Adenoviral vectors as genome editing tools : repairing defective DMD alleles

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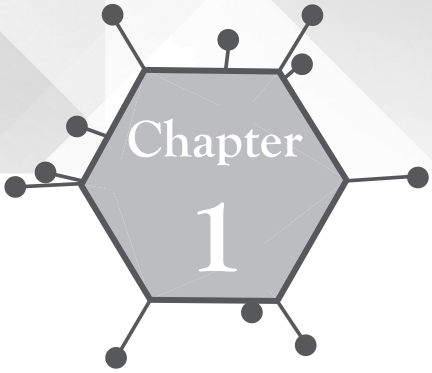

Genome editing at the crossroads of delivery, specificity, and fidelity

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Abstract

Genome editing (GE) entails the modification of specific genomic sequences in living cells for the purpose of determining, changing, or expanding their function(s). Typically, GE occurs after delivering sequence-specific designer nucleases (e.g., ZFNs, TALENs, and CRISPR/Cas9) and donor DNA constructs into target cells. These designer nucleases can generate gene knockouts or gene knock-ins when applied alone or in combination with donor DNA templates, respectively. We review progress in this field, with an emphasis on designer nuclease and donor template delivery into mammalian target cell populations. We also discuss the impact that incremental improvements to these tools are having on the specificity and fidelity attainable with state-of-the-art DNA-editing procedures. Finally, we identify areas that warrant further investigation.

Contents chapter 1

Background and scope of GE

Genetic manipulation of higher eukaryote cells plays a crucial role in basic and applied biology (**Box 1**). The advent and recent diversification of designer nuclease (DN) technologies (see **Glossary**) and their combination with nucleic acid and protein delivery systems have led to the emergence of a new field interchangeably dubbed genome engineering or GE. This biotechnology is becoming invaluable to not only interrogate but also efficiently rewrite DNA sequences in germ and somatic cells from an increasing number of organisms, including those of mammals[1,2]. Indeed, the universal role played by the genome in biological systems opens up the possibility for adapting the basic principles of GE to many disciplines and applications, including gene therapy, functional genomics, regenerative medicine, synthetic biology, and transgenesis.

Principles of DN-assisted GE

Various genetic engineering methodologies currently fall under the operative definition of GE, such as those based on site-specific recombinases (**Box 2**), single-stranded oligodeoxyribonucleotides (ssODNs), and recombinant adeno-associated viral vectors (rAAVs). However, we will focus on reviewing the strategies, parameters, and outcomes of GE procedures based on modifying target cell populations through the delivery of DNs, for which there is a growing and versatile portfolio (**Box 3**). DNs are built to generate double-stranded DNA breaks (DSBs) at predefined chromosomal positions and, in doing so, activate endogenous cellular DNA repair pathways. Indeed, the two main DNA repair pathways responsible for maintaining chromosomal integrity, non-homologous end-joining (NHEJ) and homologous recombination (HR), are activated by DSBs regardless of whether these lesions occur in a random or a site-specific fashion[3,4]. The repair of site-specific DSBs by NHEJ can create knockouts of either coding or *cis*-acting, non-coding sequences. These DSBs can also lead to knock-ins when repaired by HR events involving surrogate DSB repair substrates in the form of foreign donor DNA (**Fig. 1**). Importantly, DNs can increase HR rates from 10^{-8} to 10^{-6} events per treated cell to frequencies as high as 1–30%. These high frequencies avoid the need for complex cell selection schemes in many experimental settings broadening, as a result, the applicability of HR-mediated GE.

Although GE has a broad sphere of action in science and technology, it will be most likely in the context of improved gene therapies that GE interventions will be put to the test in the most stringent manner, both in terms of their ultimate

efficiency and safety. An initial example of such 'genome surgery', currently being tested in clinical trials, is based on zinc-finger nuclease (ZFN)-mediated *CCR5* (chemokine C-C motif receptor 5) knockout to render $CD4^+$ T cells resistant to HIV-1 infection[5]. In addition to acquired pathologies, 'genome surgery' is also being pursued and developed for tackling inherited disorders. These include the genetic complementation or correction of faulty genes underlying recessive disorders and the knockout of dominant illness-associated alleles. Crucially, several aspects linked to GE technologies require further investigation; these include devising improved methods for delivering the often large and complex GE tools as well as for increasing the specificity and accuracy of the knock-in procedures.

