mutants, among which E488Q displayed the highest level of RNA editing *in vitro*. Interestingly, the observed improvement in the catalytic rate was significantly different for cold spots and hot spots, which suggested a loss of enzyme specificity. Compared with the WT, E488Q showed a 60-fold and nearly a 3-fold increase in the catalytic rate for the 5′GAC (cold spot)- and 5′UAG (hot spot)-containing hairpins, respectively.[155] Most recently, Katrekar et al., by applying a quantitative deep mutational scan (a technique that enables a simultaneous assessment of activities of thousands of variants), identified a novel double mutant E488Q/N496F in the ADAR2 deaminase domain (ADAR2-DD) that, compared with E488Q alone, exhibited 3-fold enhanced activity at a 5′GAC motif and 1.1- to 2.1-fold enhanced activity at various 5′GAN motifs.[165] The E488 residue is located in a highly conserved region, likely involved in the base flipping step of ADAR2-mediated deamination, and corresponds to the E1008 residue in ADAR1. Unsurprisingly, Wang et al. observed a similar effect when Q was substituted for E1008 in the ADAR1 deaminase domain (ADAR1-DD). An *in vitro* deamination assay showed an 8-fold increase in the deamination rate compared with the rate in the WT.[166] In contrast to the effect in the hyperactive ADAR2 E488Q variant, the substitution of glutamate for tyrosine (E488Y), phenylalanine (E488F), or another large, hydrophobic amino acid led to ADAR2 inactivation. This inactivation was most likely caused by a steric clash between the side chains of these amino acids and the orphan base (the nucleobase that pairs with the adenine edited by the WT enzyme in dsRNA substrate).[155] The inactive ADAR2 E488Y variant has been successfully applied by Monteleone et al. to reduce the off-target activity of the enzyme and to develop a directed RNA editing approach (which the authors called the bump-hole approach).[167] The authors discovered that a replacement of the orphan base with a hydrogen atom (application of guide RNA with an abasic site opposite the targeted A) eliminates the aforementioned steric clash and restores the editing activity of the E488Y variant. Consequently, they used abasic site-containing guide RNA for directed editing by the E488Y variant

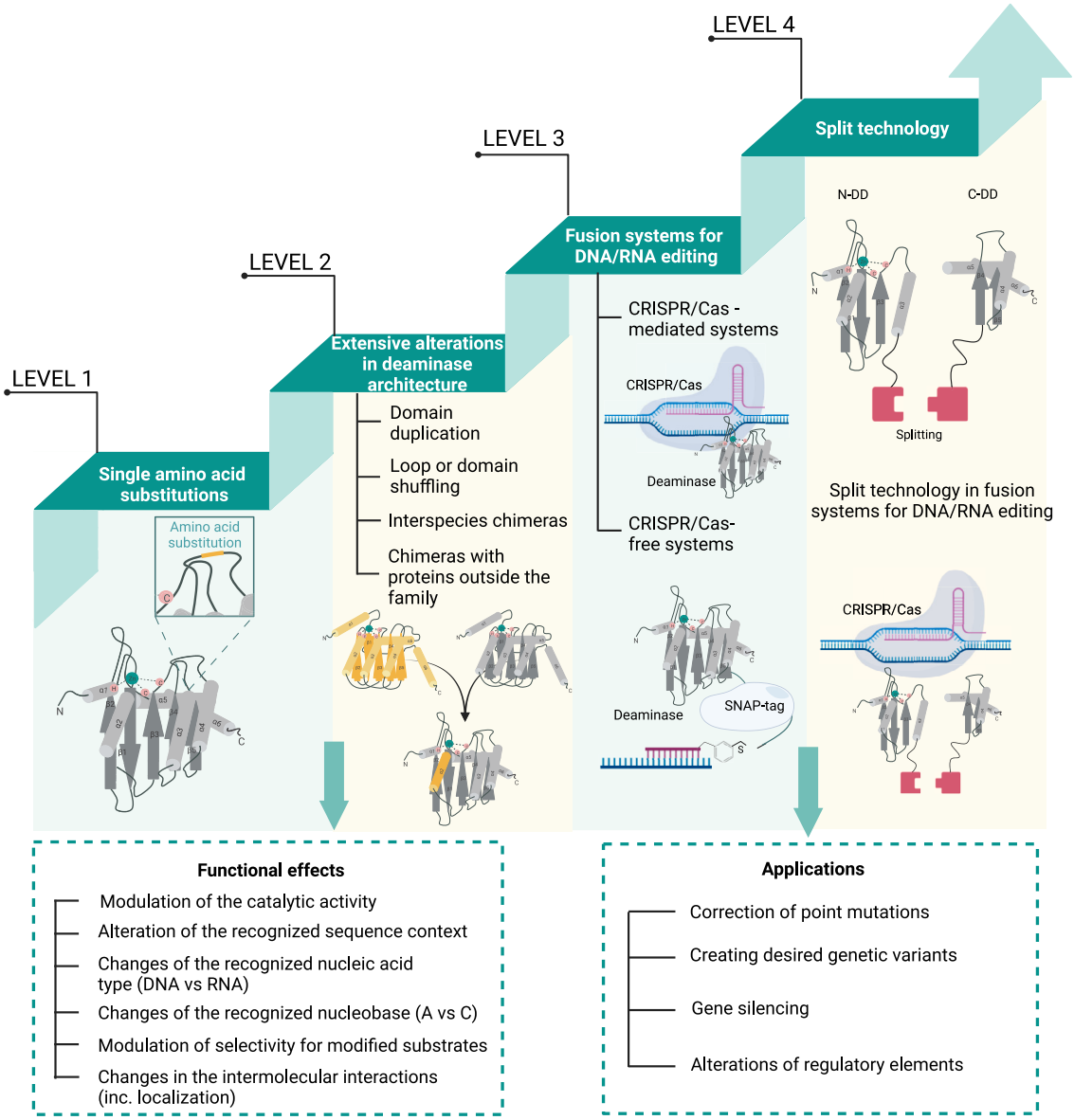

Figure 2. Approaches taken thus far to modify the structures and functions of deaminases to obtain enzymes with new or improved capabilities

Deaminase engineering can be considered at four levels of increasing complexity: (1) single amino acid substitutions; (2) region shuffling and extensive alterations in deaminase architecture; (3) fusion systems for base editing; and (4) split technology. Engineering levels 1 and 2 modify deaminase activity and functionality. Engineering on level 3 enables specific cellular applications. Split technology, on level 4, enables higher control of editing and reduction of off-target effects. Created with BioRender.com.

and obtained at least a 5-fold higher editing activity of the substrate *in vitro* compared with the WT activity in editing the A-C mismatch located within an optimal sequence motif for ADARs (5′UAG). In HEK293T cells, compared with the WT enzyme, the E488Y mutant displayed a significantly reduced off-target effect; however, its on-target activity was equal, slightly higher, or lower depending on the transcript.[167] Nevertheless, the authors demonstrated that even one or several amino acid substitutions may affect the deamination efficiency of the enzyme and could expand the spectrum of the practical applications of deamination.

Alterations of the recognized sequence context

The second type of effect caused by single amino acid substitutions in deaminases is alterations in the recognized sequence context. The simplest example is a change in specificity, as with the already mentioned ADAR2 E488Q variant, which gained the ability to efficiently edit the 5′GAC site disfavored for the WT, with a simultaneous increase in the catalytic rate at 5′UAG—the WT hot spot motif.[155] Another example is the AID S38A mutant, described by Shivarov et al., which exhibited lower activity on hot spot motifs (5′WRC; W = A/T, R = A/G), accompanied by relatively high

