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Recent advances in DSB-free kilobase-scale genome editing technologies

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Abstract

Genome editing approaches have transformed our ability to make user-defined changes to genomes in both *ex vivo* and *in vivo* contexts. Despite the abundant development of technologies that permit the installation of nucleotide-level changes, until recently, larger-scale sequence edits via technologies independent of DNA double-strand breaks (DSBs) had remained less explored. Here we review recent advances toward DSB-free technologies that enable kilobase-scale modifications including insertions, deletions, inversions, replacements, and others. These technologies provide new capabilities for users, while offering hope for the simplification of putative therapeutic strategies by moving away from small mutation-specific edits and towards generalizable kilobase-scale approaches.

Graphical Abstract

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Declaration of Interest

C.J.T. and B.P.K are inventors on patents and/or patent applications filed by Mass General Brigham that describe genome engineering technologies. B.P.K. is a consultant for EcoR1 Capital and is an advisor to Acrigen Biosciences, Life Edit Therapeutics, and Prime Medicine.

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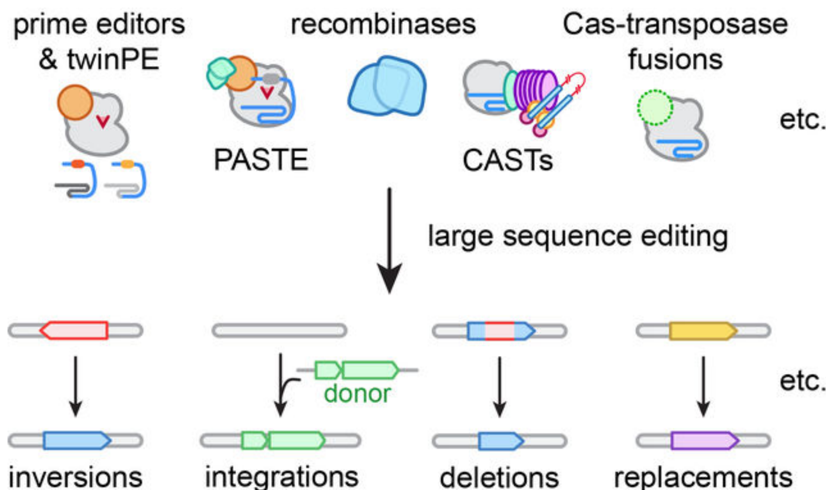

Towards large(r) genetic edits. New classes of genome editing technologies are transforming our ability to make large sequence edits ranging from dozens of base pairs to several kilobases.

The continued development of CRISPR-Cas technologies has expanded our ability to make customizable modifications to the human genome, revolutionizing the pursuit of permanent therapeutic edits. Typically, RNA-programmed CRISPR-Cas enzymes initiate genome editing events by catalyzing locus-specific DNA double-stranded breaks (DSBs) in a genome of interest. Subsequent repair of the DSBs by cellular processes can result in gene knockouts or targeted deletions via non-homologous end-joining or microhomology-mediated end-joining (NHEJ and MMEJ, respectively), or result in knock-in of small or large desired edits that are encoded on donor DNA molecules via homology-directed repair[1] (HDR; Fig. 1a). Despite these capabilities, the precise installation or deletion of small and large DNA sequences in various cells and organisms via nuclease mediated DSBs is challenging and/or can lead to unwanted side effects. For instance, DSB-based methods suffer from heterogeneous and sometimes undesired insertion or deletion mutations (indels) at the on-target site[2,3], unpredictable large-scale deletions[3], chromosomal alterations due to DSBs that co-occur at on- and/or off-target sites[4,5], toxicity resulting from cellular DSB-response[6,7], and a reliance on certain cellular factors or DNA repair pathways that may not be expressed in the target cell type. Thus, next-generation technologies that produce targeted DNA modifications directly on the sequence of interest without DSBs are critical to overcoming these caveats.

