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Base editing: advances and therapeutic opportunities

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Abstract | Base editing — the introduction of single-nucleotide variants (SNVs) into DNA or RNA in living cells — is one of the most recent advances in the field of genome editing. As around half of known pathogenic genetic variants are due to SNVs, base editing holds great potential for the treatment of numerous genetic diseases, through either temporary RNA or permanent DNA base alterations. Recent advances in the specificity, efficiency, precision and delivery of DNA and RNA base editors are revealing exciting therapeutic opportunities for these technologies. We expect the correction of single point mutations will be a major focus of future precision medicine.

Precision medicine
The development of disease prevention and treatment strategies based on a patient's individual characteristics (that is, genomic sequence).

Single-nucleotide variants
(SNVs). Major cause of genetic diseases, targetable with base editors.

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As our understanding of how the primary sequence of genomic DNA impacts human health has expanded, the therapeutic potential of genome editing has emerged. This revolution in how we think about human health and disease has, in large part, been driven by rapid advances in genome sequencing technologies, which have revealed causative mutations of genetic diseases. Furthermore, we are brought ever closer to the realization of precision medicine: the development of disease prevention and treatment strategies based on a patient's individual characteristics (such as their genomic sequence). It is therefore an exciting time for researchers in these fields as we tackle some of the most noteworthy barriers to the utilization of genome editing for the treatment and cure of genetic diseases. Approximately half of the known pathogenic genetic variants are due to single-nucleotide variants (SNVs), highlighting the need for the development of methods and tools capable of correcting SNVs with high efficiency[1]. Over 96% of observed human genetic variants are SNVs, with over 99% currently lacking a clinical interpretation[2]. Therefore, tools to introduce SNVs will also prove indispensable for improving our understanding of how human genetic variation impacts health[3–7].

To be used as a therapeutic, a genome editing tool must demonstrate high on-target efficiency and minimal harmful or undesired off-target edits, and be deliverable to the organ(s) of interest. It is important to note that the disease target will dictate the exact degree to which these criteria must be met. Early efforts in the field used platforms such as zinc-finger nucleases and transcription activator-like effector nucleases (TALENs), but these methods were hampered by the requirement of designing and validating a new zinc-finger nuclease or TALEN protein for each new target editing site[8]. However, these extensive protein re-engineering requirements were alleviated with the discovery, mechanistic elucidation and adaption for genome editing of clustered regularly interspaced short palindromic repeat (CRISPR) platforms.

CRISPR systems are a naturally occurring bacterial and archaeal defence against invading viruses, that have been harnessed for genome and transcriptome editing by inducing double-stranded DNA breaks (DSBs) or RNA cleavage at user-defined loci in living cells[9–11]. Reprogramming these systems to perform genome editing at different genomic loci simply requires changing the sequence of a piece of RNA (called a spacer, guide RNA (gRNA) or single guide RNA) via Watson–Crick–Franklin base pairing rules. DSBs are introduced at preprogrammed loci by the CRISPR-associated proteins, Cas9 and Cas12, and are typically repaired through one of two competing endogenous repair pathways in mammalian cells: non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ-resolved DSBs result in non-specific insertions or deletions (indels) at the site of the DSB, often resulting in frameshifts and gene knockout. Researchers can co-opt the HDR pathway to introduce desired and precise sequence edits into the genome by using an exogenous DNA repair template. Unfortunately, HDR efficiencies vary amongst mammalian cell types (that is, unmodified cells exhibit low HDR efficiency), the HDR pathway is only active during certain phases of the cell cycle and HDR is in constant competition with, and usually outcompeted by, NHEJ for repair of DSBs. Therefore, the development of new techniques and tools to improve HDR yields and/or suppress NHEJ rates have been primary areas of study in the field, have yielded many improvements and have been reviewed elsewhere[12–19].

In comparison with Cas9 and Cas12, Cas13 proteins function similarly as DNA targeting CRISPR systems to bind and cleave target RNA transcripts in a programmable manner. However, upon target RNA binding and

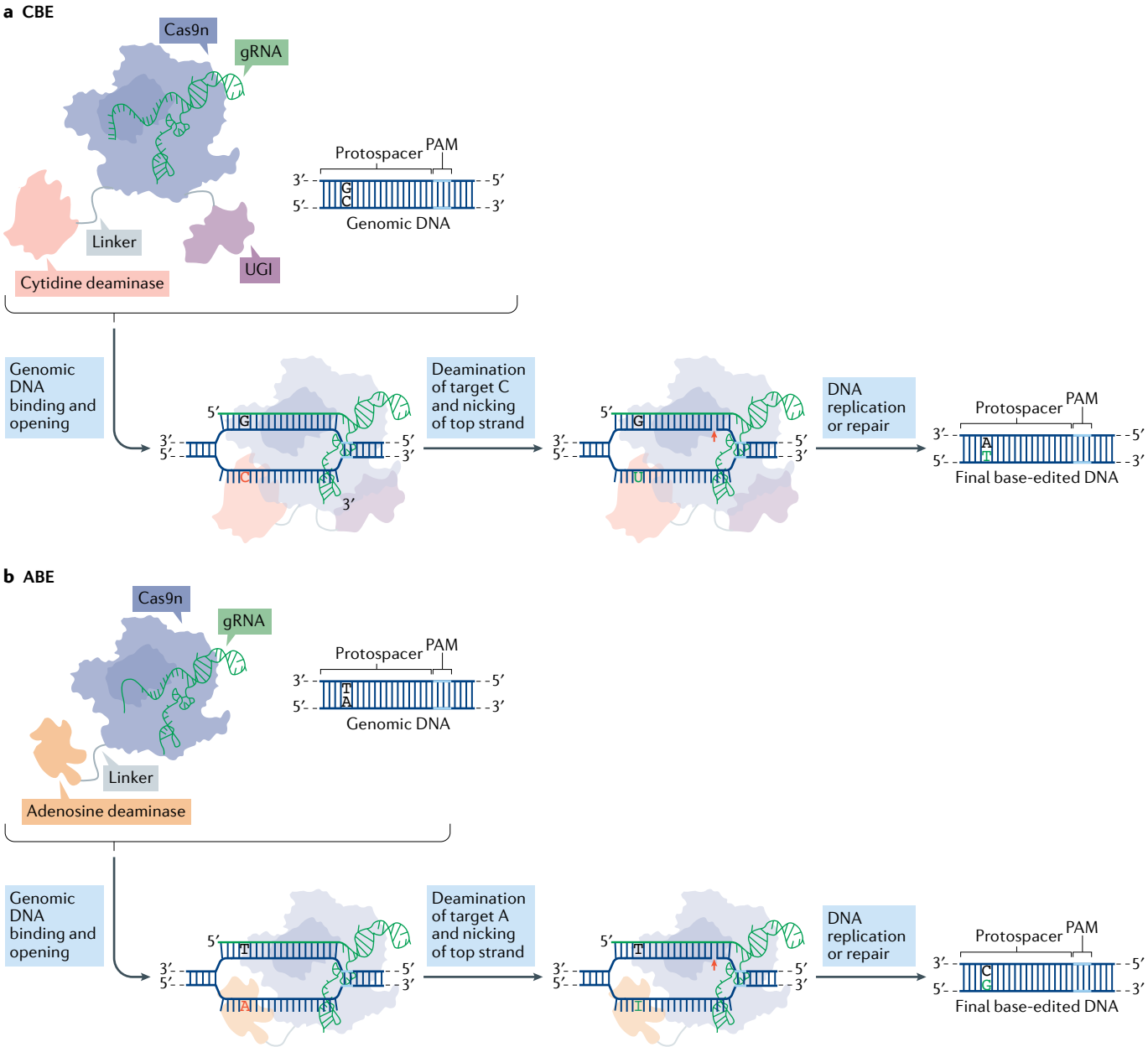

Fig. 1 | **General overview of DNA base editing technologies. a** | Cytosine base editor (CBE) mechanism. Principle components of the CBE are designated in coloured text boxes. If uracil glycosylase inhibitor (UGI) is present (an optional component), it will 'protect' the U•G intermediate from excision by uracil DNA glycosylase (UDG) to boost efficiency of the final base-edited DNA outcome. The nickase version of Cas9 (Cas9n) nicks the top strand (red arrow) whereas the cytidine deaminase converts cytosine (red) to uracil (green). Ultimate conversion of a C•G to T•A base pair is achieved through the outlined steps. **b** | The adenine base editor (ABE) mechanism is similar to that of CBE, without possible inclusion of a UGI domain in the ABE architecture. Through ABE-mediated editing, an A•T to G•C base pair conversion is achieved via an inosine-containing intermediate. gRNA, guide RNA; PAM, protospacer adjacent motif; target A, ABE desired base substrate; target C, CBE desired base substrate. Part **a** adapted from REF.[25], Springer Nature Limited. Part **b** adapted from REF.[32], Springer Nature Limited.

Guide RNA
(gRNA). A short sequence of RNA that recognizes the target DNA region of interest and directs the Cas enzyme to bind for editing to occur (also known as the spacer and single guide RNA).

cleavage, Cas13 will also non-specifically cleave nearby single-stranded RNAs in vitro, which can potentially pose complications and, thus, limit the therapeutic potential of wtCas13s (REF.[20]). It is important to note that Cas9, Cas12 and Cas13 enzymes can be catalytically inactivated to produce dCas9, dCas12 and dCas13, which maintain programmable DNA or RNA binding capabilities but do not cleave their target[21]. Nucleic acid backbone-cleaving technologies are not within the scope of this Review, but have been reviewed extensively elsewhere[22–24].

One technology developed to address the challenge of creating targeted single-nucleotide alterations in a precise and efficient manner is base editing. Base editing is unique in that it avoids nucleic acid backbone cleavage and, instead, directly chemically modifies target nucleobases in the process of genome and transcriptome editing (FIGS 1 and 2). Both DNA and RNA base editors