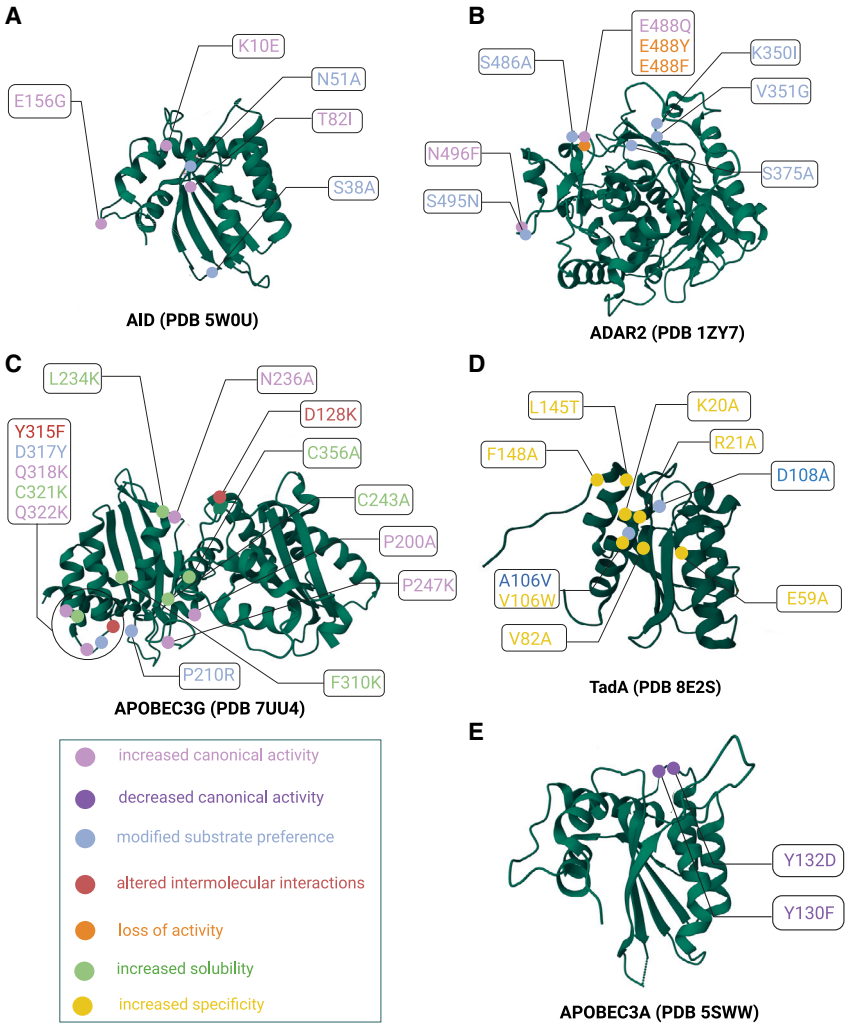

Figure 3. Three-dimensional structures of selected deaminases with the indicated main mutations that influence the enzymes' activity

The figure presents crystal structures of (A) AID (PDB:5W0U), (B) ADAR2 (PDB:1ZY7), (C) APOBEC3G (PDB:7UU4), (D) TadA (PDB:8E2S), and (E) APOBEC3A (PDB:5SWW). Different colors indicate the functional effects caused by specific mutations: pink and purple: increased or decreased canonical activity, respectively; blue: modified substrate preference; red: altered intermolecular interactions; orange: loss of activity; green: increased solubility; and yellow: increased specificity.

deamination activity on two cold spot motifs: 5′GGC and 5′CGC.[156] In the case of A3 proteins, a series of single amino acid substitutions in A3G loop 7 led to the identification of the D317Y variant with altered preference from 5′CC to 5′TC in ssDNA. This variant adopted a DNA-binding conformation similar to those of other A3 enzymes (A3A, A3B, A3C, A3G, A3F), which also have aromatic residues at corresponding positions and prefer 5′TC-containing substrates. Molecular dynamics simulations suggested a model in which the D317 residue acted as an α-helix cap by forming a hydrogen bond with the R320 backbone. This interaction restricted the mobility of loop 7 and contributed to the creation of a site capable of accommodating C (at the −1 position) but not T or larger purine nucleobases. In contrast, this helical cap was unable to form in the A3G D317Y variant. Instead, for this variant, local refolding events in loops 1 and 7 reshaped the −1 nucleobase binding pocket.[160] To determine nucleotide preferences at position +1, another A3G variant was designed by Ziegler et al. The P210R mutation in loop 1 of A3G decreased the affinity for 5′**C**A while increasing the affinity for both 5′**C**T and 5′**C**G substrates.[168] These two results provided further supporting evidence that in the AID/APOBEC architecture, loops 1 and 7 drive the selection of substrates based upon neighboring nucleotide sequences.[128]

Alterations in the recognized nucleic acid type

In addition to the alterations of the recognized trinucleotide contexts, more complex changes in enzyme selectivity are also possible. Single amino acid substitutions in deaminases can lead to changes in the recognized nucleic acid type or cause deaminases to accept both DNA and RNA. RNA-specific adenine deaminases appear to be particularly susceptible to the first of the two, since the engineered variants of both TadA and ADARs have been shown to efficiently deaminate adenine in ssDNA (see below). Importantly, an enzyme deaminating adenine in DNA is not known to exist in nature. Therefore, the engineering of DNA-specific adenine editors has been of high interest in recent years. In the case of ADARs, both the full-length protein and the aforementioned ADAR1-DD, bearing the E1008Q mutation, were successfully directed to edit specific adenosines in the ssDNA genome of the M13 bacteriophage. The specificity was conferred by 24-nucleotide guide RNAs, which formed six specific A-C mismatches with the genomic DNA, marking these sites for deamination within DNA/RNA hybrid duplexes. The same activity on DNA/RNA hybrid substrates was also observed in the case of full-length ADAR2 and ADAR2-DD bearing a corresponding E488Q mutation. The tested variants most efficiently deaminated canonical dsRNA substrates and were inactive on dsDNA substrates. Nevertheless, the authors pointed to potential applications of these variants in genome editing.[169] Moreover, through evolutionary processes, Gaudelli et al. developed *E. coli* TadA, which accepts DNA as a substrate.[157] Using a method based on directed evolution combined with a bacterial selection assay, the authors obtained, in the first round of evolution, variants enriched in A106V and D108N mutations. Transfection of plasmids expressing a construct bearing a TadA A106V_D108A double mutant in combination with Cas9 and single guide RNAs that

target six human genomic sites gave very low but observable adenine editing yields (approximately 3.2% efficiency). This result confirmed the ability of the designed editor to convert adenine in the DNA, even after the first round of evolution, during which only two amino acids were converted. The authors further improved the variant and more extensively rearranged its architecture, thus developing a programmable adenine base editor (ABE), which will be further discussed in the following sections.[157]

As mentioned in the previous chapters, A1 was originally discovered because of its highly specific RNA editing in apoB mRNA. A1 has also been shown to edit other RNA substrates, both in protein-coding regions and in 3′ untranslated regions.[170–172] Importantly, A1-mediated RNA editing is only observed when RNA-binding cofactors are present as part of the 27S editosome complex.[8] A1 is also capable of deaminating ssDNA, and this activity has been shown to restrict some viruses and retroelements.[173–177] A1 deamination activity has also been associated with somatic mutations in chromosomal DNA and therefore linked to cancer.[178–180] Notably, A1-mediated DNA deamination does not require additional cofactors, suggesting that A1 may have originally evolved to act primarily on ssDNA.[117] A1 has been shown to accept both RNA and DNA substrates *in vitro*.[181] Interestingly, as demonstrated by Shivarov et al., it is possible to partially decouple the enzyme activities on DNA and RNA by means of a single point mutation. The A1 N57A variant almost completely lost the ability to deaminate ssDNA, even with a 7.5-fold excess of the enzyme, but it retained approximately 20% of the efficiency of the WT RNA editing activity *in vitro* (on the specific C in apoB mRNA).[156]

Modulation of deaminase selectivity for modified or nonmodified nucleobases

Single amino acid substitutions in deaminases can also modulate their selectivity for modified substrates or even individual nucleobases. The deamination activity on methylated substrates *in vitro* is common among AID/APOBEC proteins. The already mentioned family-wide comparative analysis allowed to identify AID/APOBEC enzymes with the highest activity on 5mC, which were A3A, A3H, and A3B, and those with the highest 5mC selectivity factor, which were A3H, A3A, and AID.[55] Interestingly, an A3B variant with dramatically increased 5mC deamination activity was obtained by introducing several mutations and deletions in the catalytic domain that were designed to copy the architecture of A3A. Compared with the WT, the resulting variant displayed a 9-fold increased selectivity factor for 5mC, thus becoming nearly as selective as A3A and A3H. Moreover, the designed deaminase gained an activity level over two orders of magnitude higher for 5mC deamination.[182]

These results clearly show that it is possible to modulate both the activity and the selectivity of AID/APOBEC proteins on the modified substrates, expanding the range of potential applications of the enzymes. Interestingly, we showed that the N51A mutation of AID abolished the enzyme's ability to deaminate C while maintaining its activity on the 5mC substrate.[74] Based on molecular dynamics simulations, we proposed a model in which this mutation eliminates interactions essential for the deamination of C (interactions of the N51 residue with the sugar-phosphate backbone of the targeted C and the −1 nucleobase) and retains or creates interactions that enable the deamination of 5mC (between the methyl group, residues T27 and W84, and the sugar moiety of the +1 nucleobase). Thus, the network of interactions formed by the methyl group is sufficient for the proper positioning of the 5mC-containing substrates even in the absence of interactions necessary for C deamination. Importantly, in our *in vitro* studies, neither WT AID nor its N51A variant exhibited deamination activity on 5hmC, which implies that the variant can distinguish between 5mC and C/5hmC.[74]