There has been a recent expansion in the breadth of DSB-free technologies that generate nucleotide-level changes with higher precision, versatility, and programmability compared to prior approaches[8]. For example, base editors (BEs) typically facilitate the installation of A-to-G[9,10] or C-to-T changes[11,12] (ABEs and CBEs, respectively; Fig. 1b) within short sequence windows, as directed by the guide RNA (gRNA). BEs are comprised of fusions of adenine or cytosine deaminases to catalytically inactive or nicking variants of SpCas9 (nCas9) or other Cas orthologs, and they have been shown to mediate high levels of single nucleotide edits in primary human cells and *in vivo* for the treatment of human diseases[13–17]. More recently, prime editors (PEs) have been developed to permit user-defined sequence

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modifications via the fusion of nCas9 to a reverse transcriptase (RT), enabling the genetic writing of small edits that are pre-programmed on prime editor guide RNAs[18] (pegRNAs; Fig. 1c). PEs can insert, substitute, or delete short sequences[18], and have been shown to function in various cell types *ex vivo* and organisms *in vivo*, though with varying efficiencies[19–23]. Recent efforts to develop optimized prime editors[21,24,25] and pegRNAs[26], combined with future advances (e.g. understanding determinants of activity[24,27,28], cell-specific optimizations, etc.) may lead to efficient prime editing in a variety of contexts. The development of BEs and PEs highlights a concentrated effort to engineer editors capable of small sequence edits, with applicability to correct a range of disease-causing nucleotide-level mutations using bespoke enzymes and gRNAs.

Despite the promise of small sequence editors, the fact that most diseases are caused by heterogenous mutations is an obstacle for clinical translation given the time and resources required to optimize safe and effective editing approaches. One potential solution to this bottleneck is large sequence editors capable of precisely inserting, deleting, inverting, translocating, or replacing kilobases of DNA without DSBs (Fig. 1d). In contrast to nuclease-, BE-, and canonical PE-based approaches that necessitate the design and optimization of new enzyme and gRNA combinations for treating any mutation (Fig. 1e), programmable integration would enable insertion of wild-type genes or cDNAs at endogenous genetic locations (concomitantly eliminating expression of the mutant gene) or direct replacement of mutated sequences, respectively. Such capabilities could act as genotype agnostic pan-mutation genetic therapies for individual diseases caused by various heterogenous mutations (Fig. 1e) including cystic fibrosis, Duchenne muscular dystrophy, amyotrophic lateral sclerosis, neurofibromatosis, Leber congenital amaurosis, and primary immunodeficiencies. Moreover, they would facilitate insertion of engineered genes, genetic elements, or circuits at specified locations for cell engineering applications (e.g. CAR-T cells). Targeted deletions and inversions (Fig. 1d) could treat diseases caused by duplications, nucleotide expansions (e.g. Huntington's Disease, Fragile X syndrome) and certain cases of Hunter's syndrome and Hemophilia, while targeted translocations could treat certain cases of Down's syndrome and cancers. Thus, large sequence editors could obviate the burden of designing and optimizing custom enzyme and gRNA combinations for the vast diversity of heterogeneous pathogenic substitutions (Fig. 1e). Moreover, these technologies would create new basic research applications by enabling predictable cell engineering of structural variants as well as facilitating new screening and library-based approaches (including in situ saturation mutagenesis and deletion screens).

An ideal large-sequence editor would optimally have certain properties, including being: (1) able to generate a wide array of multi-kilobase edits at high efficiency and specificity without DSBs, (2) independent of HDR to improve cell-type applicability, (3) scarless without leaving residual sequences at the target locus, (4) compact in coding sequence for delivery via viruses, (5) tunable to user-defined parameters for a variety of applications, etc. Towards these ambitious goals, a suite of new technologies has begun to emerge, including programmable approaches and systems for precise deletion, integration, and inversion of genetic sequences.

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Recent developments of next-generation deletion and short sequence replacement technologies have thus far largely been based on adapted prime editing methods. Instead of using a single pegRNA as done in the canonical approach, the use of paired pegRNAs offers additional versatility to encode and generate edits. For example, in the methods PrimeDel[29] and twinPE[30], the paired pegRNAs are utilized to install 3' DNA flaps homologous to target site DNA or to each other, respectively, whose annealing and resolution leads to the programmed deletion or replacement of the intervening DNA sequence between the two nicks (Fig. 2a). Variations on this methodology have either relied on longer reverse transcription templates (RTTs) and substantial polymerase-mediated gap filling to increase insertion size[31], or utilized nuclease Cas9 to promote repair of the flap-target DNA (though inherently introducing DSBs)[32]. Despite the promise of these approaches, their efficiency decreases as the deletion or replacement sizes increase since their mechanisms rely on 3' DNA flap localization to a homologous segment of DNA or another 3' flap located at the opposite end of the intended deletion/replacement site.