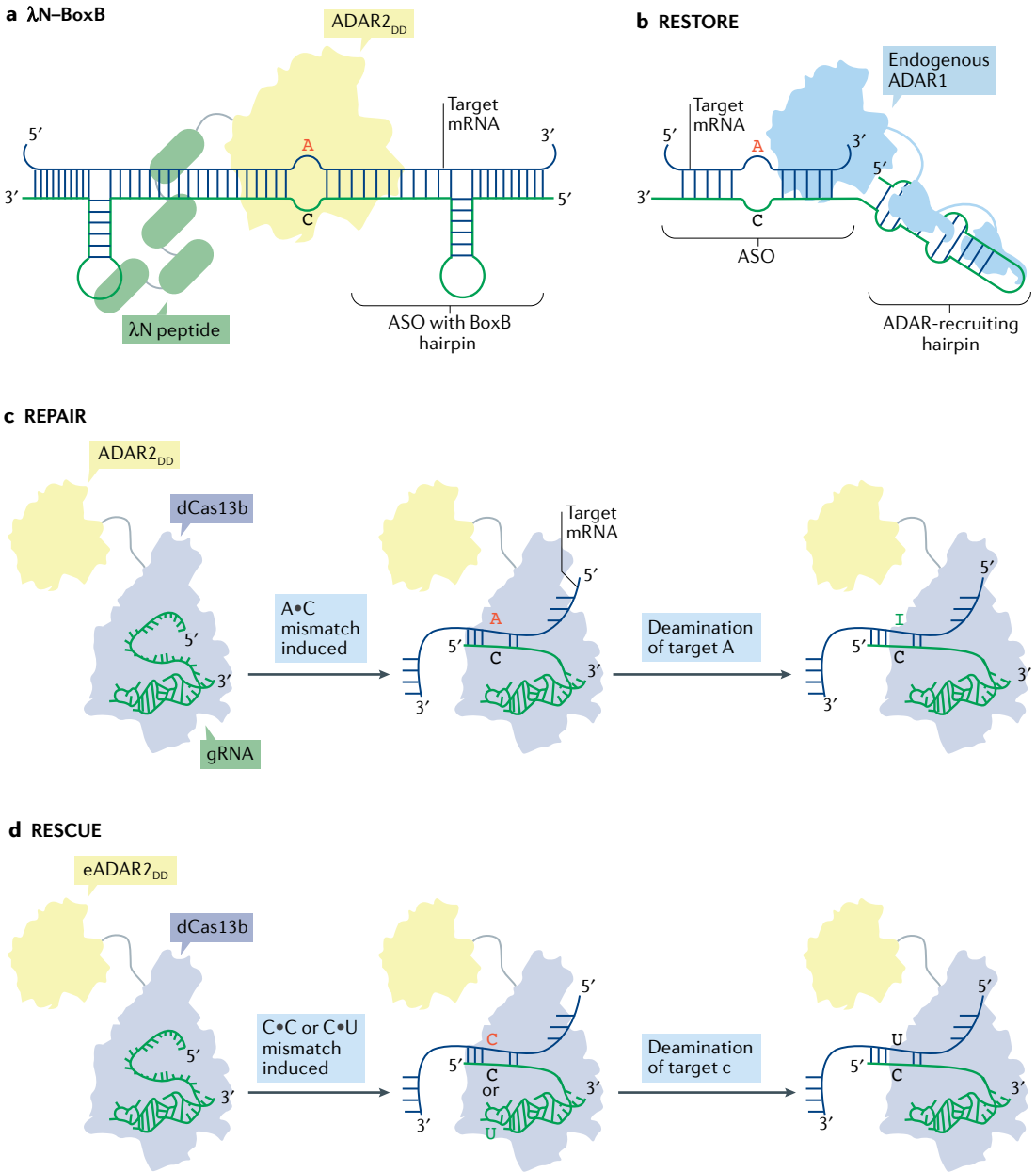

Fig. 2 | **General overview of RNA base editing technologies. a** | Antisense oligonucleotide (ASO)-mediated A-to-I RNA base editing with an engineered adenosine deaminase, using the λN–BoxB construct as an example. The catalytic domain of ADAR2 ($ADAR2_{DD}$; yellow) is fused to multiple copies of the λN coat protein (green). The ASO guide RNA (gRNA; green strand) is engineered to base pair with the target mRNA (blue strand) and contain multiple BoxB hairpins to recruit the λN peptide to the mRNA of interest. An induced A•C mismatch between the target RNA and the ASO is employed to achieve targeted adenosine deamination on mRNA by ADAR2. **b** | ASO-mediated A-to-I RNA base editing with an endogenous adenosine deaminase, using RESTORE (recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing) as an example. Endogenous ADAR1 comprises a catalytic domain and two double-stranded RNA binding domains (dsRBDs; blue). The engineered ASO gRNA (green strand) consists of a specificity domain (the 3′ end, which binds to the target mRNA of interest (blue strand) through Watson–Crick–Franklin base pairing) and an ADAR-recruiting domain (the 5′ end, which comprises the natural substrate of the dsRBD), which recruits endogenous ADAR1 to a target mRNA of interest, where an induced A•C mismatch between the target RNA and the ASO directs the catalytic domain of ADAR1 for targeted adenosine deamination. **c** | A-to-I RNA base editing through REPAIR (RNA editing for programmable A-to-I replacement). An induced A•C mismatch between the target mRNA (blue strand) and the gRNA (green strand) of Cas13b (blue) is employed to achieve targeted adenosine deamination on mRNA by ADAR2. **d** | C-to-U RNA base editing through RESCUE (RNA editing for specific C-to-U exchange). An induced C•C or C•U mismatch between the target mRNA (blue strand) and the gRNA (green strand) of Cas13b (blue) is employed to achieve targeted cytosine deamination on mRNA by a mutant version of ADAR2. ADAR, adenosine deaminase acting on RNA; dCas, catalytically dead Cas enzyme; e, enhanced; target A, REPAIR desired base substrate; target C, RESCUE desired base substrate. Part **a** adapted with permission from REF.[45], OUP. Part **b** adapted from REF.[49], Springer Nature Limited. Part **c** adapted with permission from REF.[51], AAAS. Part **d** adapted with permission from REF.[54].

have been developed, and their rapid adoption by the genome and transcriptome editing communities is a clear demonstration of their value as tools to enable both basic science research and development of human therapeutics.

This Review provides an overview of both DNA and RNA base editors and discusses therapeutically relevant advances in the development of these technologies. Emerging therapeutic opportunities and associated challenges are discussed.

Classes of base editors

Base editors, of which there are many variants, can be sorted into two main categories: those targeting DNA and those targeting RNA. Whereas the origins of base editing technology begin decades ago with RNA base editors, both categories have recently seen an explosion in development.

Base editing in DNA

DNA base editors can be further categorized as cytosine base editors (CBEs) or adenine base editors (ABEs). Both CBEs and ABEs are powerful tools for the permanent introduction of point mutations in DNA in living cells with high efficiency.

C•G to T•A base editors (CBEs). The first DNA base editor was developed as a method to perform genome editing without using DSBs. A naturally occurring cytidine deaminase enzyme was used to convert target cytosines to uracil, which has the base pairing properties of thymine. This was expected to catalyse an overall C•G to T•A base pair conversion following the cell's use of uracil as a template for repair[25] (FIG. 1a). The original prototype (named BE1, or first-generation base editor) used a catalytically dead version of the *Streptococcus pyogenes* Cas9 (dCas9) enzyme tethered to the single-stranded DNA (ssDNA) specific cytidine deaminase enzyme APOBEC1 from *Rattus norvegicus* (rAPOBEC1) (FIG. 3a). dCas9 binds to a target DNA locus of interest (the protospacer; FIG. 3b) through canonical RNA–DNA base pairing between the gRNA and the genomic DNA. Sequence complementarity between the gRNA and the protospacer, and the presence of an NGG (where N = adenine/cytosine/guanine/thymine, per standard IUPAC nucleotide codes) protospacer adjacent motif (PAM) sequence are required for dCas9 binding to the target locus. Once dCas9 finds its target sequence, it will locally denature the double-stranded DNA to generate an R-loop[26], exposing a short stretch of ssDNA (positions 4–8 if the PAM is counted as positions 21–23; FIG. 3b) on the non-complementary strand for deamination by the APOBEC1 enzyme.

BE1 could effectively convert cytosines to uracils in vitro in a programmable manner, but was significantly less effective at introducing C•G to T•A point mutations in live cells (5-fold to 36-fold decreases in efficiency were observed)[25]. The large decrease in base editing efficiency was hypothesized to be partially due to high intracellular levels of uracil excision of the U•G intermediate by the base excision repair enzyme uracil DNA glycosylase (UDG). UDG catalyses the removal of uracil in DNA to initiate the base excision repair pathway, ultimately resulting in reversion to the original C•G base pair[27,28]. To protect the uracil intermediate and boost base editing efficiencies, the phage polypeptide uracil glycosylase inhibitor (UGI) was added to the BE1 architecture, resulting in the second-generation base editor, BE2. Addition of UGI enhanced editing efficiencies approximately 3-fold compared with BE1 (REF.[25]). In a final improvement to the base editor architecture, the dCas9 portion of BE2 was replaced with the nickase version of Cas9 (Cas9n) to yield the third-generation base editor, BE3. In this new construct, Cas9n would nick the DNA backbone of the unedited, G-containing DNA strand, flagging it for removal by the eukaryotic mismatch repair pathway and forcing the cell to use the uracil as a template during downstream repair (FIG. 1a). This nicking strategy boosted efficiencies by an additional 2-fold to 6-fold compared with BE2 (REF.[25]). As the base editor toolbox expanded, DNA base editors capable of facilitating C•G to T•A base pair conversions collectively became known as CBEs. It is important to note the dependence of this strategy on the single-stranded portion of the R-loop; ssDNA-specific cytidine deaminase fusions with other classes of genome editing agents, such as zinc-finger nucleases, did not display such precision or efficiency[29]. However, very recently a double-stranded DNA-specific cytidine deaminase was discovered and repurposed into a C•G to T•A base editor using TALEs[30]. In this system, the deaminase is split in half, with each half fused to a different TALE construct. The two TALEs bind to adjacent sites in DNA, bringing the two deaminase halves together where the enzyme performs base editing chemistry. Notably, this new base editor, DdCBE, enabled efficient mitochondrial genome editing for the first time, as its reliance on TALEs instead of a Cas enzyme inherently overcame the previous challenges facing nucleic acid delivery to mitochondria[30].

A•T to G•C base editors (ABEs). Drawing inspiration from CBEs, it was quickly recognized that adenosine deamination chemistry would result in inosine, which is read by replication and transcription machinery as guanine. This theoretical ABE would therefore be capable of correcting C•G to T•A mutations, which represent the most common pathogenic SNVs reported in the ClinVar database[31]. Naturally occurring adenosine and adenine deaminase enzymes do exist, but their substrates are confined to various forms of RNA. In order to create an ABE, an adenosine deaminase acting on ssDNA needed to be generated (FIGS 1b and 3a). Various naturally occurring adenosine deaminases (such as *Escherichia coli* TadA (or ecTadA), human ADAR2, mouse ADA and human ADAT2) were assayed for ABE activity, but none yield A•T to G•C base editing above background levels[32]. Therefore, directed evolution was employed to evolve the desired enzyme from ecTadA. Similarity between the desired substrate (ssDNA) and the wild-type substrate (all contacts between ecTadA and its tRNA substrate are localized to the single-stranded loop region of the tRNA), along with its shared homology with the APOBEC enzyme used in

Cas9
(CRISPR-associated protein 9). The enzyme responsible for DNA double-stranded cutting (wtCas9), single-stranded cutting or nicking (Cas9n), or no DNA cutting (wherein Cas9 is catalytically inactive, dCas9) activity. All three Cas9 variants maintain DNA binding ability.

Base editors
Genome editing tools that allow for the direct, irreversible conversion of target cytosine or adenine bases at a specific genomic locus without relying on double-stranded DNA breaks.

Protospacer
A DNA locus of interest targeted with genome editing agent; base pairs with the guide RNA.

IUPAC nucleotide codes
N = adenine/cytosine/guanine/thymine, R = adenine/guanine, Y = cytosine/thymine, V = adenine/cytosine/guanine (in order of appearance).

Protospacer adjacent motif
(PAM). A variable region on the 5′ or 3′ end of the protospacer, required for Cas protein binding to the target locus. PAM requirements vary among different Cas enzymes (the most widely used *Streptococcus pyogenes* Cas9 requires an NGG PAM).

R-loop
A tripartite structure consisting of unpaired DNA and a paired DNA:RNA hybrid. Following R-loop formation, the unpaired or single-stranded DNA is accessible for base editing.

CBEs, were among the main reasons why ecTadA was selected as the starting point for directed evolution[33].