On rare occasions, a change in the recognized nucleobase is also possible. Abudayyeh et al. mutated ADAR2-DD to switch its activity in dsRNA from canonical A-to-I to C-to-U editing. The variant obtained was characterized by 16 mutations (distributed throughout the ADAR2 structure) that enable the fitting of either A or C to the catalytic pocket. Mutations in the catalytic core (V351G, K350I) and in the region contacting the dsRNA substrate (S486A, S495N) were crucial for the activity, while others had only minor effects. Using random mutagenesis, the authors induced this enzyme to further evolve to create a more specific variant that performed fewer A-to-I off-target edits (with the most important S375A substitution) and showed its use in the CRISPR-Cas13-based system (described below). Importantly, this study demonstrates that the catalytic center of adenosine deaminases can be relaxed to accept other bases.[183]

Changes in the intermolecular interactions of deaminases

The last type of effect caused by single amino acid substitutions in deaminases is changes in their interactome. In cells, all biomolecules are involved in complex interaction networks. Some of these interactions underlie physiological processes, while others are related to diseases.[184] Modulation of the latter interactions appears to be a promising potential therapeutic strategy. For example, A3G is well known for its antiviral activity against human immunodeficiency virus type 1 (HIV-1).[185] The N-terminal domain (NTD) of A3G is catalytically inactive, but it binds viral and cellular RNAs (Y RNA, 7SL RNA), forming ribonucleoprotein complexes that can be incorporated into HIV-1 virions during encapsidation. If viruses that carry A3G infect other cells, A3G exerts several antiviral activities, e.g., deaminating viral cDNA generated by HIV reverse transcriptase and disturbing the formation of viral particles. On the other hand, the A3G NTD is recognized by HIV-1 viral infectivity factor (Vif), which can effectively antagonize A3G. A3G-Vif binding inhibits the encapsulation of A3G into virions and promotes A3G degradation through the ubiquitin-proteasomal pathway.[186] Interestingly, it has been shown that the D128K substitution (reflecting polymorphism between A3Gs from humans and A3Gs from Old World monkeys) protects human A3G from Vif-mediated degradation while maintaining the RNA-binding capacity essential for A3G encapsulation.[187] It was suggested that the D128K substitution is located within the ^{128}DPD^{130} Vif binding motif and does not disturb the A3G-Vif interaction but changes the conformation of the complex, thereby suppressing the A3G

degradation pathway.[185,187] Consequently, the single amino acid substitution emerged as a useful tool in elucidating the mechanism of Vif-mediated degradation of A3G. Very recently, this mutant was used in cryo-electron microscopy-based studies of the complexes formed by A3G, HIV-1 Vif, and multiple components of E3 ubiquitin ligase.[188] These analyses revealed unexpected RNA-mediated interactions of Vif with A3G and illuminated the mechanism of Vif-mediated A3G ubiquitination. It has been shown that RNA acts as a molecular glue to promote Vif and A3G interaction and that Vif preferentially targets the RNA-bound form of A3G. Therefore, RNA binding-deficient A3G mutants (having one or more mutations in the -^{124}YYFW^{127}- aromatic patch within loop 7) are defective in virion packaging but also resistant to Vif-mediated degradation. A very small area of the direct interaction of A3G-Vif includes the previously reported ^{128}DPD^{130} Vif binding motif; therefore, mutations of residue D128 retain the RNA-binding capacity of A3G but disturb Vif-mediated degradation.[188] These observations were further confirmed by the newest results of cryo-electron microscopy analysis of the complexes composed of A3G, HIV-1 Vif, and the hijacked cellular proteins that promote ubiquitin-mediated degradation.[189] Notably, both studies open new avenues for the development of therapeutics against HIV-1. For example, as previously suggested,[185] the region A3G-Vif interaction can be targeted by small molecules disturbing the protein-protein interaction. These findings also provide a valuable foundation for the further engineering of deaminases in an antiviral context.

REGION SHUFFLING AND EXTENSIVE ALTERATIONS IN THE DEAMINASE ARCHITECTURE

As concluded in the previous section, one or several amino acid changes are often sufficient to significantly modulate the enzymatic activity, substrate preference, or intermolecular interactions of deaminases. Hence, the question arises whether more prominent changes can be achieved by extensive alterations of the overall protein architecture. The following sections describe examples of the large structural changes performed to engineer new properties of deaminases. We distinguish four main types of extensive alterations in deaminase structure: (1) domain duplication; (2) domain, loop, or region shuffling between deaminases; (3) interspecies chimeras within the deaminase family; and (4) chimeras with proteins outside the deaminase family. All of them are described in the following subsections.

Domain duplication

Domain duplication is one of the most important mechanisms driving protein evolution. In the AID/APOBEC family, duplication and fusion events led to the differentiation of the *A3* locus in primates. In humans, we can distinguish *A3* genes that contain a single deaminase domain (*A3A, A3C*, and *A3H* genes) or double deaminase domains (*A3B, A3D, A3F*, and *A3G* genes). In the case of double-domain AID/APOBECs, only one of the two domains is catalytically active. However, as evidenced by the A3G case, the inactive domain is highly important for protein function, mediating essential intermolecular interactions (see previous section). The evolutionary history of deaminases may thus inspire the design of new proteins through domain duplication. In an effort to stay ahead of evolution, McDonnell et al. duplicated a single-domain A3C protein that naturally and weakly inhibits HIV-1 replication, creating a synthetic tandem domain A3C-A3C enzyme, which turned out to be "a super restriction factor" against HIV-1.[163] The increase in antiviral activity was validated by increased encapsulation of the engineered protein into virions and inhibition of reverse transcription. Importantly, the observed antiviral activity was independent of deamination. Disabling both catalytic centers in the A3C-A3C enzyme resulted in antiviral activity indistinguishable from that observed for the WT enzyme.[163] Further specialization of the duplicated synthetic domain in A3C-A3C seems to be an interesting approach for future engineering.

Domain, loop, or region shuffling between deaminases

The second type of extensive alteration in deaminase architecture is domain, loop, or region shuffling between deaminases. In addition to the full-length domains, we can distinguish smaller corresponding regions in the architecture of AID/APOBECs. The similarity between corresponding fragments of AID/APOBEC proteins and the unique specialization of the particular enzymes allows the use of the region shuffling strategy to generate chimeras with altered enzymatic properties. In this strategy, the unique properties of the donor are transferred to the recipient protein by grafting of a particular region. The domain shuffling strategy for AID/APOBEC engineering was implemented for the first time by Langlois et al. in 2005.[58] The authors investigated the substrate preferences of various chimeric variants of A3 deaminases involved in virus restriction (i.e., A3C, A3F, and A3G). To alter the target specificity of A3F, the authors replaced the whole C-terminal domain of A3F with that of A3G or with A3C. All resultant double-domain chimeras gained the target specificity associated with the inserted C-terminal domains. Therefore, the authors concluded that the C-terminal domain of A3G and A3F is mainly responsible for deamination specificity. Interestingly, chimeric variants of the A3F C-terminal domain exhibited novel substrate specificity. In these variants, the N or C terminus of the A3F C-terminal domain was replaced with the corresponding part of A3C. Surprisingly, the novel substrate specificity was closer to that of A3G than that of either parent protein.[58]

In AID/APOBECs, loops 1, 3, 5, and 7 drive the selection of substrates based on neighboring nucleotide sequences. Consequently, transferring smaller regions, such as loops, between deaminases is also an interesting approach for generating chimeras that show altered substrate preferences. This approach has been successfully applied by Kohli et al., who, by grafting A3G (Ile^{314}-Gln^{322}) and A3F (Leu^{306}-Gln^{315}) loops to the AID scaffold, obtained a change in the substrate selectivity to mimic the one displayed by the donor.[158] Thus, the AID sequence preference, which was 5′ATC, was replaced by 5′CCC and 5′CGC, which are characteristic of A3G and A3F, respectively. Moreover, grafting of the loops responsible for the hot spot recognition between APOBEC3 members, for example, from A3G to the A3A scaffold, resulted in an expansion of the target sequence repertoire. The A3A-A3G chimera was able to deaminate C when it was preceded

not only by the canonical T but also by A, C, and G (at the −1 position).[159]

The region shuffling strategy has also been employed to alter the subcellular localization of deaminases. Within the AID/APOBEC family, some enzymes (e.g., A3G) show predominantly cytoplasmic localization, while others (e.g., A3B) are mainly nuclear, depending on their biological functions (see Table 1). Stenglein et al. replaced the first 60 amino acids of A3G with the corresponding fragment of A3B, which resulted in a change in A3G protein localization from the cytoplasm to the nucleus. Analogously, a reverse chimera, created by the replacement of the first 60 amino acids of A3B with the corresponding portion of A3G, resulted in the adoption of the A3G localization pattern (mainly cytoplasmic localization).[161] Although this study aimed to identify the determinants of subcellular localization, it has opened up new perspectives for deaminase engineering by directing these enzymes to different compartments and thus to new targets.