Targeted integration and inversion technologies have largely focused on recombinases and transposases, though other recombineering-like approaches have also recently been developed[33]. Tyrosine recombinases have been evolved to recognize new target sites for targeted inversion and integration (Fig. 2b), expanding their therapeutic potential[34–37]. However, their reversible recombination mechanisms can limit their activity and utility. In contrast, site-specific serine recombinases act through an irreversible mechanism, renewing interest in their discovery and application in single-step reactions to integrate donor-encoded sequences into the human genome[38] (Fig. 2c). However, reprogramming the target specificity of recombinases to defined loci is laborious, and there are specificity concerns about intentionally targeting predicted pseudosites given the number of times these occur in the genome[39,40]. Furthermore, dCas9-recombinase fusions are less efficient, and the recombinase catalytic domains can retain moderate sequence preferences, limiting reprogrammability to any user-defined site[41]. Despite these potential limitations, the discovery and characterization of additional recombinases has begun to yield new enzymes with unique and useful properties. Leveraging these datasets could lead to a machine-learning framework that can *ab initio* predict high probability recombinase enzyme variants with user-defined sequence specificities[42].

In another recent approach for generating large sequence insertions, site-specific serine recombinases have been coupled with PEs in a multi-step process (Fig. 2d). PEs first are utilized to install recombinase attachment sites at defined genomic locations, and then site-specific recombinases are co-expressed or directly fused to the PE to recombine desired sequences into the genome[30,43]. While these PE-based approaches hold promise for targeted DNA integration (as well as targeted inversions and potentially replacements and translocations), these experiments require the extensive design and optimization of multiple parameters (i.e. target site, pegRNA components, recombinase, donor, etc.), the coding sequences of current enzyme complexes are very large (>8 kb coding size), the edits are not scarless, and current integrases remains less efficient compared to generating small sequence edits with other leading-edge technologies.

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Transposons are another class of technology that offer unique properties for large DNA insertions (Fig. 2e). CRISPR-associated transposases (CASTs) are Tn7 or Tn5053-like transposons that have co-opted type I or type V CRISPR-Cas systems, respectively, for genetic element mobilization[44–47]. CASTs have garnered attention due to their high-efficiency RNA-guided DNA integration in bacteria. Beyond the expanded exploration of the aphylogenetic diversity of CASTs to uncover useful new characteristics[48,49], derivative systems have been engineered that offer more streamlined use and optimal editing properties and capabilities[50,51]. For example, HELIX employs a nicking homing endonuclease fusion to TnsB of type V-K CASTs, enabling these systems to achieve comparable simple insertion product purity, specificity, and efficiency to type I systems[51] (Fig. 2e). Furthermore, recent structural studies have provided unique insights into mechanisms of CASTs[52–57], potentially motivating new engineering approaches to enhance their properties. While CAST-based technologies hold tantalizing potential to enable facile and efficient transposition as human therapeutics and for cell engineering, translation into eukaryotic cells has yet to be demonstrated. Alternatively, transposase enzymes have been directly fused to Cas DNA binding domains to localize transposition events to defined target sites. While the initial development of piggybac-dCas9 or sleeping-beauty-dCas9 fusions displayed significant off-target editing and lower efficiency[58,59], recent adaptations have shown promise by fusing evolved piggybac variants with a Cas9 nuclease[60] though this approach generates DSBs. Still, there remains a major need to optimize the efficiency, deliverability, and translatability of transposase-based technologies in therapeutically relevant contexts.

Despite this progress towards developing optimal next-generation kilobase editors, there exist several challenges for DSB-free technologies including: their relatively low editing efficiency, their complex design determinants, poorly characterized mechanisms, that certain types of kilobase-scale changes remain nascent or largely unexplored (e.g. translocations, replacements, etc), that most approaches leave undesirable sequence scars from recombined sites or transposon ends in gene insertion products, the large sizes of current-generation machinery, and the necessity to co-deliver donor molecules encoding genetic cargoes. To overcome these challenges, there exists potentially transformative opportunities for technological advances. For example, retrotransposases, which naturally integrate a reverse-transcribed RNA template, could be harnessed for direct insertion of RNA-encoded sequences into the genome[61]. However, some challenges include the fidelity of some retrotransposase RTs[62,63], the potential for integration of 5' truncated templates resulting from insufficient RT processivity and/or transcript degradation[64], the programmability of retrotransposase specificity[65–67], and others. Together, the discovery and engineering of other integration enzymes or methods with capabilities for scarless gene products and facile and efficient multiplexing would increase utility and create new approaches to cell engineering and gene therapy.