A total of seven rounds of directed evolution were performed, identifying 14 mutations in TadA to create the final ABE7.10 construct, consisting of a heterodimeric wtTadA–TadA* (* indicates the presence of mutations, the wild-type enzyme acts as a dimer to perform its chemistry on tRNA) complex fused to Cas9n. ABE7.10 was demonstrated to introduce A•T to G•C point mutations in live cells with average editing efficiencies of 58% across 17 genomic loci, with an editing window of positions 4–7 within the protospacer[32] (FIG. 3b). Unlike CBE, no DNA repair manipulation component (such as UGI) is required due to the infrequent nature of the inosine intermediate (intracellular inosine excision is much less efficient than that of uracil). Additionally, various subsequent studies have suggested that the wtTadA component of ABEs is unnecessary and can be

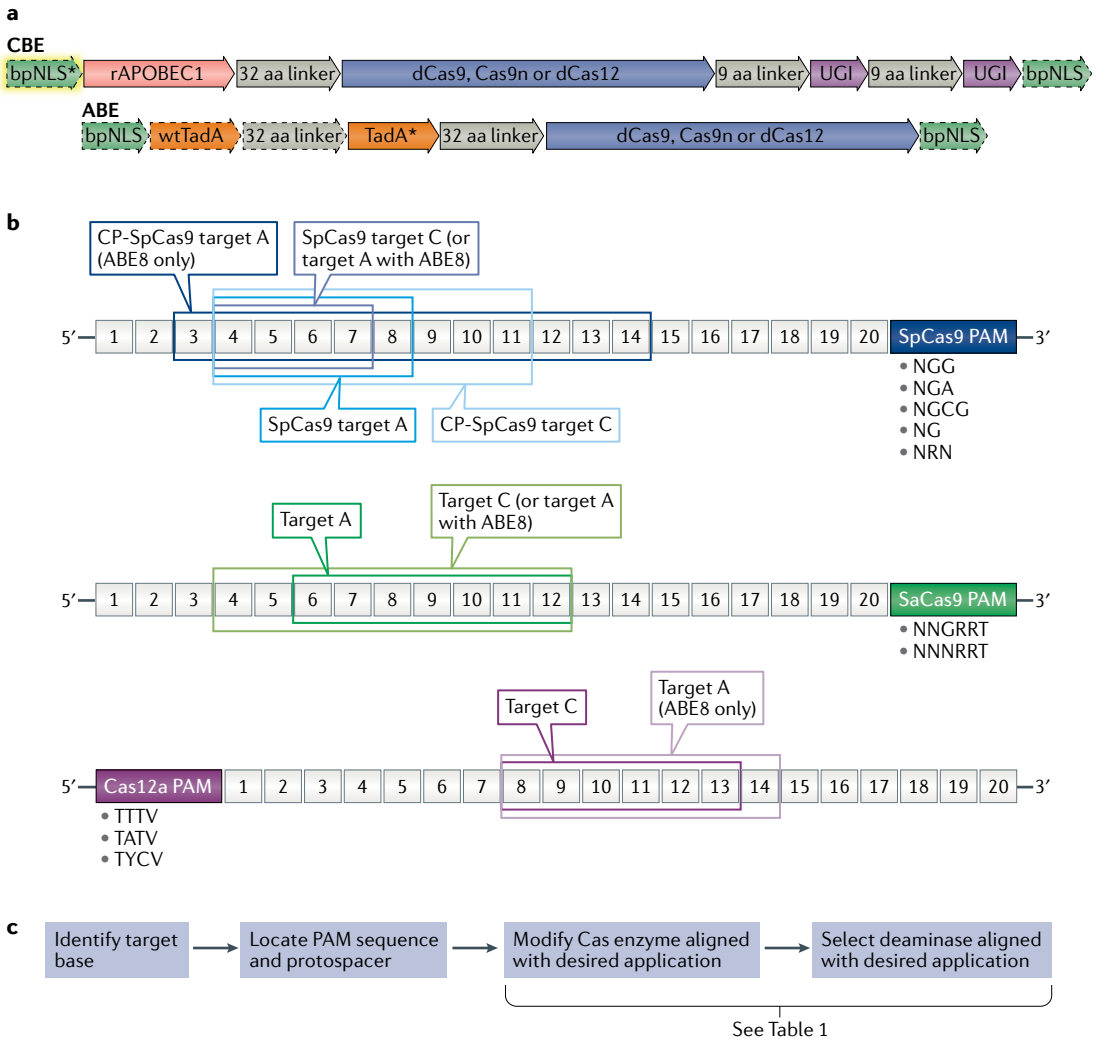

Fig. 3 | **DNA base editor and protospacer design scheme. a** | Construct maps of basic cytosine base editor (CBE) and adenine base editor (ABE) architectures. In the CBE architecture (top), solid line components make up the basis for the fourth-generation CBE, BE4, whereas dotted line components (bipartite nuclear localization signal (bpNLS); green) can be optionally added, to produce BE4max. The amino-terminal bpNLS* component is FLAG-tagged (yellow haze). In the ABE architecture (bottom), all solid line and dotted line components make up the basis for ABE7.10; the dotted lined components (wtTadA (orange) and one of the two 32-amino-acid (aa) linkers (grey)) are optional, and removal of these components results in a monomeric ABE construct with no reduction in on-target efficiency. For both CBE and ABE architectures, use of an appropriate nickase Cas variant is only possible with Cas9 (Cas9n; blue). **b** | Activity windows of base editors with the basic architecture from part **a** with the indicated Cas proteins (*Streptococcus pyogenes* Cas9 (SpCas9; blue), *Staphylococcus aureus* Cas9 (SaCas9; green) and Cas12a (purple)). Protospacer adjacent motifs (PAMs) associated with each Cas enzyme are listed. Base editor activity windows are shown over the 20-nucleotide protospacer sequence (corresponding coloured box outlines). **c** | Simplified workflow of protospacer and DNA base editor design strategy for user-defined adaptation. Once a protospacer, Cas enzyme variant and basic architecture are chosen, modifications can be incorporated for each specific application according to TABLE 1. CP, circular permutant; dCas, catalytically dead Cas enzyme; rAPOBEC1, APOBEC1 from *Rattus norvegicus*; TadA*, mutated TadA (contains ABE7.10 or ABE8 mutations as indicated); target A, ABE desired base substrate; target C, CBE desired base substrate; UGI, uracil glycosylase inhibitor; wt, wild-type.

Antisense oligonucleotides (ASOs). Small pieces of DNA or RNA that bind to specific molecules of RNA.

$ADAR_{DD}$ (Deaminase domain of adenosine deaminase acting on RNA enzyme). The first reported case explored for A-to-I RNA base editing.

omitted without decreases in editing efficiency, indicating a fundamental difference in the enzyme's mechanism for performing chemistry on RNA versus DNA[34,35]. Together, ABEs and CBEs are theoretically capable of correcting 63% of pathogenic SNVs reported in ClinVar.

Base editing in RNA

RNA base editors are further classified according to the modification that they introduce. Unlike DNA base editors, these modifications are not further processed by the cell in the lifetime of the transcript.

A-to-I RNA base editors. Whereas DNA base editing provides the means for irreversible, permanent changes to the genome, RNA base editing offers researchers the opportunity to make reversible modifications to a cell's genetic material or install epitranscriptomic modifications into RNA. Chronologically, RNA base editing predates both DNA base editing and the use of CRISPR for genome editing purposes. Conceptually, adenosine to inosine (A-to-I) RNA base editing was explored more than 25 years ago, when the adenosine deaminase acting on RNA (ADAR) enzyme from *Xenopus* oocyte nuclear extracts was used to correct a premature stop codon in a synthetic mRNA construct in vitro. ADAR was recruited to the nucleobase of interest using complementary RNA oligonucleotides, which would create a localized region of dsRNA (the substrate of ADARs)[36,37]. Subsequent work has focused on directly tethering ADARs to antisense oligonucleotides (ASOs), allowing for targeted, precise A-to-I editing in live cells[38–42] (FIG. 2a,b). Canonical Watson–Crick–Franklin base pairing between the ASO and the target RNA transcript increased the specificity of the deaminase domain of ADAR ($ADAR_{DD}$), particularly when the target adenosine is imbedded within a bulged A•C mismatch[38,39,43–48]. The λ-phage N protein–BoxB system is one such system for site-directed adenosine deamination using this strategy in various sequence contexts[45] (FIG. 2a).

Concerns about high levels of unintended off-targets, due to the introduction and overexpression of exogenous ADAR enzymes, led to newer strategies using ASOs that leverage endogenously available ADAR enzymes to target transcripts. One such strategy, referred to as RESTORE (recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing)[49] (FIG. 2b), was used to edit endogenous RNA transcripts both in cancer cell lines and in primary human cells using an optimized ASO variant with chemical modifications[49]. Another example of this is LEAPER (leveraging endogenous ADAR for programmable editing of RNA), which uses genetically encodable 70-nucleotide-long ADAR-recruiting RNAs to recruit native ADAR1 or ADAR2 enzymes to a target adenosine for conversion to inosine[50]. LEAPER enables ADAR-recruiting RNA-mediated RNA base editing in various cell lines, albeit with varying levels of efficiency, potentially due to differences in endogenous ADAR expression levels. The ability of LEAPER to be delivered to cells in various different ways (via plasmid or viral vector delivery, or after in vitro chemical synthesis) makes it a flexible therapeutic strategy.

Since the discovery of Cas13 enzymes (RNA-guided, RNA-targeting CRISPR effector complexes), several CRISPR-derived RNA base editing systems have been engineered. The first example of these was REPAIR (RNA editing for programmable A-to-I replacement), in which a Cas13b variant derived from *Prevotella* sp. P5-125 (PspCas13b) was fused to the catalytic domain of ADAR2 containing the hyperactivating mutation E488Q ($ADAR2_{DD}$)[51] (FIG. 2c). Again, a cytidine was programmed into the gRNA to form an A•C mismatch upon target binding to increase editing efficiency and precision[51–53]. This first-generation construct (REPAIRv1) was capable of performing A-to-I editing at endogenous transcripts, but displayed poor specificity and was therefore further optimized[51]. REPAIRv2 incorporated an additional R455E point mutation in $ADAR2_{DD}$ (identified through rational mutagenesis), resulting in increased on-target efficiencies (up to ~40% editing) with a 900-fold decrease in global off-target editing as compared with REPAIRv1 (REF.[51]). REPAIRv2 demonstrates promising potential as an RNA base editor therapeutic.

C-to-U RNA base editors. The variety of A-to-I RNA base editor options is a product of the natural preference of wild-type ADAR enzymes for adenosines imbedded in A•C mismatches. Although RNA cytosine deaminase enzymes naturally exist, their high activity for any cytosine present in single-stranded RNA has precluded their use in the development of precise RNA base editors. RESCUE (RNA editing for specific C-to-U exchange) has been developed to address this challenge[54] (FIG. 2d). Starting with the A-to-I REPAIR system, $ADAR2_{DD}$ was mutated into a cytosine deaminase enzyme acting on dsRNA. RESCUEr16 was generated from 16 rounds of evolution and allows for C-to-U editing at C•C and C•U mismatches with minimal sequence preferences[54]. RESCUE maintains A-to-I activity, allowing for multiplexed adenine and cytosine RNA base editing. To avoid unwanted A-to-I activity, the gRNA can be designed to incorporate potential off-target adenines within A•G mismatches. One last round of rational mutagenesis was performed to reduce transcriptome-wide off-targets while maintaining on-target efficiencies and yielded a mutant with the highest specificity, RESCUE-S[54]. RESCUE-S maintained ~76% on-target editing and minimal C-to-U and A-to-I off-target editing[54]. The RESCUE method demonstrates a key step forward in the field of RNA base editing, opening up the possibility for additional types of RNA base editors to be developed.

Therapeutically relevant advances

Based on the foundations laid for the first-generation base editors described above, many laboratories have markedly improved CBEs and ABEs. These advances offer a wide array of benefits for their therapeutic application.

Decreasing off-target activity

As with all therapeutic applications of genome editing agents, potential off-target editing needs to be taken into account. In the original reports of CBEs and ABEs, off-target editing was detected at a subset of known Cas9

Activity window
A defined region of single-stranded DNA accessible for base editing activity. Activity windows vary among different base editor variations.

Ribonucleoprotein complexes
(RNPs). Macromolecular structures containing both Cas9 protein and guide RNA molecules.

IUPAC amino acid codes
V = valine, W = tryptophan, R = arginine, A = alanine, K = lysine, G = glycine, Y = tyrosine, E = glutamic acid, P = proline (in order of appearance).

off-target sites[25,32,55]. Further studies using modified Digenome-sequencing protocols confirmed these results but also found that CBEs and ABEs would recognize a small number of additional, unique off-target sites as compared with Cas9 (REFS[56–59]). These sites were dependent on the sequence of the gRNA, and the off-target C•G to T•A or A•T to G•C edits were localized to the same five-nucleotide activity window of these off-target protospacers. Overall, these types of off-targets are referred to as 'gRNA-dependent off-targets', and both ABEs and CBEs display fewer of these gRNA-dependent off-targets than does wild-type Cas9.

To combat gRNA-dependent off-targets, high-fidelity Cas9 variants have been incorporated into the classic CBE architecture, replacing wild-type Cas9n (FIG. 3c; TABLE 1). Previous work had employed structure-guided protein engineering on SpCas9 to quench non-specific electrostatic interactions between the Cas9 protein and its target DNA. These efforts resulted in Cas9 variants with more favourable off-target profiles, including enhanced Cas9 (eCas9), further eCas9 (FeCas9), high-fidelity Cas9 (Cas9-HF), hyper-accurate Cas9 (HypaCas9), evolved Cas9 (evoCas9), Sniper-Cas9 and a separate high-fidelity Cas9 (HiFi Cas9)[60–66]. When Cas9-HF was incorporated into the BE3 architecture, a 3-fold decrease in gRNA-dependent off-target base editing was observed, highlighting the utility of these variants for therapeutic base editing[67]. Additional studies in rice using CBEs with eCas9, Cas9-HF and HypaCas9 variants have confirmed these results[68]. Although no such study has been performed with ABEs, we anticipate due to the modular nature of base editors that this strategy will be equally effective with ABEs. An additional strategy that has been shown to be successful in reducing gRNA-dependent off-target editing is to deliver base editors as mRNA constructs or as purified ribonucleoprotein complexes (RNPs), which are discussed in greater detail in the 'Base editor delivery strategies' section below. Although these and other delivery methods have helped alleviate gRNA-dependent off-target editing, additional types of base editor off-targets have recently been reported[69].