Interspecies chimeras

Similarities between corresponding domains of AID/APOBEC homologs from different species enable the construction of active interspecies chimeras. It was shown that A1 enzymes from humans and rabbits have 75% amino acid sequence identity. However, they show markedly different abilities to restrict HIV-1 infection. Rabbit A1 (rA1) efficiently inhibits HIV-1 through deamination-dependent and deamination-independent mechanisms, whereas human A1 (hA1) has very weak antiviral activity. To improve the latter, Ikeda et al. generated a series of chimeras combining human and rabbit A1 and tested their activity against HIV-1 in a mutation assay of viral cDNA and RNA in 293T cells.[162] Chimeras with the highest capacity to restrict HIV-1 contained a large C-terminal region of rA1: two dimerization domains, a leucine-rich motif, and a nuclear export signal. This region has been shown to confer efficient encapsulation of rA1 into HIV-1 virions and to cause cytoplasmic localization of the modified enzyme (hA1 predominantly localizes to the nucleus).[162] These data also confirm the previous conclusions that biological functions of AID/APOBECs can be modulated by altering the localization pattern or oligomerization status. Different functional properties of AID/APOBEC homologs from relatively closely related species seem to reflect the structural plasticity of these enzymes. Therefore, one can conclude that a deeper functional characterization of AID/APOBECs in different species may be highly useful in the future engineering of deaminases.

Chimeras with proteins outside the deaminase family

The most promising approach, however, seems to be the fourth type of extensive alteration in deaminase structure—constructing chimeras with proteins outside the deaminase family. One such example, designed to acquire completely new features, is the chimera formed by the fusion of a single-domain A3A protein with viral protein R (Vpr) of HIV-1 (a small protein incorporated into the viral core). Aguiar et al. hypothesized that native A3A does not restrict HIV-1 because it is not targeted to viral particles. To overcome this problem, they fused Vpr (as a guide to target the chimera into HIV-1 particles) and A3A, which is not sensitive to Vif-mediated degradation. The engineered Vpr-A3A protein, in contrast to WT A3A, was efficiently incorporated into the viral core and was able to block HIV-1 replication to the background level in the presence or absence of Vif. In conclusion, the authors successfully engineered a novel HIV-1 restriction factor using the viral protein as a guide to target deaminase.[190] Another group of proteins that can be used to guide deaminases to their targets are those containing DNA-binding or RNA-binding domains. The resulting fusion proteins are already being used to repair genomic mutations. For example, to obtain an efficient genome editor, Yang et al. tested several combinations of deaminase, DNA-binding module and linker. The highest editing efficiency was achieved by the variant consisting of AID and the zinc finger motif, linked by a stretch of eight amino acids (SGGGLGST) to prevent steric hindrance. The optimized variant displayed a 13% editing efficiency. The authors noted the need for further optimization of the system due to the fairly common off-target activity.[191] However, this was one of the first attempts to use deaminases for site-specific genome editing. The idea has become highly developed in recent years, leading to the rapid expansion of base editing technology, which is further discussed in the following section.

CONSTRUCTION OF FUSION SYSTEMS FOR PRECISE BASE EDITING

The mutational potential of deaminases and their engineered variants has recently been widely exploited in base editing systems developed for precise and programmable genome or transcriptome modification. Base editors are a group of tools that allow the introduction of point mutations at specific sites in either DNA or RNA. In general, these systems comprise two modules: (1) a modifying enzyme (the editor), e.g., deaminase, which introduces a point mutation, and (2) a targeting module that delivers the deaminase to the specific site in the genome. Although they raise ethical questions, these systems are particularly promising for the precise correction of disease-related single-nucleotide polymorphisms, which underlie 58% of all human pathogenic genetic variants.[192] Moreover, they enable the modulation of gene expression, for example, by generating a premature stop codon or altering a start codon.[193–195] Representative examples of base editing system applications are summarized in Table 2. The rate of development of these tools has increased rapidly in recent years, as evidenced by the number of reports on new base editors published each year, which has ultimately led to the construction of artificial enzymes with multipoint editing capacity of different bases. Among the editing systems containing deaminases, one can distinguish CRISPR-Cas-mediated base editors and CRISPR-Cas-free systems. In this section, we summarize the current knowledge about them. Importantly, several base editors have been developed through mutations of deaminases. However, the effects of the mutation on the deaminase itself (not fused to the targeting module) have rarely been investigated, and the results of the few studies on the subject have been described in previous chapters.

CRISPR-Cas-mediated DNA base editors

The first CRISPR-Cas-mediated base editor was proposed by Alexis Komor et al. in 2016 and has since revolutionized gene editing technology, becoming an alternative to the conventional CRISPR-Cas system.[204] The first base editor was able to convert cytosine to uracil (and was therefore also called cytosine base editor; CBE) in ssDNA bubbles generated within the Cas9 R-loop complex. The first generation of cytosine base editors (CBE1) was a fusion of APOBEC1 from *Rattus norvegicus* (rAPOBEC1) with a catalytically dead version of *Streptococcus pyogenes* Cas9 (dCas9) guided in an RNA-dependent, programmable way.[204] The use of a catalytically inactive dCas9 variant eliminated dsDNA backbone cleavage, which, in conventional CRISPR-Cas systems, often leads to random insertions, deletions, or even chromosomal translocations through the activation of endogenous repair pathways, such as non-homologous end-joining or homology-directed repair (HDR).[205] CBE1 was capable of correcting six of seven tested disease-relevant mutations *in vitro,* showing ∼44% editing efficiency (on average for the six regions); however, in a cell line, this efficiency was 5- to 36-fold lower. The authors hypothesized that uracil DNA glycosylase (UDG), as a part of the cellular DNA repair response, removes U from DNA and therefore is responsible for the observed decrease in editing efficiency in cells. To overcome this problem and increase the editing efficiency in cells, UGI was added to CBE1. This new complex, called the second-generation CBE (CBE2), elevated the level of cytosine conversion to approximately 20% in human cells. Next, further improvements were added to boost editing efficiency. In CBE3, dCas9 was replaced with Cas nickase (nCas9), which resulted in a 2- to 6-fold increase in editing efficiency in human cells compared with what had been achieved with CBE2. Generating single-strand breaks (nicks) in the nonedited DNA strand, containing G opposite the edited U, further triggers cellular DNA repair machinery to correct the nonedited strand and ultimately resolve the U:G mismatch into the desired U:A and T:A base pairs. More specifically, Komor et al. reasoned that nicking the DNA strand containing the unedited G would induce mismatch repair (MMR) or long-patch base excision repair. However, further studies on repair pathways induced by nCas9 pointed out the importance of HDR pathways in DNA nick repair with little accompanying mutagenic end-joining (mutEJ).[206–209] Importantly, both CBE2 and CBE3 caused very few insertions and deletions (<0.1% and $\leq$1%, respectively) in the primary experiments. A year after publishing data on the first three CBE generations, Komor et al. engineered fourth-generation base editors (CBE4) by optimizing linker length and adding a second UGI domain. These modifications further increased the efficiency of C:G to T:A editing by approximately 50%.[210] Further engineering carried out by Koblan et al. improved CBE4 by modifying NLSs, codon usage, and ancestral reconstruction of the deaminase sequence (introducing 36 or 45 amino acid substitutions), resulting in the BE4max and AncBE4max systems.[211]

Although APOBEC1-based CBEs have been the most popular systems, there have also been attempts to utilize other cytidine deaminases, e.g., AID and A3s, to obtain higher editing efficiency and expanded editing scope. In 2016, Nishida et al. developed a Target-AID system combining nCas9 and an AID ortholog, PmCDA1, from sea lamprey. In this system, PmCDA1 is fused to the C terminus of Cas9 instead of the N terminus as in APOBEC1-based CBEs, and the main editing window is within one to five instead of four to eight nucleotides. The proposed combination was highly efficient in yeast, whereas in mammalian cells, it induced deletions as well as point mutations. Therefore, the authors added UGI to the complex, by analogy to APOBEC1-based CBEs, which improved mutation frequency and reduced indel formation.[212] Since then, Target-AID has been further improved. Intensive truncation of the deaminase has reduced off-target activity, and a combination with the smaller Cas9 ortholog (SaCas9) has minimized the size of the system to the limit of an adeno-associated virus vector.[213] A3 deaminases have also been identified as candidates for developing CBEs. Lee and coworkers engineered A3G variants (including truncated NTD) when fused to the Cas9 nickase (referred to as A3G-BE).[164] The authors engineered A3G with several sets of mutations, starting with those improving catalytic activity (P200A + N236A + P247K + Q318K + Q322K), solubility (L234K + C243A + F310K + C321A + C356A), and ssDNA-binding affinity (partial replacement of loop 3 with A3A's loop 3: H248N + K249L + H250L + G251C + F252G + L253F + E254Y), and ending with some additional mutations in later variants (e.g., T311A + R320L—maximizing editing efficiency, or Y315F—modulating interaction with ssDNA backbone). The most potent variants (A3G-BE5.13 and A3G-BE5.14) were characterized by high editing efficiency and precision in the context of the 5′-CC motif. The authors demonstrated the applicability of these variants to efficiently correct mutated alleles associated with pathogenic phenotypes. The most active variant (A3G-BE5.13) has been proven to outperform the BE4-max tool and induce baseline levels of genome and transcriptome off-targeting.[164]