Similar to other genome editing technologies, the delivery of kilobase-scale editors for *in vivo* applications represents a challenge for the field[68]. Many of these nascent technologies editors have very large coding sequences and can require multiple sgRNAs or pegRNAs, which together can constrain delivery by size-constrained viral vectors[69]. Moreover, codelivery of donor molecules is required for edits involving integration or replacement of large sequences. Continued innovation in viral, nanoparticle, exosome, and virus-like

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particle technologies that package DNA, RNA, and RNP cargos, enable selective targeting to diseased tissues, and minimize immunogenicity will be crucial for all *in vivo* genome editing approaches[68], particularly kilobase-scale alterations. Advances in the composition and size of the editing technologies themselves will also facilitate more effective delivery strategies.

Together, kilobase-scale DSB-free and HDR-independent technologies represent an exciting new frontier of genome modification. These tools hold promise for high impact as research reagents and for various applications. Continued optimization of these technologies might unlock their potential as blanket therapies to treat diseases caused by dispersed heterogeneous mutations within patient populations (Fig. 1e), as treatment strategies for currently intractable structural variants, as methods to facilitate engineering of therapeutic cells, and as tools for biological studies through variant modelling and screening approaches. Further metagenomic discovery will continue to reveal the vast diversity of enzymes that can be harnessed as kilobase-scale editors either on their own (e.g. new classes of RNA-guided systems), as more optimal components in current technologies (e.g. novel types of RNA-guided nucleases[70–74]), and/or in tandem with new approaches (e.g. PE-based systems). The continued interest in and development of these diverse but nascent technologies will refine and simplify our ability to precisely edit genomes at the kilobase scale.

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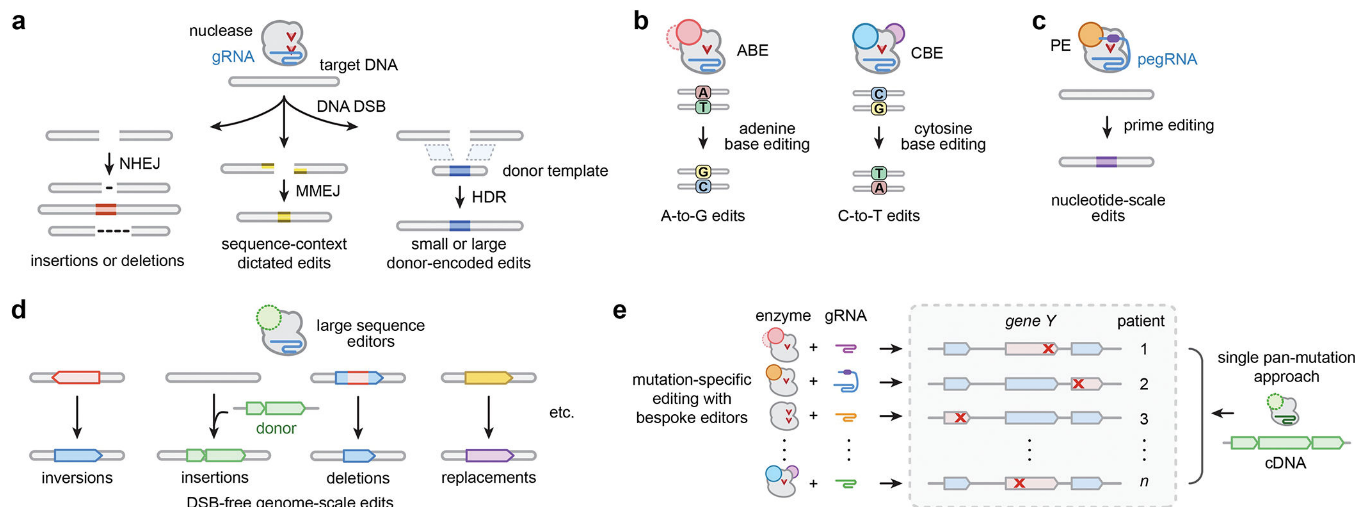

Fig. 1. CRISPR-based technologies for small and large genome edits.

a, Summary of major edit outcomes following nuclease-mediated editing. CRISPR-Cas enzymes paired with guide RNAs (gRNAs) generate DNA double-strand breaks (DSBs) to initiate editing events, which are generally repaired by non-homologous end joining (NHEJ), micro-homology-mediated end joining (MMEJ), or homology-directed repair (HDR). **b,** Adenine and cytosine base editors (ABEs and CBEs) generate A-to-G and C-to-T changes, respectively, without intentionally causing DSBs or requiring HDR. **c,** Prime editors (PEs) generate short insertion, deletion, and/or substitution edits that are encoded on a prime editing guide RNA (pegRNA). **d,** Large sequence editors can generate targeted inversions, insertions, and deletions of multi-kb sequences, which would enable novel editing approaches for previously inaccessible classes of diseases. **e,** Compared to the requisite suite of mutation-specific gene editing approaches for diseases caused by numerous heterogeneous mutations, multi-kilobase integration and replacement technologies could act as single pan-mutation therapies for all genotypes.