Whereas global DNA off-target C•G to T•A editing was not observed in the initial CBE report, later studies using more sensitive methods did observe genome-wide, gRNA-independent off-target edits due to CBE, but not ABE, treatment[70–75]. Recent efforts have resulted in a suite of CBE variants with 10-fold to 100-fold lower levels of gRNA-independent off-target editing and 5-fold to 50-fold lower levels of gRNA-dependent off-target editing[76]. These variants all incorporate specific mutations into the rAPOBEC1 domain that decrease the kinetics of ssDNA deamination. Additionally, eight next-generation CBEs with lower frequencies of gRNA-independent off-target editing using wild-type and engineered rAPOBEC1 homologues have recently been developed[77]. These novel CBEs (derived from the cytidine deaminases PpAPOBEC1, RrA3F, AmAPOBEC1 and SsAPOBEC3B) maintain on-target editing efficiencies comparable with their rAPOBEC1 counterparts and display up to a 45-fold decrease in gRNA-independent off-target DNA editing[77]. Given their low propensity for off-target editing, improvements of ABE7.10 therefore focused on increasing on-target editing efficiency. Two recent reports have described eighth-generation ABEs with up to 6-fold increases in on-target editing efficiencies and slight increases in both gRNA-independent and gRNA-dependent off-target editing, which were both mitigated by incorporation of the V106W (where V = valine and W = tryptophan, per standard IUPAC amino acid codes) mutation into the deaminase domain[78,79].

In addition to DNA off-targets, it was recently observed that both CBEs and ABEs can induce gRNA-independent off-target editing in RNA transcripts[80,81]. In one particular study, whole-transcriptome RNA sequencing revealed both CBE-induced and ABE-induced deamination in many transcribed genes in HepG2 cells, with efficiencies ranging from 0.07 to 81.48%[80]. Rational engineering of the cytosine deaminase domain was used to develop SECURE-CBE (Selective Curbing of Unwanted RNA Editing) variants that incorporated the rAPOBEC1 mutations R33A or R33A/K34A (where R = arginine, A = alanine and K = lysine). These SECURE-CBEs displayed off-target RNA editing equivalent to background levels. Soon after, SECURE-ABEs were also reported, in which the wtTadA portion of ABE7.10 was omitted from the construct and the mutations K20A/R21A or V82G (G = glycine) were introduced into TadA* to eliminate off-target RNA editing[34]. Concurrently, off-target RNA deamination induced by CBEs and ABEs was described, which were both eliminated through deaminase engineering efforts of their own[81,82]. Collectively, these studies have afforded a spectrum of CBE and ABE variants that can be used with reduced or eliminated off-target profiles for therapeutic base editing. Notably, previous studies have identified anti-CRISPRs and Cas9 variants exhibiting 'switchable' and photo-inducible properties as potential control factors for CRISPR/HDR-mediated genome editing[47,83–89]. We acknowledge the potential of these devices to assist in further decreasing off-target activity displayed by base editors when deployed in a therapeutic setting.

Enhancing product purity of CBEs

C•G to non-T•A conversions at the target cytosine have been observed when using CBEs[90–92]. This mixture of editing outcomes, which occurs in an unpredictable and locus-dependent manner, reduces the precision of CBEs and thus represents a hurdle to their use in certain therapeutic applications. Early studies to probe the mechanism of this mutagenesis demonstrated UDG to be responsible for the mixture of products following cytosine deamination[93]. A fourth-generation CBE, BE4, was developed to address this issue, which incorporated a second UGI domain and additional architecture engineering efforts to decrease C•G to non-T•A conversions by a factor of 2 (REF.[93]). It was additionally shown that overexpression of free UGI with BE3 can result in increased product purity[94]. Although these methods demonstrate increased on-target editing in addition to increased product purity, it is important to take into consideration the fact that UGI overexpression may lead to increased genome-wide C•G to T•A editing, which would be problematic for therapeutic applications.

Table 1 | **Compilation of DNA base editor component modifications for various desired outcomes**

Goal	Component modified	User-defined modification	Refs
Decrease gRNA-dependent DNA off-target editing	Cas protein	HF-SpCas9n (D10A, N497A, R661A, Q695A, Q926A)	62,67
	Cas protein	eSpCas9n(1.1) (D10A, K848A, K1003A, R1060A)	68
	Cas protein	SpCas9n-HF2-pBE (D10A, N497A, R661A, Q695A, Q926A, D1135E)	68
	Cas protein	HypaCas9n-pBE (D10A, 2A, N694A, Q695A, H698A)	68
	Delivery	mRNA or RNP delivery	131,132
Decrease gRNA-independent DNA off-target editing	ssDNA cytidine deaminase	AALN APOBEC1 (R33A, K34A, H122L, D124N)	76
	ssDNA cytidine deaminase	YE1 rAPOBEC1 (W90Y, R126E)	82
	ssDNA cytidine deaminase	PpAPOBEC1 (wild type, H122A, and R33A)	77
	ssDNA cytidine deaminase	RrA3F (wild type and F130L)	77
	ssDNA cytidine deaminase	AmAPOBEC1	77
	ssDNA cytidine deaminase	SsAPOBEC3B (wild type and R52Q)	77
	ssDNA adenosine deaminase	ABE8e TadA* (V106W)	78,79
Decrease RNA off-target editing	ssDNA cytidine deaminase	SECURE rAPOBEC1 (R33A or R33A and K34A)	80
	ssDNA adenosine deaminase	SECURE ABE7.10 TadA* (V82G or K20A and R21A)	34
	ssDNA cytidine deaminase	YE1 rAPOBEC1 (W90Y, R126E)	82
	ssDNA cytidine deaminase	Human APOBEC3A (R128A or Y130F)	82
	ssDNA adenosine deaminase	ABE7.10 TadA* (F148A)	82
	ssDNA adenosine deaminase	ABE7.10 TadA-TadA* (E50A-V106W)	82
Enhance product purity	Architecture	Add additional UGI domain(s) (1 or 9) to the carboxy terminus	93,97
	Architecture	Add Gam to the amino terminus	93
	ssDNA cytidine deaminase	CDA1	93
	ssDNA cytidine deaminase	AID	93
Broaden activity window via engineered Cas9 variants	Cas protein	VQR-Cas9n (D10A, D1135V, R1335Q, T1337R)	99,101
	Cas protein	VRER-Cas9n (D10A, D1135V, G1218R, R1335E, T1337R)	99,101
	Cas protein	dxCas9(3.7) (D10A, A262T, R324L, S409I, E480K, E543D, M694I, H840A, E1219V)	104
	Cas protein	SpCas9n-NG (D10A, R1335V, L1111R, D1135V, G1218R, E1219F, A1322R, T1337R)	103
	Cas protein	SpG-Cas9n (D10A, D1135L, S1136W, G1218K, E1219Q, R1335Q, T1337R)	105
	Cas protein	SpRY-Cas9n (D10A, A61R, L1111R, D1135L, S1136W, G1218K, E1219Q, N1317R, A1322R, R1333P, R1335Q, T1337R)	105
	Cas protein	SpCP-Cas9 (CP1012 and CP1028 or CP1041)	102,241
Broaden activity window via SpCas9 homologues	Cas protein	SaCas9n (D10A)	93,101,106,107
	Cas protein	SaKKHCas9n (D10A, E782K, N968K, R1015H)	100,101,107
	Cas protein	LbdCas12a (D832A)	79,242
	Cas protein	enAsdCas12a (E174R, S542R, K548R)	79,243
Minimize bystander editing	ssDNA cytidine deaminase	YE1 APOBEC1 (W90Y, R126E)	101
	ssDNA cytidine deaminase	YE2 APOBEC1 (W90Y, R132E)	101
	ssDNA cytidine deaminase	EE APOBEC1 (R126E, R132E)	101
	ssDNA cytidine deaminase	YEE APOBEC1(W90YM R126E, R132E)	101
	ssDNA cytidine deaminase	YFE APOBEC1 (W90Y, Y120F, R126E)	113
	ssDNA cytidine deaminase	CDA1	93
	ssDNA cytidine deaminase	APOBEC3G	93
	ssDNA cytidine deaminase	Human APOBEC1 (N57G)	112
	Architecture	Use PAPAPAP linker	114
	Architecture	Truncate the C terminus of CDA1 (nCDA1Δ195 or nCDA1Δ195)	114
Increase DNA on-target editing	Codon optimization	Use GenScript to select optimal DNA sequence	78,79,137,243
	Architecture	Add NLS with a FLAG epitope tag at the N terminus (FNLS)	78,79,139,243

AID, activation-induced deaminase; As, *Acidaminococcus* sp.; Cas9n, nickase version of Cas9; CDA1, cytidine deaminase 1; CP, circular permutant; e, enhanced; en, engineered; gRNA, guide RNA; HF, high fidelity; Hypa, hyper-accurate; Lb, *Lachnospiraceae bacterium*; NLS, nuclear localization signal; rAPOBEC1, APOBEC1 from *Rattus norvegicus*; RNP, ribonucleoprotein complex; Sa, *Staphylococcus aureus*; SECURE, Selective Curbing of Unwanted RNA Editing; Sp, *Streptococcus pyogenes*; ssDNA, single-stranded DNA; TadA*, mutated TadA (contains ABE7.10 or ABE8 mutations as indicated); UGI, uracil glycosylase inhibitor.

CBEs (and, to a lesser extent, ABEs) will also produce low levels of indels in a locus-dependent fashion. Knockout of UDG decreased, but did not eliminate, CBE-induced indels; therefore BE4-Gam was developed to address this issue[93]. Base editing-induced indels were hypothesized to occur as a result of a base excision repair enzyme, DNA lyase (AP lyase), which converts abasic sites to ssDNA nicks. Due to the use of Cas9n and presence of a single copy of UGI in BE3, the induced ssDNA nick could turn into a DSB following UDG and AP lyase activity (FIG. 1a). The Gam protein from the bacteriophage Mu binds to the ends of DSBs in DNA and can protect them from extensive end processing[95]. Fusing Gam to the amino termini of BE3 and BE4 resulted in creation of BE3-Gam and BE4-Gam, which displayed 1.2-fold and 2.1-fold decreases in indel formation compared with their respective BE3 and BE4 counterparts[93]. In rabbit embryos, BE4-Gam was shown to reduce indel frequency by ~5-fold and increase product purity by ~8-fold across two target genes when compared with BE3 (REF.[96]). A reduction in indel formation by CBEs was also achieved by engineering BE-PLUS (base editor for programming larger C-to-U (T) scope), in which 10 copies of UGI are recruited to CBE via a SunTag system[97,98]. In this study, BE-PLUS demonstrated an average 5.6-fold decrease in indel formation and 3.6-fold decrease in C•G to non-T•A editing across seven target sites as compared with BE3, albeit with an increased activity window[46]. These studies demonstrate the importance of understanding the cellular DNA repair mechanisms involved in base editing outcomes. A more thorough understanding of how our cells process base editing intermediates may additionally inform new therapeutic strategies using base editors.