Conjugated A3A variants in CBEs have also been shown to efficiently edit C in multiple sequence contexts, including CpG sites in highly methylated regions.[214] In the latter case, the editing efficiency induced by hA3A-BE3 was significantly higher than that caused by BE3 on cytosines in the CpG context in either highly methylated or low-methylated regions. However, hA3A-BE3 was characterized by higher indel frequencies and a much wider editing window (∼12 nt) than BE3 (∼5 nt), presumably due to the high deaminase activity of A3A. However, the Y130F or Y132D mutation in the subsequent variants of this tool successfully minimized the above effects.[214] hA3A-BE3 has been tested for editing efficiency in various organisms, e.g., rabbits,[199] pigs,[215] mice,[200] and agricultural plants,[201] and an additional strategy for its further improvement has been proposed by Gehrke and coworkers.[216]

ABEs have been developed almost in parallel to CBE. In 2017, Nicole Gaudelli et al. developed the first CRISPR-Cas-mediated base editor able to convert adenosine to inosine in ssDNA.[157] As mentioned in the previous sections, the authors developed *E. coli* TadA to accept DNA as a substrate when fused to the dCas9 variant. Multistage directed evolution resulted in seven generations of ABEs (57 different genotypes). The final ABE7.10 showed the highest editing activity at

Table 2. Representative examples of base editing applications

Application	Base editing system/strategy	Deaminase	Example	Model organism/cell type	Reference
Correction of point mutations	A3G-BE	APOBEC3G	Correction of pathogenic mutation in *SPTA1* gene (c.620 T>C) associated with hereditary pyropoikilocytosis, and in *CFTR* gene (c.4004 T>C) associated with cystic fibrosis	HEK293T cell line	Lee et al.[164]
	ABE7.10	TadA variant which accepts DNA as a substrate	Correction of pathogenic mutation in *HFE* gene (c.845 G>A) associated with hereditary hemochromatosis	Lymphoblastoid cell line	Gaudelli et al.[157]
	ABEmax-VRQR	TadA variant which accepts DNA as a substrate	Correction of pathogenic mutation in *LMNA* gene (c.1824 C>T) associated with Hutchinson-Gilford progeria syndrome	Patient-derived fibroblasts and mouse model	Koblan et al.[196]
	i-stop (BE3)	APOBEC1	Silencing of *Tyr* gene to mimic albinism; silencing *Pdcd1* gene to study autoimmunity	Mouse	Jia et al.[197]
	CRISPR Start-Loss (BE4max, ABEmax)	APOBEC1 and TadA variant which accepts DNA as a substrate	Silencing defected genes by disruption of start codons (*Otc* gene associated with hyperammonemia, *Hbb2* gene associated with erythrocytosis)	Rabbit	Chen et al.[198]
Creating genetic variants	Variant of hA3A-BE3	APOBEC3A	Generation of a model to mimic human oculocutaneous albinism by introducing mutation in *Tyr* gene	Rabbit	Liu et al.[199]
	Variant of hA3A-BE3	APOBEC3A	Generation of a model of androgen insensitivity syndrome by introducing pathogenic mutations in GpC context	Mouse	Li et al.[200]
	A3A-PBE	APOBEC3A	Generation of genetic variant with nicosulfuron resistance by introducing mutation in acetolactate synthase gene	Wheat	Zong et al.[201]
	STEMEs	APOBEC3A and TadA variant which accepts DNA as a substrate	Multipoint editing of *OsACC* gene for herbicide resistance	Rice	Li et al.[202]
Alteration of regulatory elements	A3A-PBE	APOBEC3A	Disruption of transcription factor-binding sites in the promoter of *TaVRN1-A1*	Wheat	Zong et al.[201]
	A&C-BEmax	APOBEC1 and TadA variant which accepts DNA as a substrate	Generation of new binding sites for the transcription activator GATA1 by introducing mutations in *HBG1* and *HBG2* genes	HUDEP-2 cell line	Zhang et al.[203]

five genomic *loci* in HEK293T cells (above 50%) and significantly reduced off-target editing (to less than 0.1%). The most efficient variant was a heterodimer consisting of the WT TadA domain, and the TadA* variant evolved to accept ssDNA as a substrate. The ABE7.10 structure, therefore, follows the example of the native TadA homodimer, in which one monomer catalyzes deamination and the other monomer works as a docking system for tRNA. As a proof-of-concept, the authors used the system to correct two pathogenic mutations related to human diseases. For example, using ABE7.10, they reversed, with 28% efficiency, the G-to-A missense mutation at position 845 in the human *HFE* gene. This mutation is responsible for the iron storage disorder called hereditary hemochromatosis (HHC). In one of the latest reports, ABEmax-VRQR, which combines an optimized ABE7.10 variant[211] with an engineered Cas9-VRQR variant,[217] was used to reverse Hutchinson-Gilford progeria syndrome (HGPS) *in vivo*.[196,211] HGPS is associated with a single mutation (c.1824 C>T) in the *LMNA* gene that encodes nuclear lamin A. A mutation at this site causes RNA mis-splicing that produces progerin, a toxic protein responsible for accelerated aging. To reverse this mutation in the mouse model of progeria, ABEmax-VRQR was delivered using adeno-associated virus (AAV) vectors. The applied strategy resulted in approximately 20%–60% conversion of the pathogenic mutation, which lasted up to 6 months after injection.[196] Efforts have recently focused on enhancing ABE system performance. Point mutations were introduced into WT TadA/TadA* to create improved versions of ABEs with reduced off-target activity. These include ABEmax-F148A7 (TadA F148A and TadA* F148A mutations),[218] ABEmax-AW8 (TadA E59A and TadA* V106W mutations),[219] and SECURE-ABEs (TadA* K20A/R21A or V82G mutations).[220] Molecular evolution of the TadA* monomer led to the development of two new groups of ABE variants, ABE8e[221] and ABE8s,[222] characterized by improved editing efficiency (3- to 11-fold improvement compared with ABE7.10) but also significant off-targeting. The latter presumably results from increased deamination activity and expanded editing windows. The high editing efficiency of ABE8e and ABE8s has been demonstrated in mice, nonhuman primates,[223] and hematopoietic stem cells from sickle cell anemia patients,[224] highlighting the therapeutic potential of these enzymes. Very recently, the expanded editing window problem of ABE8e was overcome by the AB9 system (ABE8e with N108Q and

L145T mutations), which precisely catalyzed A-to-G conversions within a 1–2 nt editing window. According to the authors, ABE9 induced minimal RNA off-target effects and undetectable DNA off-target effects in mouse and rat embryos, leading to the efficient generation of disease models.[225]

The traditional CRISPR-Cas9 system has been widely used for gene-knockout studies. However, as mentioned above, this may cause uncontrolled DNA damage and cell death since it relies on the generation of DSBs. CRISPR-Cas-mediated DNA base editing systems (both CBE and ABE) are currently recognized as an attractive, less deleterious alternative to WT Cas9-mediated knockouts. Both CBE and ABE systems have been applied in technologies known as CRISPR-STOP, i-STOP, i-Silence, and CRISPR Start-Loss (CRISPR-SL). These technologies are capable of eliminating the expression of multiple genes simultaneously without DSBs by introducing premature stop codons or altering start codons.[193–195,198] For example, CRISPR-STOP and i-STOP utilize CBEs to target CGA (Arg), CAG (Gln), and CAA (Gln) codons and therefore to create in-frame stop codons TGA, TAG, and TAA, respectively. Similarly, the TGG (Trp) codon can be modified into TGA, TAG, and TAA stop codons by mutating C-to-T in the noncoding strand. Moreover, the i-Silence approach is mediated by the ABE system and was designed to introduce point mutations to the start codon and consequently convert the ATG codon to GTG or ACG (if the noncoding strand is mutated). The applicability of these approaches to the simultaneous inactivation of multiple genes has been demonstrated *in vivo* in mice[197] and pigs,[226] proving that these systems can be successfully used for gene silencing, identifying gene functions, and mimicking disease-associated nonsense mutations. Moreover, the simultaneous disruption of multiple genes allows the testing of compensatory mechanisms and multigene interactions revealed during gene knockouts. To elaborate, the CRISPR-STOP approach, utilizing the BE3 complex,[204] has proven to be feasible to introduce early stop codons in multiple endogenous *loci* in two different cell lines and appears to be safer than WT Cas9, as well as comparably efficient, particularly in targeting high copy number genomic regions.[193] Similar to the above approach, the i-STOP system (also utilizing the BE3 complex) was adapted to model and study human disease nonsense mutations on a genome-wide scale.[194] To facilitate the use of iSTOP, the authors provided an online database of single guide RNAs (sgRNAs) dedicated to iSTOP that is possible to use for eight eukaryotic species, showing that 94%–99% of analyzed genes can be targeted by this tool. Notably, the database includes sgRNAs for iSTOP that could be used to mimic over 32,000 nonsense mutations associated with human cancer.[194]