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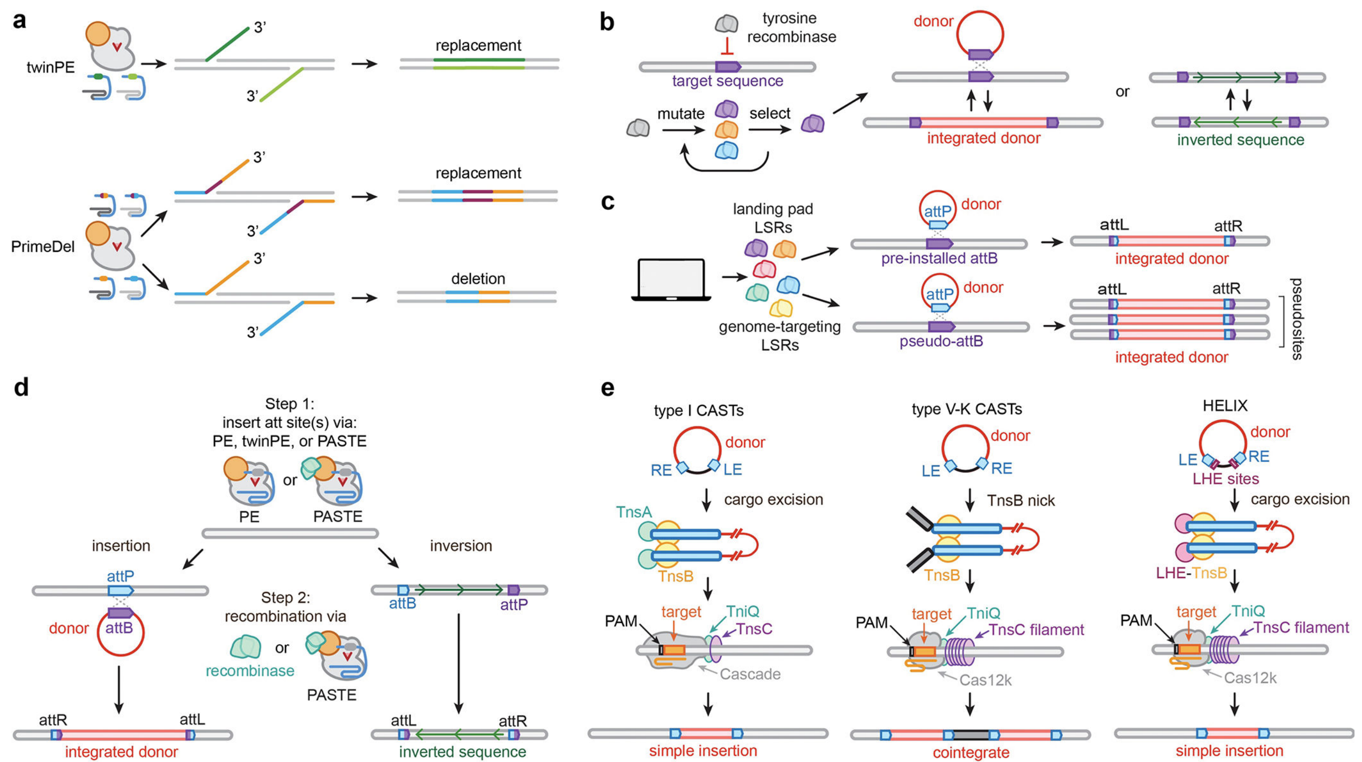

Fig. 2. Emerging approaches for kilobase-scale edits.

a, Paired pegRNA prime editing approaches, such as twinPE and PrimeDel, enable precise deletion and replacement of target sequences. **b,** Tyrosine recombinases can be evolved to recognize new therapeutically relevant target sites for site-specific donor recombination or inversion, which is a reversible process. **c,** Novel large serine recombinases (LSRs) have been discovered and characterized for use with pre-installed genomic landing pads (landing pad LSRs) or to integrate into predicted pseudosites (genome targeting LSRs). **d,** Two-step large sequence edits by combining PE or twinPE with site-specific serine recombinases (directly fused to the PE or co-expressed separately) can install programmable targeted integrations or inversions. **e,** RNA-guided DNA integration with type I or V-K CRISPR-associated transposases (CASTs), or engineered versions, such as HELIX.