Broadening the targeting scope

Because of the strict requirement for the target cytosine or adenine to occur in an accessible stretch of ssDNA, there are tight restrictions for gRNA design with base editors. Due to these restrictions, the majority of base editors employ the SpCas9 enzyme that has the simplest PAM requirement of NGG on the 3′ end of the protospacer. The architecture of the SpCas9 R-loop is such that the target cytosine or adenine must occur exactly 12–16 bases away from the NGG PAM, which can make certain target bases inaccessible. To broaden the targeting range of base editors, engineered SpCas9 variants with alternate PAMs were incorporated into the BE3 and ABE7.10 architectures[99–104] (FIG. 3c; TABLE 1). These variants collectively increased the targeting space of base editors to include PAMs of NGAN (VQR-Cas9), NGAG (EQR-Cas9), NGCG (VRER-Cas9) and NG (xCas9 and Cas9-NG). SpG (which recognizes 5′-NGN PAMs) and SpRY (which recognizes 5′-NRN and, to a lesser extent, 5′-NYN PAMs, where R = adenine/guanine and Y = cytosine/thymine) SpCas9 variants were recently developed, which afford efficient base editing when incorporated into both BE4 and ABE7.10 architectures[105]. Additionally, circular permutant SpCas9 variants (CP-Cas9) were successfully incorporated into both CBE and ABE architectures to afford base editors with expanded editing windows[102]. In these CP-Cas9 variants, the location of the N-termini in relation to the R-loop varies, allowing the deaminases better access to the ssDNA and thus a broadened available target space.

In addition to using engineered SpCas9 variants to expand the number of targetable sites, researchers have incorporated other Cas9 homologues as well as Cas12 enzymes into base editor architectures (FIG. 3c; TABLE 1). Specifically, Cas9 from *Staphylococcus aureus* (SaCas9) is another widely used Cas9 homologue that requires a NNGRRT PAM on the 3′ end of the protospacer and is smaller (1053 versus 1368 amino acid residues) than SpCas9. A smaller Cas9 homologue is an attractive candidate for base editor development to ensure easier packaging for therapeutic delivery (discussed later). Incorporating SaCas9 into CBE and ABE architectures resulted in CBEs with higher average base editing efficiencies and wider targeting windows (FIG. 3b), whereas the resulting ABE7.10 construct displayed lower overall editing efficiencies[93,101,106,107]. The increase in editing efficiency for the CBE variants may be due to a larger stretch of exposed ssDNA in the SaCas9 R-loop[108]. The recently developed ABE8 variants were shown to be compatible with SaCas9n and afforded base editing efficiencies comparable with the SpCas9-derived ABE8s (REFS[78,79]). Additionally, incorporation of mutations into SaCas9 to relax the PAM to NNNRRT (referred to as KKH SaCas9) were also compatible with these SaCBEs and SaABEs. In fact, successful editing in mouse and rat embryos with the KKH SaABE variant has been reported[107].

In an example of a non-Cas9-derived base editor, a CBE using Cas12a (previously known as Cpf1), a type V CRISPR effector, has been successfully developed[109,110]. Unlike Cas9 enzymes, Cas12 enzymes have T-rich PAM sequences on the 5′ end of their protospacers (Cas12a recognizes a TTTV PAM sequence, where V = adenine/cytosine/guanine) and cleave the DNA backbone in a staggered manner using a single amino acid residue[110]. As such, generating appropriate nickase Cas12a variants to increase base editing efficiency is not currently possible. Two dCas12a-BE3 variants using the *Acidaminococcus* sp. (As) and *Lachnospiraceae bacterium* (Lb) homologues were therefore engineered, which demonstrated an activity window of positions 8–13 (counting the PAM as positions −4 to −1; FIG. 3b), with editing efficiencies comparable with BE3 (REF.[110]). The first example of Cas12-derived ABEs was recently reported with LbABE8 and AsABE8 variants, which afforded A•T to G•C editing efficiencies up to 29%[78]. This collection of CBE and ABE variants made from different Cas variants demonstrates the modular nature of base editors and their compatibility with a wide range of R-loop structures. Furthermore, Cas12b enzymes, which are often smaller than Cas12a enzymes and thus likely to be more compatible with delivery vehicles, may add to the collection of non-Cas9-derived base editors. We acknowledge early genome editing success using an engineered Cas12b variant isolated from *Bacillus hisashii* (BhCas12b v4) and believe this would be an interesting avenue to explore for future base editors[111]. Most importantly, these established variants collectively allow researchers to design and test multiple gRNA–base editor combinations per target base, which can be

important for balancing high on-target editing with low bystander editing (discussed below) to identify the most appropriate combination for therapeutic applications of base editing.

Minimizing bystander editing

For therapeutic SNV correction, it is necessary for a base editor to only target a single cytosine or adenine within the activity window or induce a synonymous bystander mutation. The most commonly used and well-characterized base editors incorporate SpCas9, which result in a five-nucleotide editing window for CBEs and a four-nucleotide editing window for ABE7.10. It is also important to note that the sequence context of the target cytosine or adenine can affect the size of the activity window. 'Bystander editing' occurs when additional cytosines or adenines beyond the desired target base are present in the base editor activity window and become edited[112]. Whereas bystander edits can be acceptable in cases of gene disruption via the introduction of a premature stop codon or mutation of splice donors and acceptors, in many other instances they must be avoided. Therefore, to minimize bystander editing, several strategies have been employed.

An analysis of bystander editing outcomes revealed that the majority of these edits occurred within the same DNA strand as desired target edits, potentially due to multiple deamination incidents per Cas9 binding event[93]. This led to the hypothesis that bystander edits could be reduced by making the deaminase enzymes less processive. In the first example of protein engineering to reduce bystander editing, mutations were incorporated in the deaminase domain of APOBEC1 to reduce its processivity and, thus, narrow the activity window of BE3 (REF.[101]). The mutations tested were those that were predicted to reduce APOBEC1 activity, alter substrate binding, alter the conformation of bound DNA or decrease substrate accessibility[101]. These studies identified the CBE variants YE1-BE3 (with the mutations W90Y and R126E in rAPOBEC1), YE2-BE3 (W90Y and R132E), EE-BE3 (R126E and R132E) and YEE-BE3 (W90Y, R126E, and R132E; where Y = tyrosine and E = glutamic acid), which collectively display comparable (YE1) or slightly decreased (YE2, EE and YEE) on-target activity to BE3 but with varying levels of decreased bystander editing activities. These variants were later demonstrated to have greatly reduced (and, in some cases, undetectable) levels of both gRNA-independent off-target DNA editing and RNA off-target editing[76]. The YE1 variant was further engineered in a later study to produce YFE-BE4, which maintains high editing efficiency only at positions 4–6 (REF.[113]). It is important to note that the incorporation of these mutations can impart a sequence specificity on the deaminase domain that can affect which position within the editing window is edited with the highest efficiency. This was later employed as a strategy to generate sequence-specific base editors for improved precision[101].

During the initial characterization of BE1, the natural sequence preference of rAPOBEC1 for YC (where Y = thymine/cytosine) motifs was observed to be relaxed; the increased local concentration of rAPOBEC1 relative to the ssDNA of the R-loop as afforded by dCas9 resulted in editing at non-YC motifs, but with relative efficiencies in the order of TC > CC ≥ AC > GC[25]. Subsequent studies on CBEs with different cytidine deaminases (such as CDA1, AID, APOBEC3A, APOBEC3B, APOBEC3H and APOBEC3G) revealed similar trends among their sequence preferences[93]. These natural sequence context preferences were combined with rational engineering to produce eA3A-BE3, which displays a >40-fold higher precision than BE3 with a strong sequence preference of TCR > TCY > VCN[112]. In contrast, most engineering efforts on the deaminase domain of ABEs have focused on eliminating sequence motif preferences, as early-generation ABEs displayed low base editing efficiencies at non-YAC motifs. As such, ABE7 and ABE8 variants have very little sequence context preference, with each variant displaying a slightly different activity window. In general, ABE8 variants display slightly broadened activity windows as compared with ABE7.10, including the SaCas9-derived ABE8s (REFS[78,79]).

Additional engineering strategies that can alter the base editing activity window are linker and architecture modulation. For example, incorporation of the seven-amino-acid-long rigid linker PAPAPAP (where P = proline) between the deaminase and Cas9n afforded a CBE with an activity window of only two bases (positions 5 and 6)[114]. Additionally, tethering the deaminase to the carboxy terminus of Cas9n has been shown to shift the activity window to positions 3 and 4 (REF.[114]). These extensive engineering efforts underscore the importance of selecting the correct gRNA–BE variant combination for each individual application. If bystander editing must be avoided, it is recommended to test alternative Cas enzymes with differential PAM sequences in combination with deaminase mutants to 'push' the bystander base(s) out of the activity window. In particular, SpRY-derived base editors can be used to test multiple gRNAs that position a given target base in nearly all possible window positions by 'tiling' protospacers. The development of sequence-specific deaminases in combination with this tiling method would allow for the identification of the optimal gRNA–BE combination for a given target. Again, the extent to which bystander editing is acceptable will be dependent on the specific application.

New and emerging subcategories

Recent reports have described the development of 'dual base editors', in which the deaminase domains of ABEs and CBEs are combined into a single base editor that can introduce C•G to T•A and A•T to G•C mutations simultaneously[115–118]. Three of these dual base editors (synchronous programmable adenine and cytosine editor (or SPACE), A&C-BEmax and Target-ACEmax) were engineered for use in mammalian cells, and one (saturated targeted endogenous mutagenesis editor, or STEME) for use in plants. The mammalian dual base editors reported similar overall average on-target editing efficiencies to each other, with varying levels of simultaneous editing events. Furthermore, it is important to note that, depending on the number of target adenosines and cytosines within the editing window, many editing outcomes are possible. In a demonstration of their therapeutic potential, A&C-BEmax was used to

mutagenize the *HBG1* and *HBG2* promoter to reactivate fetal haemoglobin as a strategy to treat β-thalassaemia[116].

A final expansion of the base editor toolkit are a set of C•G to G•C base editors[119–121]. The C-to-G base editor 1 (CGBE1) consists of the UDG enzyme from *E. coli* (eUNG) and the rAPOBEC1 (R33A) variant fused to the N terminus of Cas9n (REF.[119]). The same study also yielded a miniCGBE1 (in which the eUNG component was omitted), which showed a slight decrease in C•G to G•C editing efficiency as compared with CGBE1, with a concurrent significant decrease in indel formation. CGBE1 on-target editing efficiency was tested across 25 genomic loci and showed that the editing window for this base editor is also protospacer positions 5–8, with position 6 being optimal. It is important to note that on-target editing efficiency varied drastically from one locus to another[119]. Another CGBE uses the BE3 architecture, with the UGI component replaced by the DNA repair protein rXRCC1 (REF.[120]). Data show that this CGBE has a preference for protospacer positions 5 and 6 and the sequence motifs WCW, ACC and GCT for the most efficient and precise C•G to G•C editing[120]. The final CGBE variant (named GBE, for glycosylase base editor) comprised a rAPOBEC–Cas9n–UNG fusion and also displayed a preference for on-target editing at protospacer position 6, with a range of efficiencies depending on the locus[121]. The same study also reported a C•G to A•T editor (AID–Cas9n–UNG) for use in bacterial cells with an average on-target editing efficiency of 87.2%. All three of these new editors rely on efficient excision of the uracil intermediate followed by mutagenesis across the resulting abasic site. These promising results demonstrate the importance of follow-up studies to evaluate their off-target effects, as well as the 'rules' governing the drastic differences in on-target editing observed at different loci.

Further expanding the suite of base editor tools to provide researchers with multiple options is a logical and significant avenue to explore in future studies. To aid in this expansion, several groups have developed an array of computational tools to increase the accessibility of base editor technology (BOX 1). This vast array of DNA base editor variations that have been engineered and implemented (TABLE 1), along with future tools not yet reported, to improve on-target editing efficiencies and production distributions and to decrease off-target editing efficiencies, demonstrates the potential for deployment of base editors as therapeutic devices. A further means of varying base editors comes from the incorporation of nuclear localization signals and codon optimization, which are discussed below. A final step in that deployment is selection of the appropriate delivery method.

Box 1 | **Computational tools to aid in base editor experimental design**

To further the accessibility of base editor technologies, efforts have been taken by several groups to aid other laboratories with guide RNA (gRNA) design, data analysis and base editor outcome predictions. Specifically, gRNA design tools such as BE-Designer, beditor and BE-Hive have been developed to identify the most appropriate gRNA to use to introduce a single-nucleotide variant of interest[246–248]. gRNA selection is a key step to ensure optimal base editor activity, and, in many instances, multiple base editor–gRNA combinations can theoretically be used to install the same edit. These open-sourced software programs provide the user with key information such as predicted on-target efficiencies, gRNA-dependent off-targets and C•G to non-T•A editing efficiencies, which help ensure the most optimal desired outcome. Additionally, CRISPResso was recently updated to enable facile quantification of base editing efficiencies from next-generation sequencing data. These online-based tools are powerful resources not only for laboratories beginning their respective forays into base editor-based studies but also for experienced laboratories looking to streamline their experimental timelines.