Importantly, CBE systems are generally characterized by a higher rate of off-target effects than ABEs.[227,228] Therefore, Wang et al. proposed the i-Silence strategy based on ABEmax-mediated start codon mutation. Efficient gene silencing (60%–80%) by this system was demonstrated for four endogenous genes (*HDAC1*, *SEC61B*, *PIGH*, and *FTL*) in HEK293T cells. After successful optimization in the cell line, i-Silence technology was used to silence the P1 gene in 10 mouse zygotes with efficiencies ranging from 31.8% to 73.6% in different embryos. An investigation of the human variation database (ClinVar) revealed 247 human diseases associated with start codon mutations, of which 147 can be modeled using i-Silence technology.[195] A good complement to the above strategies is CRISPR-SL technology utilizing both ABEs and CBEs to disrupt the start codon. In this approach, each of three bases in the ATG codon can be modified (into GTG, ACG, or ATA) by either rA1-BE4max or ABEmax (targeting the coding or noncoding strand). In addition, their cotransfection significantly increases the chances of efficient gene knockout. This strategy has proven to be feasible in cell lines and rabbit embryos (with editing efficiencies up to 30.67% and 73.50%, respectively) as well as in two rabbit models.[198] However, a specific limitation of the systems altering the start codon is the fact that other codons may initiate translation, though at very low efficiency.[229]

Recently, the repertoire of CRISPR-Cas-mediated base editing tools has been extended by those capable of simultaneous C-to-U and A-to-I deamination (ACBE, A&C-BEmax, SPACE, and STEMEs).[202,203,230,231] Programmable multipoint editing is particularly useful when a conversion of two different nucleobases within the same editing window is needed. This new class of base editors, known as dual base editors, has been generated by fusing cytidine and adenine deaminases to the N and C termini of nCas9. For example, the ACBE system is a fusion of the evolved TadA heterodimer (the previously engineered variant capable of DNA deamination) and AID to the N and C termini of nCas9, respectively.[230] Multipoint editing by ACBE was successfully verified in HEK293 cells as well as in primary somatic cells, including mouse embryonic fibroblasts and porcine fetal fibroblasts.[230] Other recently developed dual base editor systems (A&C-BEmax, SPACE, and STEMEs) differ slightly in their general architecture. For example, they use various deaminases, differ in the terminus of deaminases attachment to Cas9, or use constructs optimized for better efficiency (for example, by codon optimization or modulation of the linker length). As in the case of single-base editors, dual base editors have been shown to have several powerful practical applications. For example, the therapeutic potential of the A&C-BEmax system was validated in the β-hemoglobinopathy model. β-Hemoglobinopathies (including β-thalassemia and sickle cell disease) are caused by defects in β-globin production, which ultimately lead to abnormal structure of adult hemoglobin (composed of two α and two β subunits). A potential treatment strategy for β-hemoglobinopathies involves the reactivation of the production of fetal hemoglobin (composed of two γ and two α subunits) in an adult organism. To achieve this goal, A&C-BEmax was used to introduce two point mutations (114 C-to-T or −113 A-to-G) into the promoter of the γ-globin genes (*HBG1* and *HBG2*) in an erythroid progenitor cell line. The introduced mutations disrupted the binding site of the transcription inhibitor BCL11A and generated a new binding site for the transcription activator GATA1. Consequently, the introduced mutations reactivated the production of fetal hemoglobin (due to γ subunit production).[203,232] Moreover, Li et al. proposed an application of STEME editors (consisting of A3A, the previously described fused TadA heterodimer to the N terminus of nCAs9-UGI) to facilitate

the directed evolution of plant genes and therefore to improve their agronomic performance.[202] For example, STEME technology has been successfully used to introduce mutations (C-to-U and A-to-I) in the gene encoding acetyl-coenzyme A carboxylase (OsACC), which yielded herbicide-resistant genetic variants of rice.[202]

CRISPR-Cas-mediated RNA base editors

The discovery of the nuclease Cas13 acting on ssRNA has opened up a wide range of new possibilities for using deaminases for programmable RNA editing, which is an essential complement to DNA base editing for both research and therapeutic applications.[233] The effects of manipulations at the RNA level are as durable as RNA is stable; therefore, it can be considered safer than genome editing. This approach might also be particularly useful for lethal mutations at the DNA level. In recent years, extensive efforts have led to the development of many versions of A-to-I and C-to-U CRISPR-mediated RNA base editors, all exploiting ADAR or A3A deaminases. As the first to succeed, Cox et al. took advantage of the ability of the dCas13b ortholog from *Prevotella* sp. to target specific transcripts. As the editing module, they used ADAR2-DD with the E488Q mutation (hyperactive variant). The dCas13b-ADAR2-DD fusion, referred to as the REPAIR system, has been applied in RNA knockdown and correction of full-length transcripts containing pathogenic mutations by programmable A-to-I editing. REPAIR achieved substantial editing at 33 sites of 34 tested disease-related mutations with up to 28% editing efficiency. Since the system was characterized by a significant number of dCas13-independent off-target effects, the authors applied structure-guided engineering of ADAR2-DD to finally obtain a much higher specificity of REPAIRv2 utilizing the ADAR2-DD E488Q/T375G variant.[234] The same group induced further evolution of ADAR2-DD to enable it to accept C as a substrate and developed the first C-to-U RNA editing system (referred to as RESCUE). Rational mutagenesis of ADAR2-DD resulted in the C82R variant exhibiting 15% editing efficiency when fused to dCas13b on C in a luciferase reporter transcript. Further enhancement of C deamination was obtained after 16 rounds of directed evolution in a yeast system, resulting in a number of additional mutations (of which V351G, K350I, S486A, and S495N were indispensable to RESCUE activity). Notably, RESCUE retained its enzymatic activity on A and generated a significant number of both A-to-I and C-to-U off-target mutations. Therefore, its specificity was further increased by an additional S375A mutation, and the improved system has been referred to as RESCUE-S. The final RESCUE-S system was characterized by ~76% on-target C-to-U editing efficiency as well 103 C-to-U and 139 A-to-I off-target mutations in transcriptome-wide analysis.[183] More recently, the editing arsenal has been expanded by CURE—C-to-U RNA editase utilizing the A3A enzyme. In this case, both activity and specificity were improved (in CURE-X generation) by the use of the dCasRx variant.[235] dCasRx was also utilized in REPAIRx developed by the same group. However, in this case, the deaminase domain was inserted into the middle of CasRx, and the complex was directed to the nucleus.[236] Finally, minimal RNA base editors for A-to-I or C-to-U editing (termed xABE and xCBE) were developed by a fusion of deaminases with a significantly truncated dCas13X.1 variant.[237]

Recently, the repertoire of CRISPR-mediated RNA base editors has been expanded through the development of a novel Cas protein, referred to as Cas7-11, from *Desulfonema ishimotonii* (*Di*Cas7-11 is a fusion of Cas7 with Cas11).[238] One of the remarkable advantages of the Cas7-11 system is minimal cell toxicity compared with Cas13. Building upon these findings, Özcan et al. engineered a catalytically dead version of *Di*Cas7-11 (d*Di*Cas7-11) and proposed its application as a novel targeting module for ADAR-mediated RNA editing. The fusion system composed of d*Di*Cas7-11 and the hyperactive variant of ADAR2-DD (E488Q) has been proven to be applicable for effective RNA editing in HEK293T cells.[238]

CRISPR-Cas-free systems

Although CRISPR-Cas is currently the most widely used targeting module, alternative approaches to deliver deaminase to specific sites in the genome or transcriptome had been developed even before the CRISPR-Cas revolution. Therefore, we describe the CRISPR-Cas-free systems as the second group of editors in which deaminases are involved. Pioneers in this area, Stafforst and Schneider[239] and Rosenthal's group,[240] independently developed two ADAR-mediated A-to-I RNA editing strategies. The common concept for these two strategies was to remove the dsRNA-binding domains from ADAR and replace them with an antisense RNA oligonucleotide that served a dual purpose in the RNA editing process. First, it acted as a module guiding the ADAR catalytic domain to the target sequence. Second, it was directly involved in the formation of the dsRNA structure essential for ADAR-mediated editing. However, these two groups proposed different approaches for linking the antisense RNA oligonucleotide to the ADAR catalytic domain. Stafforst and Schneider used a fusion of hADAR1-DD with the C terminus of SNAP-tag protein (an engineered variant of O^6-alkylguanine-DNA-alkyltransferase). The SNAP-tag was covalently conjugated with 5′-O-benzylguanine-modified gRNA in a chemoselective reaction and thus guided the system to the target sequence. This system efficiently (at a rate of 60%–90%) repaired nonsense mutations (UAG) in a fluorescent reporter gene in an *E. coli* plate assay, with very little overediting.[239] On the other hand, Rosenthal's group constructed a targeting module using the λ-phage N protein that interacts with boxB hairpin RNA to regulate antitermination during the transcription of λ-phage mRNAs.[240] A peptide from the λ-phage N protein (mediating the binding of the N protein and RNA) was fused to the N terminus of hADAR2-DD, while boxB hairpin RNA was fused to the gRNA. These two parts were encoded separately, but when expressed in cells, λ-phage N peptide-boxB RNA interaction allowed restoration of the entire complex (λN–DD). This strategy has been proven to be applicable *in vitro* in correcting a premature termination codon in mRNA encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (W496X mutation responsible for a genetic disorder—cystic fibrosis). The system also yielded promising results in *Xenopus* oocytes.[240] Both SNAP-tag and λN–DD technologies have been systematically improved in terms of specificity and efficiency. For example, to enhance editing efficiency within the cellular environment, Montiel-González et al. added λ-phage peptides and boxB RNA hairpin to the λN–DD system, as well as the E488Q mutation in