Online tools for gRNA design are perhaps even more useful for the prime editor-equivalent pegRNAs. Despite the similarities between base editor and prime editor technology, one standout difference is the complexity of pegRNA design for successful prime editor experiments. Given the young age of this technology, the rules of pegRNA design for optimal outcomes are not fully understood. However, recent tools such as multicrispr and PrimeDesign are welcome additions to the suite of base editor gRNA design software[249,250].

Base editor delivery strategies

Efficient in vivo delivery of base editors to the proper tissue(s) of interest is essential for their use as therapeutics (FIG. 4; TABLE 2). Furthermore, the delivery method must demonstrate a fine balance between maximizing on-target efficiency and minimizing (or eliminating if possible) any and all off-target activity. It has been extensively reported that various base editor delivery methods can have a profound effect on genome editing specificity. Specifically, in cell lines, prolonged base editor expression results in higher off-target editing outcomes, highlighting the importance for timely control of base editor expression during in vivo genome editing[67,78]. There are several additional considerations to be made when choosing the optimal delivery vehicle for therapeutic base editing, including the type of cargo to be delivered (that is, base editors delivered as DNA, mRNA or RNP), target environment (that is, the liver versus crossing the blood–brain barrier), desired outcome (that is, gene knockout or inducing a targeted point mutation to reverse a disease SNV) and compatibility between the chosen base editor modality and potential immune responses to the treatment (both to the delivery agent and to the base editor itself). To date, base editors have successfully been delivered to various organisms including mice, rats, zebrafish and discarded human embryos[122–127].

Nucleic acid-based delivery methods

Direct delivery of nucleic acids. The most commonly used delivery methods for base editors directly deliver a nucleic acid encoding for the base editor and the gRNA using cationic lipids, electroporation or direct injection (FIG. 4). These non-viral nucleic acid delivery methods are advantageous as there are minimal size restrictions on the payload and the risk of genomic integration is nearly zero[128] (TABLE 2). The nucleic acid can be in the form of a plasmid or linear DNA encoding both base editor and gRNA, or as base editor mRNA and synthetic gRNA[129]. It is important to note that plasmids, which are primarily composed of bacterial DNA containing unmethylated CpG motifs, can stimulate a multifaceted immune response in vivo[130]. Delivering base editors as DNA requires both transcription (and, thus, nuclear delivery) and translation prior to generation of active base editor–gRNA complex, resulting in a slightly longer time frame between delivery and onset of genome editing compared with mRNA-based and RNP-based delivery[131].

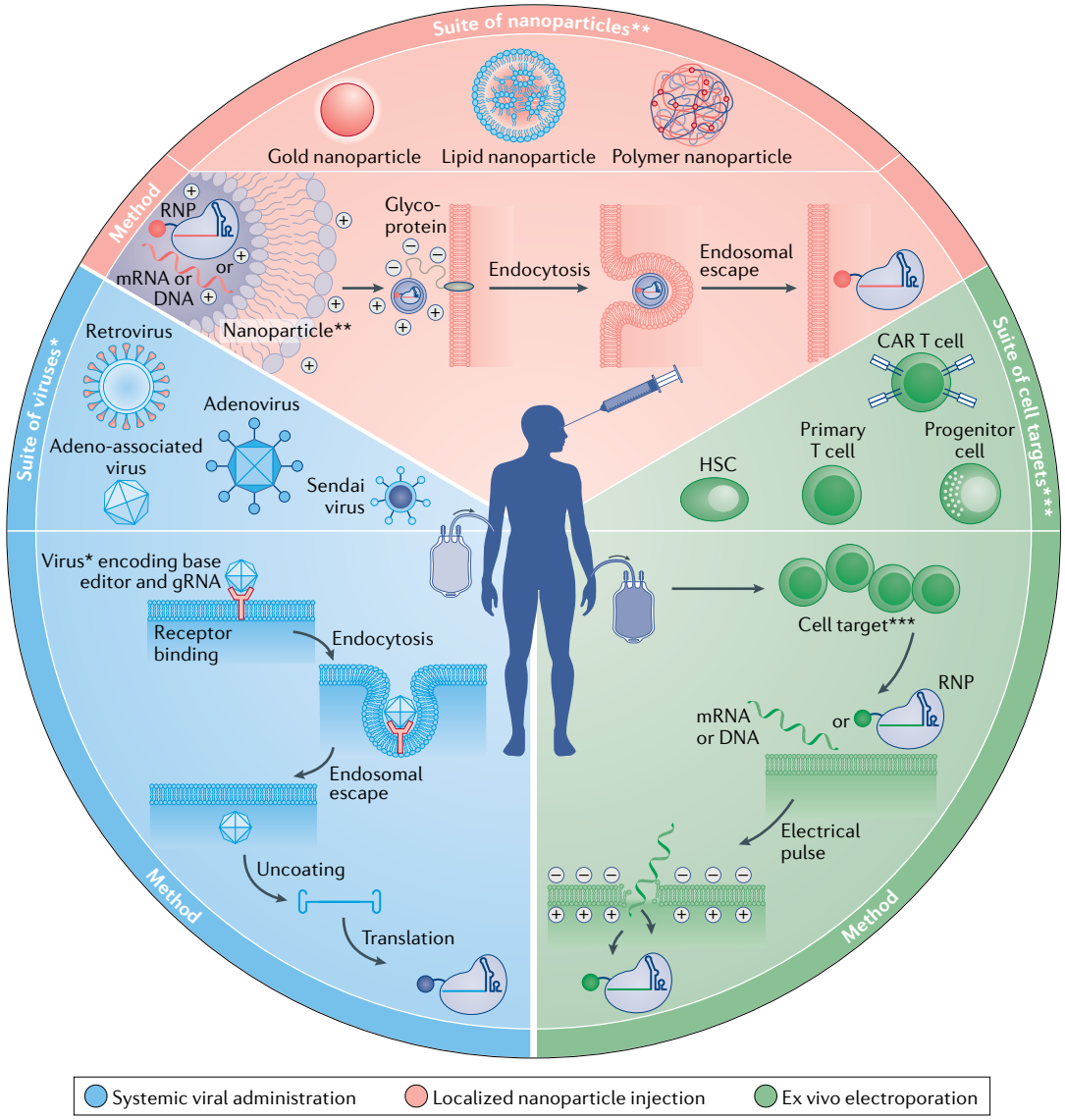

Fig. 4 | **Base editor delivery strategies.** Localized nanoparticle injection (red), systemic viral administration (blue) and ex vivo electroporation (green) collectively make up the three primary means to potentially administer base editor therapeutics. Each methodology is shown with the corresponding range of delivery vehicles or target cells. Red and green panels show base editor delivery through nucleic acid (i.e. mRNA or DNA) or RNP format. *, suite of virus options; **, suite of nanoparticle options; ***, suite of cell target options; CAR, chimeric antigen receptor; gRNA, guide RNA; HSC, haematopoietic stem cell; RNP, ribonucleoprotein complex.

Additionally, due to the increased inherent stability of DNA compared with RNA, DNA-based delivery of base editors results in prolonged expression of the base editor, often causing higher levels of off-target editing. However, DNA-based delivery is time and resource-effective, making it an attractive delivery method. Delivery of base editors via mRNA has the advantage of being able to express protein quickly as transcription is not required[132]. This initial burst of activity is followed by relatively quick degradation of the mRNA, which reduces off-target editing rates. However, its decreased stability can sometimes be problematic, requiring chemical modifications to maintain desired levels of on-target activity. Additionally, if left unmodified, mRNA can illicit an immune response[133,134].

For both DNA and RNA, codon optimization can be a major determinant of intracellular protein expression levels following delivery, even among different cell types or tissues of a given organism. As such, care must be taken to employ the most appropriate codon usage for the given organism and desired application[135,136]. Together with the incorporation of nuclear localization signals into base editor architecture, codon optimization greatly affects base editing rates. Base editing efficiency by BE4 and ABE7.10 was shown to be hampered by poor nuclear expression levels[137]. A bipartite nuclear localization signal (bpNLS) at both the N and C termini of the BE4 construct resulted in a 1.3-fold average increase in editing efficiency[137]. Subsequently, codon re-optimization of the bpNLS-BE4 construct using

various methods demonstrated additional improvements in base editing efficiencies as compared with the original BE4 construct[137,138]. Ultimately, the BE4 construct using the GenScript codon optimization method displayed the highest editing efficiency, and was termed BE4max (REF.[137]). The same optimization was applied to ABE7.10 to generate ABE7.10max, which also displayed enhanced base editing efficiency and modest increases in indel formation[137]. Concurrently, re-codon optimized BE3 has been generated to afford higher editing efficiency across a wide range of mammalian cell types and organoids[139]. The new BE3 construct demonstrated an

Table 2 | **Overview of base editor delivery strategies**

Class	Delivery vehicle/ method	Summary of technical aspects	Benefits	Challenges	Refs
Nucleic acid (plasmid DNA or mRNA)	Cationic lipid	Encapsulation of DNA or mRNA in lipid spheres Relies on endocytosis and endosomal escape	Relatively easy production Many possible formulations	Immunogenic Mostly ends up in the liver	142,183,184
	Lipid nanoparticles	Encapsulation of DNA or mRNA in lipid nanoparticles Relies on endocytosis and endosomal escape	Relatively easy production Many possible formulations	Immunogenic Mostly ends up in the liver	141,230
	Electroporation	Direct delivery to the cytoplasm via membrane disruption	Very efficient Minimal size restriction	Low throughput Only practical for ex vivo therapeutics Requires specialized equipment	143,144
	Direct administration	Systemic delivery of naked DNA or RNA	Simple administration	Only works for targeting hepatocytes Immunogenic	139–141
	Cell injection	Direct injection into the cell nucleus or cytoplasm	Effective	Extremely low throughput and viability	91,146–149
Viral (cell surface receptor mediated entry by viruses)	Adenovirus	Double-stranded DNA virus Non-enveloped ~8–10-kb maximum packaging limit	Large cargo size	Immunogenic	152,164,165
	Adeno-associated virus	Single-stranded DNA virus Non-enveloped 4.7-kb maximum payload size	Comes in many engineered serotypes	Immunogenic, cargo size limited Requires inteins for base editor delivery	129,150, 151,153
	Retrovirus	Broad class that includes lentivirus	Large cargo size Broad targeting and pseudo-typing	Non-specific DNA integration creates an oncogenic risk, immunogenic	129,163
	Lentivirus	Retrovirus Enveloped 9.4-kb packing limit	Large cargo size Broad targeting and pseudo-typing	Non-specific DNA integration creates an oncogenic risk, immunogenic	129,163
	Sendai	Minus-strand RNA virus Unclear packaging limit ~5 kb	RNA virus removes risk of DNA insertion	New viral vector requires more validation and characterization	129,163
Protein (base editor protein pre-complexed with guide RNA)	Lipid nanoparticles	Encapsulation of protein and RNA in lipid nanoparticles	Controlled dose and timing of base editor minimizes off-targets	Immunogenic	129,141,196
	Cationic lipids	Encapsulation of protein and RNA in cationic lipids	Controlled dose and timing of base editor minimizes off-targets	Immunogenic	142,183,184
Other (emerging or potential strategies for base editor delivery)	DNA cages	Particles built using DNA origami	Engineerable DNA-based scaffold	Preliminary technologies have not been shown to work with base editors	205–207
	Virus-like particles	Protein cages from viral sources or computationally designed	Engineerable protein-based scaffold	Preliminary technologies have not been shown to work with base editors	205–207,238
	Cell-penetrating peptides	Direct fusions of peptides that facilitate cell entry to cargo	Simple implementation	Preliminary technologies have not been shown to work with base editors	244,245
	Physical	Physical disruption of cells using nanowires, hydrostatic injection or cell squeezing to help base editors enter the cell	Physical methods bypass endosomal entry pathways	Preliminary technologies have not been shown to work with base editors	244,245

increased on-target editing efficiency of up to 30-fold, while maintaining low indel formation[139]. This same study found that positioning the NLS at the N terminus with a FLAG epitope tag (creating FNLS-BE3) increased editing efficiency an additional 2-fold[139]. These two optimized variants were used in vivo in mice to model hepatocellular carcinoma[139]. The improvements of BE4max and ABE7.10max demonstrate the importance of obtaining sufficient expression levels of genome editing agents to achieve desired editing levels.