hADAR2-DD.[241] While on-target editing efficiency was significantly improved by the abovementioned modifications, extensive off-target editing occurred, both near the target sequence and across the entire transcriptome of the transfected cells. To eliminate these effects, Vallecillo-Viejo et al. redirected the λN–DD system from the cytoplasm to the nucleus by adding a nuclear localization signal to the construct. The redirection resulted in a significant reduction in off-target events without compromising the on-target editing efficiency.[242]

In further development of SNAP-tag technology, Vogel et al. adapted SNAP-ADAR technology to cellular applications by chemical modifications of the gRNA. 2′-O-methylation and phosphorothioate modification improved the covalent conjugation between gRNA and SNAP-ADAR inside the cell, enhanced editing selectivity, provided resistance to nucleases, and supported penetration into the cell membrane. The improved SNAP-ADAR system was used to repair the Factor V Leiden missense mutation (1746 G-to-A) *in vitro*, which is the most common genetic risk factor for hypercoagulability.[243] In the following years, SNAP-ADAR technology was refined. Vogel et al. (2018) generated four constructs: SNAP-ADAR1 (SA1), SNAP-ADAR2 (SA2), and their hyperactive variants SA1Q and SA2Q (bearing the previously mentioned E1008Q and E488Q mutations, respectively). In the preliminary tests, SA1Q showed the best balance of efficiency and specificity. Therefore, this variant was further validated by the simultaneous targeting of two disease-relevant signaling transcripts of KRAS and STAT1, showing a significantly higher level of adenine conversion (46%–76% for different target sites) than the maternal editase SA1 (18%–31%). The researchers also demonstrated improved performance of this system compared with the dCas13b-ADAR tool (see above), positing that SNAP-ADAR off-target activity was reduced by the chemical modifications (2′-methoxy, 2′-fluoro) of gRNA and its shorter sequence (compared with the gRNA in dCas13b-ADAR). It is also worth mentioning that the human origin and the small size of SNAP-ADAR can provide additional advantages over CRISPR-Cas-mediated systems due to lower immunogenicity and more effective transformation.[244] SNAP-tag technology has been adapted to be controlled by extracellular factors such as light and chemicals. Hanswillemenke et al. constructed SNAP-ADAR triggered by light, which allowed light-induced RNA editing *in vitro*, in mammalian cell culture, and in *Platynereis dumerilii*.[245] Stroppel et al. modified the SNAP-ADAR system to be activated by chemically induced dimerization. The authors used a plant hormone, gibberellic acid (GA3), that induces the heterodimerization of two plant proteins: GAI (gibberellic acid insensitive) and GID1A (gibberellin insensitive dwarf 1A). To control SNAP-ADAR-based editing by GA3-induced dimerization, SNAP-tag and ADAR1 were expressed as two separate fusion proteins with GAI and GID1A, respectively. Restoration of the complex after GA3 induction allowed tight control and editing yields up to 44% in human cell culture.[246] Recently, Stafforst's group extended their editing toolkit by combining the SNAP-tag tool with HALO-tag (a self-labeling protein derived from the haloalkane dehalogenase enzyme DhaA) in one system designed for the orthogonal and concurrent recruitment of two pairs of editing effectors within the same cell: ADAR1 and ADAR2 or ADAR1 and APOBEC1. The selective recruitment of ADAR1 and ADAR2 activity enabled site-directed A-to-I editing with improved editing efficiency, and the selective recruitment of ADAR1 and APOBEC1 activity allowed concurrent A-to-I and C-to-U editing within the same cell.[247]

In addition to SNAP-ADAR and λN–DD, other CRISPR-Cas-free systems have been developed, for example, the CRISPR-Cas-Inspired RNA Targeting System (CIRTS)[248] and editors that do not use gRNA (see below). CIRTS is a universal platform of programmable RNA effector proteins with a modular structure composed of RNA hairpin binding protein (a high-affinity binder such as TBP or SLBP protein), gRNA (which both forms the hairpin and targets the selected transcript), ssRNA binding protein (the nonspecific protector of gRNA unstructured parts, e.g., β-defensin 3, ORF5), and effector protein (e.g., deaminase or ribonuclease). The authors used hADAR2 and its hyperactive mutant (E488Q) as examples of possible effector proteins, showing efficient repair of a G-to-A mutation that causes a premature stop codon in the luciferase transcript. The small size and human origin are the advantages of the CIRTS system.[248] Another category of CRISPR-Cas-free tools are those that are also free of gRNA. In these editors, substrate specificity is ensured by an RNA-binding protein. For example, the RNA-binding scaffold of Pumilio/fem-3 mRNA binding factor homology (PUF) proteins has been proposed as a programmable domain to specifically bind RNAs and function as a targeting module for deaminases.[249–251]

Parallel to the RNA editing strategies based on the expression of exogenous deaminases, approaches utilizing endogenous enzymes (mainly ADAR) have been intensively developed.[252–255] The latter strategy aims to recruit the endogenously expressed ADAR enzyme for deamination. The recruitment is facilitated by gRNA, antisense to the target sequence. Since these systems use native rather than engineered enzymes, discussing them is beyond the scope of this article. However, they have recently been extensively reviewed elsewhere.[256–260]

SPLIT TECHNOLOGY APPLICATION

Despite the constantly increasing efficiency and specificity of base editing tools, the problem of their off-target activity is still valid. Very recently, so-called split technology (also called split-protein reassembly or protein fragment complementation) has been used to overcome this issue. We propose to consider this technology as the next level of deaminase engineering since it is often based on the division of previous fusion. Split technology makes use of the fragmentation of an enzyme followed by its reconstitution at the target site, which can also be controlled by light or small-molecule treatment.

Splitting deaminases or whole fusion systems into conditionally reconstituting fragments has been shown to be a powerful strategy for controlling base editing in both CRISPR-Cas-mediated and CRISPR-Cas-free systems. For example, Mok et al. invented a novel CRISPR-Cas-free and gRNA-free mitochondrial DNA editing tool.

In this tool, engineered transcription activator-like effectors (TALEs) were used as the targeting module. The researchers determined the crystal structure of interbacterial deaminase-like toxin (named DddA) and found its structure-based homology with AID/APOBEC deaminases. In contrast to the AID/APOBECs, in activity tests, DddA showed unexpected 5′-TC deamination in dsDNA and no detectable activity on ssRNA and dsRNA. Additionally, the researchers used split technology to divide the cell-toxic deaminase into two inactive parts. The enzymatic activity on dsDNA was restored when the inactive halves met in the vicinity of the targeted mitochondrial DNA (mtDNA). The final editase included split-DddA, engineered TALE array proteins, and a uracil glycosylase inhibitor. Thanks to this construction, the editor has overcome two previous limitations of base editing within mtDNA: the challenge of delivering gRNA into the mitochondria as well as the toxicity of both nucleases and deaminases, including deaminase off-target activity.[261] Lee et al. adapted this technology to precisely edit mtDNA in mouse embryos and create models of mitochondrial diseases (such as Leigh disease, MELAS syndrome, and LHON syndrome).[262] Most recently, CRISPR-Cas-free split-engineered ADAR2-DD variants were developed by Katrekar et al. and showed a 1,000- to 1,300-fold reduction in the number of off-target mutations compared with the full-length ADAR2-DD or ADAR2-DD(E488Q). The researchers also reported that in the split-ADAR2-DD systems, highly edited off-target sites were mainly gRNA dependent. In the case of classic (not split) deaminase overexpression, off-target effects are predominantly deaminase-driven.[165] Split technology has also been applied to CRISPR-Cas-mediated base editors. Berrios et al. used split technology to create a more controllable CRISPR-Cas-mediated genome editing tool, adding rapamycin-controlled reconstruction of the split halves.[263] Clearly, the new generations of both CRISPR-Cas-free and CRISPR-Cas-mediated base editors are designed to better control off-target activity, and in this context, split engineering is starting to be recognized as a powerful solution. It can be expected that the coming era of tightly regulated DNA/RNA editing tools will further facilitate the clinical applications of DNA/RNA editing technology.