Injection of naked plasmid DNA into the tail vein of animals is sufficient for in vivo delivery into hepatocytes. This method was employed to correct an adult mouse model of tyrosinaemia using ABEs and to model cancer-relevant mutations in the *CTNNB1* gene in mice[139–141]. For delivery to other cell types and organs, however, a delivery vehicle is necessary. Delivery of base editor-encoding nucleic acids can be achieved by packaging in ionizable cationic lipid nanoparticles (cationic lipids), targeted electroporation and direct injection (FIG. 4). Cationic lipids will form liposomes with the negatively charged nucleic acid complexed inside, facilitated by electrostatic interactions with the cationic headgroup. The lipid bilayer protects the packaged cargo from degradation and is taken up into cells via endocytosis. Delivery of the nucleic acid into the cytosol then occurs upon endosomal escape[142]. This delivery method is mostly employed in vivo to deliver base editors as RNPs, and is discussed below. Electroporation is a method that uses pulses of electric currents to create transient breaks in cellular membranes to allow for entry of foreign material[143]. In vivo electroporation is rare, given the invasive nature of the delivery method, but has been used to deliver Cas9 to mouse and rat embryos[144]. However, electroporation has been used to deliver BE3 and BE4 mRNA with chemically modified gRNAs ex vivo into primary T cells, resulting in over 90% multiplexed C•G to T•A editing at three different genes simultaneously[145]. In fact, a rigorous off-target analysis of both wtCas9 and BE4 revealed that when using three optimal gRNAs that resulted in >90% on-target editing efficiencies by both wtCas9 and BE4, indel formation by wtCas9 was observed at only two sites (with efficiencies of 0.2% and 13.1%) and C•G to T•A base editing was observed at only one site (with 0.9% efficiency)[145]. This same method was also used to deliver ABE8 variants both into human haematopoietic stem cells to induce targeted A•T to G•C mutations into the promoter regions of the *HBG1* and *HBG2* genes and into primary human T cells to induce knockdown of six different genes through splice site disruption[78]. Finally, direct microinjection of nucleic acids is an option at the single-cell stage and has been successfully demonstrated with both CBEs and ABEs to generate disease models[91,146–149].

Viral vector delivery method. Viral-based delivery vehicles are a popular modality for delivery of genome editing agents[12,129,150–162]. Viral vectors used for delivery of genome editor tools include adenovirus (AdV), adeno-associated virus (AAV), lentivirus, Sendai virus and retrovirus[129,163]. Viral transduction requires recognition between the viral particle and target cell receptor, followed by viral vector uncoating, cargo transport and release, and transgene transcription and translation[129] (FIG. 4). AdV has successfully been used to deliver base editors in utero and in adult mice; however, given the inclination to induce an innate immune response, AdV is not an ideal delivery vehicle for human therapeutics[152,164,165]. AAVs are the most promising choice due to their low immunogenicity and toxicity, and their transient gene expression[129] (TABLE 2). Additionally, owing to their popularity, multiple AAV serotypes have been identified with varying tissue tropisms, which can provide tissue specificity[166]. AAVs have been used to deliver genome editors to various human tissues, including the brain, cardiac and non-cardiac muscle, and the eye[150,151,153,166]. AAVs have also been used to deliver antisense oligo-derived RNA base editors in vivo in mice to correct models of ornithine transcarbamylase deficiency and Duchenne muscular dystrophy (DMD)[167,168]. Despite these advantages, using AAVs as a therapeutic delivery tool for DNA base editors is problematic given their low packaging capacity of 4.7 kb[169]. The size of wtSpCas9 (the most commonly used variant for DNA base editors) is 4.3 kb, making packaging of the base editor, gRNA, appropriate promoters and necessary viral elements impossible due to exceeding the size limit. Therefore, split-intein base editors have been developed by multiple groups to circumvent the limited AAV packaging capacity[170–174]. In these studies, the base editor is divided into N-terminal and C-terminal fragments that are then each fused to half of the fast-splicing split inteins[171]. Split-CBE and split-ABE variants have been delivered to the brain, liver, retina, heart, skeletal muscle and inner ear of mice to correct amyotrophic lateral sclerosis, phenylketonuria, Niemann–Pick disease type C and genetic deafness[170–175]. Additional efforts to treat hearing loss using base editors are discussed below.

It is important to note that despite their reputation as low-immunogenic delivery agents, antibodies against both AAV capsids and the transgenes that they deliver can still be elicited by the host, which would prove particularly problematic for therapeutic applications in which multiple doses of a base editor are required[176,177]. Furthermore, recent studies have demonstrated that healthy humans harbour pre-existing antibodies against SpCas9 and various AAV serotypes, which may significantly reduce the efficacy of AAV-mediated delivery of base editors via neutralization of the vector and/or the base editor protein[178–181]. Additionally, a pre-existing effector T cell response against SpCas9 has been reported[182]. These immune response challenges, which exist regardless of the delivery method, must be taken into consideration when developing strategies for therapeutic base editing. We would also like to emphasize that no studies have been published examining the extent to which the deaminase domains of base editors, or the UGI component of CBEs, affect immune response(s).

Protein-based delivery methods

Lipid-based delivery of ribonucleoprotein complexes. The final format for base editor delivery is as a pre-complexed RNP, which allows for the quickest in vivo response. Cho et al. report the first instance of Cas9

RNP delivery via direct injection into the germline of *Caenorhabditis elegans*, and Cas9 RNP delivery to somatic cells was achieved 2 years later, facilitated by cationic lipids[142,183,184]. The negative charge of the gRNA allows the RNP to become complexed inside the liposomes formed by these reagents, resulting in efficient intracellular delivery. Importantly, RNP delivery of genome editing agents affords even faster initial rates of editing than mRNA-based delivery. Due to the reduced intracellular residence time of the genome editing agents, RNP-mediated delivery also affords better specificity profiles. Finally, delivery of base editors as RNPs ensures temporal coordination of the base editor system components[16,185–187]. In vivo RNP delivery of base editors to postmitotic cells of the inner ear was recently reported, with editing efficiencies high enough to observe a phenotypic response[67,188]. Importantly, this highlighted the ability of base editors to perform genome editing in non-replicating cells in vivo. In addition, electroporation of BE3 RNP has been used for ex vivo editing of haematopoietic stem and progenitor cells to introduce targeted C•G to T•A mutations into the *BCL11A* erythroid enhancer and correct a pathogenic mutation in the *HBB* promoter[189].

Nanoparticle delivery method. Nanoparticle delivery vehicles have been gaining popularity to address the pitfalls associated with use of viral vectors as genome editor delivery vehicles[132,142,190–195]. Whereas lipid-derived nanoparticles display a certain degree of similarity to liposomes, lipid-derived nanoparticles can take various forms, making them an attractive delivery vehicle for genome editing tools (FIG. 4). Particularly, nanoparticles can be specially designed to optimally suit their cargoes and their immune-compatibility can be optimized through design changes to the size, shape and coating[129,196]. The unique biological fingerprint assigned to the nanoparticle (the protein 'corona') will have specialized, albeit potentially unpredictable, interactions with the native cellular environment[197,198]. For safe therapeutic use, interactions at the nanoparticle–biological interface need to be well understood and rigorously tested to pre-emptively determine the in vivo outcome of these nanoparticles[199]. Several varieties of nanoparticles exist for genome editor delivery: gold nanoparticles, polymer nanoparticles, lipid and viral nanoparticles, and magnetic nanoparticles. Depending on the type of payload being delivered (that is, as DNA, mRNA or protein), different nanoparticles would have optimal compatibility. Recently, lipid nanoparticle-mediated delivery was used to deliver ABE mRNA and chemically modified gRNA for in vivo delivery to the liver of mice[141].

Additionally, poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles have long been established as an FDA-approved viable tool for the delivery of drugs and materials in the human body given their highly compatible toxicity profile[200,201]. In their native negatively charged state, PLGAs are not well suited for cellular uptake. Similar to the cationic lipid delivery methods discussed previously, cationic lipids can be incorporated into the PLGA system to assist with cellular uptake. Cationic cholesterol-assisted PLGA nanoparticles have been successfully used to deliver Cas9 mRNA and plasmid DNA[202,203]. Various additional nanoparticle delivery methods have been used to deliver Cas9 RNPs, such as nanocrystals, CRISPR–gold and Cas9–gRNA crosslinked in polyethyleneimine hydrogels[89,192,195,204]. However, these methods have yet to be explored for base editors. Protein cages that could be used as synthetic virus-like particle delivery vehicles and DNA cages created by DNA origami, in their nanoparticle-like capacity, could potentially be used to deliver base editors[205–207] (TABLE 2).

Therapeutic opportunities of base editors

Although the sheer number of base editor variants and delivery strategies may seem inordinate, each disease target will require a unique combination of base editor, gRNA or ASO and delivery method. Specifically, the requisite level of on-target efficiency to afford a phenotypic response will vary drastically according to the disease. Additionally, due to the transient nature of RNA editing, it must be paired with a delivery method that is amenable to a repeated dosage. The disease will also dictate to which tissue or organ the base editor must be delivered, which in turn will determine what delivery method must be used. The genetic diversity of humans adds yet another layer of required customization: it is possible that two individuals who require correction of the same SNV will need different gRNA sequences[208]. Below, we illustrate these points by discussing specific therapeutic opportunities for DNA and RNA base editors.

Cancer immunotherapy

One exciting therapeutic opportunity for which base editors have already established their application is in ex vivo engineering of allogenic chimeric antigen receptor (CAR) T cells for use in cancer immunotherapy. In allogenic CAR T cell therapy, T lymphocytes are collected from a healthy donor and genetically engineered (either through retroviral transduction or Cas9/HDR-mediated genome editing) to produce CAR T cells[209]. These CAR T cells are engineered to activate the T cell's immune response upon recognition of specific tumour cell antigens, thus resulting in tumour cell-specific cytotoxicity[210]. Manufacturing CAR T cells from healthy donors (allogenic CAR T cells) rather than patients (autologous CAR T cells) is preferred as it is cheaper (multiple therapeutic doses can be generated from a single donor), faster (the patient could receive a treatment from a CAR T cell 'bank' immediately upon requiring one) and safer (the quality of autologous T cells is often compromised due to cancer and/or previous chemotherapeutic treatments)[211]. However, autologous CAR T cell treatments can cause immune responses in the patient, either by the patient's immune cells attacking the CAR T cells or the CAR T cells attacking the patient's healthy cells[212]. Knockout of the endogenous T cell receptor and major histocompatibility complex (MHC) class I and class II molecules of the CAR T cells can alleviate these issues[213]. Ex vivo multiplexed gene knockout (that is, knockout of multiple genes at a time within the same cell) of T cells is a unique therapeutic opportunity, as delivery can be achieved relatively easily via

Box 2 | **Base editor screens further expand technologies' utility**

The use of cytosine base editors that lack a uracil glycosylase inhibitor (UGI) component have been used as targeted random mutagenesis tools. Two such strategies to generate localized sequence diversity have been developed: targeted activation-induced deaminase (AID)-mediated mutagenesis and CRISPR-X[90,92]. Both systems maintain a slight preference for C•G to T•A editing outcomes but do produce a distribution of products, with mutagenesis rates in the order of ~1/500–1/1,000 bp^{-1} (REFS[90,92]). These methods have shown great promise for generating genetically encoded libraries without relying on homology-directed repair[251]. Large-scale 'base editor screens', in which a library of guide RNAs (gRNAs) is combined with a base editor to introduce thousands of single-nucleotide variants into a population of cells, have also been developed recently and utilized in the functional genomics space. These screens range from low-resolution genome-scale studies (in which individual genes are knocked out via the introduction of premature stop codons) to higher-resolution 'saturation mutagenesis-like' studies (where gRNAs are tiled across a gene to probe protein function, in both human cells and yeast)[215,252–255]. However, as discussed in BOX 1, optimal base editor–gRNA selection is imperative to achieve efficient base editing. We therefore anticipate that additional studies that design, test and validate base editor–gRNA combinations in high throughput (as has been done with CRISPR interference and CRISPR activation screens)[256–260] will significantly expand the utility of base editor screens. Recent advances such as the growing suite of base editor gRNA design tools and the constant generation of new base editor tools have aided researchers greatly in these regards, but there is still much work to be done towards true saturation mutagenesis using base editing, with the ultimate goal of dissecting gene function.

electroporation of mRNA or RNP[214]. Multiplexed gene knockout using traditional, DSB-reliant genome editing agents can result in high levels of toxicity and large-scale chromosomal rearrangements, although these have not prevented advancement of clinical trials (PMID: 32029687). Base editors circumvent chromosomal rearrangements by introducing a premature stop codon or splice site disruption to knock out the gene of interest[215]. Indeed, proof-of-concept studies have already demonstrated high-efficiency multiplexed gene knockout with no large-scale chromosomal rearrangements in T cells using both CBEs and ABEs[78,145]. Because gene knockout is the ultimate outcome, bystander mutations do not need to be rigorously avoided. Importantly, cell sorting can be used to isolate only the cells with all desired genes knocked out, resulting in 100% editing efficiency. This is an exciting therapeutic avenue in which we see base editing making an immediate and significant impact.