RNA: REGULATOR OF DEAMINASE ACTIVITY AND POTENTIAL TARGET OF ENGINEERING

RNA can be both a substrate for deaminases and a regulator of their catalytic activity. As mentioned in the previous sections, it is believed that AID/APOBECs and ADARs originate from TadA/ADAT2, which edit adenosine to inosine at the anticodon loop of tRNAs.[4] Over the course of evolution, some of these proteins preserved (or regained) the ability to edit RNA (e.g., ADARs), while others have specialized in ssDNA targeting (e.g., AID) or have targeted both DNA and RNA substrates (A1, A3A, A3G). Importantly, almost all AID/APOBECs bind RNAs, not necessarily at the catalytic center, and a regulatory role of these interactions has been proposed.[3,142]

In the classical view, RNA-binding proteins (RBPs) are defined as proteins that bind RNAs through well-defined RNA-binding domains (RBDs) to regulate RNA metabolism and functions. ADARs meet this definition: they bear dsRNA-binding domains and act on RNA (playing a key role in RNA editing, converting adenosine to inosine). Since AID/APOBECs do not have typical RBDs, they could be viewed as an example of unconventional RBPs, so-called "enigmRBPs," whose existence has been proposed by Beckmann et al.[264] and Hentze et al.[265] These proteins lack typical RBDs, yet they bind RNA. Unconventional RNA binding assumes the possibility that RNAs can act as aptamers and interact with proteins in a specific manner to affect their activity and functions. AID/APOBECs can be perceived as an example of unconventional RBPs since RNA can affect their subcellular localization, intermolecular interactions, and activity. For example, many AID/APOBECs interact with a variety of cellular RNAs that bridge AID/APOBEC monomers to form megadalton-sized ribonucleoprotein particles that also contain a variety of other RBPs.[3] Each AID/APOBEC shows a different oligomerization status, which is critical for regulating their subcellular localization and impacts their functions at different levels. Large RNA-bound multimeric complexes have been found for single-domain AID/APOBECs, such as A1, AID, A3H, and double-domain A3s (A3B, A3D, A3F, and A3G), but interestingly not for A3A, for which RNA editing activity has been proposed.[141,266,267] For example, A3H forms catalytically inactive, high-molecular-weight complexes with a molecular weight exceeding 500 kDa. The size of the complex can be reduced to 30–100 kDa by RNase A treatment. The treatment also restores the deamination activity of the protein on ssDNA, indicating that RNA binding mediates both oligomerization and enzyme inhibition.[140] RNA binding can also regulate the enzymatic activity of single-domain AID/APOBECs more subtly. Abdouni et al. tested the binding and deaminase activity of purified AID on DNA/RNA hybrid bubbles (simulating immunoglobulin loci, particularly GC-rich switch regions, which often form R loops in the transcription bubbles). Surprisingly, AID exhibited significantly higher binding affinity and deamination activity on GC-rich DNA/RNA hybrids than substrates composed entirely of DNA. Moreover, the DNA/RNA hybrids modulated the deamination activity of AID in a sequence-dependent manner. The authors further supported this observation by identifying a putative RNA-binding groove on the AID surface opposite of the ssDNA-binding region, which additionally supported the hypothesis of the modulating role of RNA-AID interaction and its potential influence on AID specificity.[144] RNA-binding modes and their functional consequences are more complex for double-domain AID/APOBECs, which all form megadalton-sized ribonucleoproteins. RNA-binding ability is also one of the major functional requirements for the anti-HIV activity of A3s, their encapsidation into HIV virions, and their anti-retroelement activity. A3G has been thought to bind RNA mainly through its catalytically inactive NTD. ssDNA deamination is catalyzed by the Zn-active center of the C-terminal domain. However, the simple division of functions does not give the full picture. Nonsubstrate RNAs can displace ssDNA from the C-terminal catalytic center in a concentration-dependent manner and are competitive inhibitors of its deaminase activity.[146] Simultaneously, the inactive NTD can enhance the deamination efficiency of the C-terminal domain by two to three orders of magnitude and is critical for the processivity of the full-length enzyme.[268] Recently, different RNA-binding modes have been suggested for particular

A3G functions based on the full-length rhesus macaque A3G structure.[269]

Regulatory RNAs have not yet been used in deaminase engineering despite their undeniable potential in this regard. Synthetic RNAs have previously been proposed for similar applications. For example, RNA has been used as a scaffold to colocalize enzymes and increase local enzyme concentrations[270] or induce proximity oligomerization.[271] A more direct approach is the design of RNA aptamers that recognize and bind to specific enzymes to either activate or inhibit their functions.[272,273] Therefore, one can speculate that regulatory RNAs can also be utilized to modulate deaminase functions, localization, or intermolecular interactions. The stable complex of RNA and a deaminase can be formed by the disulfide bridge cross-linking approach typically used to stabilize protein-RNA complexes for structural and biochemical studies. In this method, a single amino acid substitution to cysteine allows the formation of a disulfide bond between the protein and P-cystamine incorporated into chemically modified RNA.[274] Computational modeling allows precise design of the site of protein-RNA conjugation. The future will show whether this or other RNA-involving strategies could be used to modulate the functions of AID/APOBECs or to further study the role of RNAs in the regulation of deaminase activity and/or localization.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In recent years, deaminase engineering has significantly promoted the development of new therapeutic gene editing tools. DNA alterations can correct disease-causing point mutations, create desired genetic variants, and modulate gene expression by modifying start/stop codons. Targeting RNA could also be a promising therapeutic strategy owing to the transient and regulated effects of transcriptome modification. As recently proposed, site-directed RNA editing could be used to tune cell physiology to achieve temporary outcomes that are therapeutically advantageous, particularly in the nervous system.[275] Despite unquestionable advances in the engineering of DNA/RNA editing systems, there is still much to be done to improve the existing tools. In particular, there is a need to eliminate their off-target activities with a simultaneous increase in editing efficiency. Split engineering is currently emerging as the most powerful method for the precise control of editing. However, deaminase engineering to obtain higher sequence specificity could also help to reduce off-target effects. Importantly, the long-term side effects of editing system activity in animal models have just begun to be studied. For example, comprehensive testing of ~400 transgenic mice over 15 months with CBE3 revealed *de novo* genomic mutations in the offspring and transcriptome-wide mutations across various tissues. In contrast, in an analogous experiment, ABE7.10 (with the F148A mutation in TadA) showed no detectable off-target effects at either the DNA or RNA level.[276] The difficulty of effective delivery and the immunogenicity and toxicity of CRISPR-Cas also remain challenging. The most robust approaches for editing system delivery *in vivo* use viruses, such as AAVs, to deliver DNA encoding the editing system.[277–279] This strategy results in prolonged expression of the system and consequently a greater risk of off-target effects and viral vector integration into the genome. Last year, an alternative to the above has emerged since DNA-free virus-like particles were adapted to deliver base editor nucleoproteins.[280]

Moreover, in the near future, the currently available editing toolset could be further developed. For example, an RNA-specific cytidine editor seems to be attainable, taking into account the latest report of Tang et al. showing RNA-specific APOBEC3A variants.[281] Although the hAP3A-Cas9 fusion has been shown to efficiently edit methylated regions,[214] systems for the selective targeting of modified nucleotides such as 5mC or 5hmC remain to be developed. Direct 5mC to T deamination could result in a wider spectrum of modifications or the possibility of creating C-to-T genetic variants without employing UDG activity. Furthermore, editors that are selective for modified cytidines in DNA could improve the currently used sequencing methods by enabling two additional letters to be read, namely, 5mC and 5hmC—two of the most important epigenetic markers. Variants with selective deaminase activity on modified nucleobases in RNA, m5C and hm5C, would be even more promising. In this case, deamination would have two effects: (1) the erasure of m5C or hm5C modifications (relatively abundant in RNA molecules), and (2) the generation of m5U and hm5U modifications (relatively rare in RNA), enabling functional or structural studies of four modifications in total.

Despite the numerous current and proposed applications of deaminases, our knowledge of their functions is still limited. More effort needs to be put into broadening our understanding of the basic biochemical and structural aspects of deaminase biology, such as (1) their activity on modified substrates; (2) the processivity of their action; (3) the impact of oligomerization on their subcellular localization, substrate binding, and functions; and (iv) interactions with regulatory and substrate RNAs, as well as the influence of RNA modifications on these interactions. Basic research in all of these areas will vigorously stimulate deaminase engineering and the further development of deaminase applications.

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AUTHOR CONTRIBUTIONS

L.B. - conceptualization, original draft preparation, responsible for the final version of the manuscript; K.H. - original draft preparation and figure preparation; P.J. - reviewing and editing; M.F. - supervision, conceptualization, reviewing and editing, responsible for the final version of the manuscript. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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