Hearing loss

A major distinction between DNA and RNA editing is the transient nature of RNA editing; prolonged RNA editing would require repeated dosage or integration of a transgene encoding the RNA editor. However, there are certain therapeutic targets in which the reversibility of RNA editing is desirable. For example, sensorineural hearing loss is a common disease caused by ageing, exposure to loud noise, congenital abnormalities and ear infections. These types of ear trauma can damage the sensory hair cells and cochlear supporting cells of the ear, which are postmitotic and thus cannot regenerate once damaged[216]. Stimulation of the Wnt signalling pathway has been shown to facilitate reprogramming of supporting cells into sensory hair cells in multiple studies[188,217]. Furthermore, Wnt signalling can be stimulated either by deleting exon 3 of β-catenin or by mutation of key phosphorylation residues of β-catenin[218,219]. However, it is important to note that permanent or widespread upregulation of Wnt signalling can be oncogenic[220]. Thus, precise spatial and temporal control over editing of β-catenin is essential to ensure sufficient proliferation of supporting cells for hearing loss reversal, with no possibility of oncogenesis. This therapeutic opportunity would therefore benefit from an RNA base editing approach. Additionally, strategies for localized delivery of otoprotective drugs, mRNA and RNPs to inner ear cells via nanoparticles have been established and shown to be effective[221,222]. Mutation of key phosphorylation residues using A-to-I RNA base editors or RESCUE (which has been used to stimulate Wnt signalling in HEK293TF cells via editing of residue Thr41), or skipping of exon 3 by mutating the splice acceptor (AG to IG) using an A-to-I RNA base editor, would result in temporary upregulation of Wnt signalling, without any extreme alterations to overall β-catenin levels, and generation of sensory hair cells from supporting cells[54]. Once the RNP or mRNA has degraded, Wnt signalling levels would be back to normal, thus minimizing the likelihood of oncogenesis. In addition to affording spatial and temporal control of editing, RNA editing offers an opportunity to fine-tune intracellular editing levels due to the presence of multiple mRNA transcript copies per cell. Titration of editing levels in model systems would allow for identification of the ideal editing levels for therapeutic applications. This demonstrates the utility of an RNA base editing approach when permanent alterations to the genomic sequence would result in potentially deleterious consequences.

Duchenne muscular dystrophy

As mentioned previously, a key issue that faces any protein-based therapeutic (particularly those that are not derived from humans, such as Cas proteins) that requires repeated administration is immunogenicity. After initial administration, the patient's immune system will activate and generate neutralizing antibodies, rendering any future treatments ineffective[223]. Whereas DNA base editors introduce permanent changes and, thus, will only require a single treatment once optimized, CRISPR-derived RNA base editors may face this additional immunogenicity hurdle in the clinic if repeat dosages are needed. However, most ASO-dependent RNA base editors are derived from human ADAR enzymes and therefore would evade this issue. Additionally, ASO-dependent RNA base editors have relatively small construct sizes and could therefore be packaged into a single AAV vector. For these reasons, we believe ASO-dependent base editors could be well suited to tackle diseases that require targeted delivery to large organs or tissues. For example, DMD is a neuromuscular disease caused by mutations in the *DMD* gene, the largest known human gene. *DMD* encodes for the protein dystrophin, which connects the cytoskeleton to the extracellular matrix in muscle cells[224]. DMD patients generally die by age 25 years due to degeneration of skeletal and cardiac muscles[224]. Nonsense mutations in *DMD* account for 10% of cases, and a current treatment for these cases of DMD is stop codon read-through therapy[225]. A-to-I RNA base editors can be used to convert premature stop codons to Trp, and this was recently demonstrated

Prime editing
A recently developed genome editing technology that, like base editing, does not rely on double-stranded breaks.

in vivo in a mouse model for DMD, where dystrophin restoration was demonstrated[168]. An additional 70% of cases can be treated via exon-skipping strategies, which could also be accomplished with A-to-I RNA base editors[226]. Furthermore, several AAV serotypes exist that afford delivery to muscle cells and have already been validated in human patients[227]. Furthermore, DMD is a monogenic recessive disease, and it is estimated that correction levels as low as 15% can restore dystrophin expression levels to normal[228,229]. There is currently a dire need for the development of treatments for this devastating disease, and we believe RNA base editors are ideally poised to help.

Rare liver diseases

Another exciting therapeutic opportunity for base editors is rare liver diseases. Lipid nanoparticle-mediated delivery of nucleic acids to liver hepatocytes in vivo has been established and offers several advantages to viral delivery methods, such as larger payloads, cheaper and faster manufacturing options, and reduced likelihood of an immune response[230]. For example, Crigler–Najjar syndrome type I is a rare autosomal recessive genetic disorder caused by mutations in the *UGT1A1* gene, which encodes for the liver enzyme uridine diphosphate-glucuronosyltransferase family 1 member A1 (UGT-1A)[231]. Mutations that inactivate this enzyme result in build-up of bilirubin (a component of bile), causing death within the first year of life unless diagnosed and treated[231]. The current treatment options for Crigler–Najjar syndrome are aggressive phototherapy, plasmapheresis and liver transplantation[232]. UGT-1A normally converts bilirubin to its water-soluble form in liver cells, and thus its expression is mostly limited to the liver[233]. Various mutations in *UGT1A1* have been identified to cause this disease, many of which can be corrected with a CBE or ABE. We envision that lipid nanoparticle-mediated delivery of DNA base editor RNPs may afford correction at sufficient levels to alleviate symptoms of this autosomal recessive disease. Although unique base editor–gRNA combinations will need to be designed and validated for each case, we believe this is an important disease target to tackle, given its severity combined with the lack of treatment options that do not severely reduce the quality of life of the patients.

Future outlook

The relevance of SNVs to human health cannot be overstated; there are currently over 685 million human SNVs identified in the dbSNP database and this number will only increase with time[234]. Along these lines, we believe that base editor technologies can aid in therapeutic development beyond in vivo single base pair correction. Particularly, base editor screens are a promising way to interrogate the functional consequences of point mutations, which would further aid in genetic disease diagnosis, and the identification of potential treatments (BOX 2). As such, significant resources have been devoted to the development of tools and methods for introducing this type of genetic variation, including both DNA and RNA base editors. The large number of improvements and iterations on the original DNA and RNA base editors highlights the enthusiasm of the field for these genome editing tools, as well as their value as both basic research tools and therapeutics (TABLE 1). It is particularly important to note that widespread engineering efforts from various groups have addressed many of the limitations of the technology almost as quickly as they have been identified (such as gRNA-dependent and gRNA-independent DNA off-targets, RNA off-targets, bystander editing and limitations to the types of SNVs that can be introduced). One major limitation that has yet to be solved is the inability of base editors to catalyse transversion mutations. We anticipate that creative strategies based on DNA repair manipulation (given the observance of C•G to non-T•A mutations with CBEs) and/or new nucleic acid modification chemistries will combat this limitation in the future. This wide array of base editor tools nicely illustrates their relevance to the field of precision medicine; genetic disease treatments must be tailored to the genomic sequence of the individual whom they are intended to cure. However, we would like to acknowledge the ethical implications of genome editing technology with respect to clinical treatment (BOX 3).

To further underscore the importance of non-DSB-inducing genome editing tools, a new method has been developed, termed 'prime editing'[235]. Prime editing can mediate targeted insertions and deletions and all possible SNVs in human cells[235]. The prime editor architecture consists of a reverse transcriptase (RT) fused to Cas9n and requires a modified gRNA (termed a prime editing gRNA, or pegRNA) that contains a 3′ extension on the canonical gRNA. Prime editors show great therapeutic potential, but more studies are needed to assess off-target concerns, delivery options and immunogenicity responses.

Box 3 | **Ethical implications of base editing technology**

The accuracy and precision at every step, from tool design to delivery vehicle selection, when deploying a base editor as a therapeutic cannot be understated. However, in addition to the basic science development of base editors at the bench, the transition of this technology to a clinical setting poses additional challenges. Precision medicine as a field has paved the way for a multitude of scientific advancements in the search for cures of various genetic diseases. Through base editing, these precise and accurate somatic cell genome modifications can mitigate the symptoms of, or cure, a patient of a disease. Curing a disease caused by a single-nucleotide variant, such as sickle cell anaemia, in an afflicted individual is a unifying goal. However, this same technology could also be used to correct base mutations in the germline, in which case these genome modifications would be passed on to subsequent generations[261]. Human germline editing has been voraciously rallied against, making it the strongest point of contention in the field. This contention presents psychological, societal and ideological conflicts for all involved parties: scientists, clinical patients, medical doctors, policy-makers, patients and the general public.

In addition to ethical questions being raised surrounding the technology itself, ancillary questions are being asked with regards to the field of precision medicine as a whole. With the rise in popularity of determining one's own personalized genomic information through companies such as 23andMe, we must stop and ask what is entailed after providing our DNA to these institutions. Who retains ownership of the resulting data and what will they be used for? Could this information affect one's ability to receive health insurance benefits? The link between knowledge and economic value grows stronger, leading to the possibility that this technology could be used negatively. There are no conclusions to be made from this debate but, rather, the need to point out base editing technology is just that — a technology with limitless potential. Implications of the technology will be user-dependent, and, as such, the need to keep the ethical debate at the forefront of this developing field is more important than ever.

Although prime editing is outside the scope of this Review, a recent review further details prime editor technology[236]. A wide assortment of follow-up studies from various laboratories in the future is anticipated, which will help determine the translatability of this technology.

Just as the genome editor tool must be tailored for each application (FIG. 3c), an appropriate and effective delivery method must also be chosen that is compatible with the desired outcome (TABLE 2). In addition to the delivery methods discussed here, strategies extend beyond viral-derived and particle-derived means. These strategies include, but are not limited to, lentivirus virus-like particles, vesicles developed with the envelope glycoprotein of the vesicular stomatitis virus and the 'molecular Trojan Horse' — all of which have been used for Cas9 delivery[237–240]. Collectively, there exists a wide range of delivery methods in varying stages of development for CRISPR–Cas9-mediated genome editing tools. We anticipate that future studies will repurpose some of these cutting-edge and creative delivery methods for base editors. Together, improving and expanding base editor technologies as well as establishing various systems suitable for human in vivo delivery of base editors will collectively represent the next step forward in the field of precision medicine.

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Author contributions
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Competing interests
A.C.K. is a member of the scientific advisory board (SAB) of Pairwise Plants, and is an equity holder for Pairwise Plants and Beam Therapeutics. I.M.S. is an employee and shareholder of Beam Therapeutics. G.W.Y. is co-founder, member of the Board of Directors, on the SAB, equity holder and paid consultant for Locana and Eclipse BioInnovations. G.W.Y. is a visiting professor at the National University of Singapore. A.C.K.'s and G.W.Y.'s interests have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. The authors declare no other competing financial interests.

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