Glossary

Adverse genome-modifying event (AGE): undesirable alteration(s) to the cellular DNA or to the integrant structure resulting from a genome editing (GE) intervention.
Ad.iting: designer nuclease-induced GE based on adenoviral vector donor DNA templates.
Designer nuclease (DN): engineered sequence-specific biomolecules (also known as programmable nucleases) consisting of nucleic acid binding and cleaving domains. The most commonly used are ZFNs, TALENs, and RGNs.
Genome editing (GE): the purposeful manipulation of the DNA content of living cells by adding to or removing from specific genomic sequences one or more nucleotides.
GE fidelity: the level of integrant accurateness following a targeted genomic DNA insertion event.
GE specificity: the relative frequencies of on-target versus off-target DN chromosomal cleavage or exogenous DNA chromosomal insertion.
Homologous recombination (HR): cellular DNA break-repairing mechanism involving the copying of genetic information from a donor DNA template (e.g., sister chromatid or homologous chromosome) whose sequence is identical (homologous) to the acceptor, lesion-containing, chromosomal region. HR occurs during the G2 and late S phases of the cell cycle and, in the presence of exogenous donor DNA, can be exploited for introducing genomic changes with nucleotide-level precision.
Integrant: exogenous DNA sequences once integrated in the genome of a transfected or transduced cell.
Non-homologous end-joining (NHEJ): cellular DNA break-repairing mechanism involving end-to-end ligation of DNA termini. NHEJ takes place throughout the cell cycle and can be exploited for disrupting and restoring reading-frames following DN-induced DSBs.
Protein transduction domain (PTD): a peptide (also known as a cell penetrating peptide) whose net positive charge favors plasmalemma interactions and ensuing cellular uptake of a linked cargo (normally a protein).
Transduction: the introduction of foreign nucleic acids into cells by means of viral vector particles.
Transfection: the introduction of foreign nucleic acids into cells by means of chemical or physical methods.

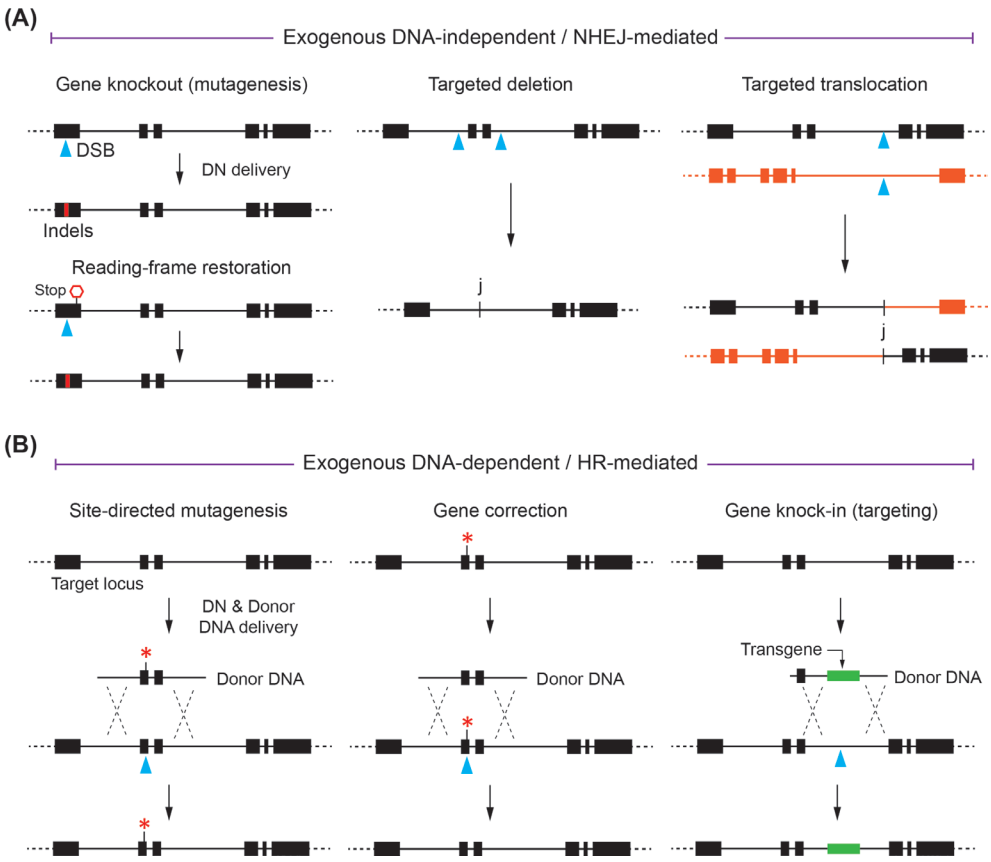

Figure 1. Illustration of the main GE strategies based on DNs.

(**A**) NHEJ-mediated GE. Site-specific DSBs (cyan arrowheads) activate NHEJ repair pathways. These processes can be exploited for obtaining different GE outcomes. Left panel, site-specific DSBs can yield reading-frame shifts resulting from indels (red boxes) that disrupt and restore, respectively, in-frame and out-of-frame sequences. Middle panel, simultaneous induction of tandem DSBs by DN pairs (multiplexing) can result in the deletion of the intervening sequence following end-to-end ligation of the distal chromosomal breakpoints. Alternatively, re-insertion of the intervening sequence in an 'antisense' orientation can also occur (not drawn). Of note, indel formation is, in this case, less likely because the newly formed junction (j) should yield a sequence that is not susceptible to DN activity. Right panel, DN multiplexing applied to sequences in different chromosomes can be exploited for studying well-defined translocations. (**B**) HR-mediated GE. Site-specific DSBs can also activate the HR pathway. In the presence of foreign DNA flanked by nucleotide sequences identical to those framing the target site (donor DNA) the HR process yields well-defined junctions between the endogenous and the chromosomally integrated exogenous DNA. By using judiciously constructed donor DNA templates, this DN-induced homology-directed gene targeting can be exploited to introduce or remove specific mutations or single-nucleotide polymorphisms (asterisks) or, alternatively, insert a gene tag (not drawn), ▶

◀ a complementary DNA (not drawn), or an entire transgene at a predefined chromosomal position (e.g., a 'safe harbor' whose prototypic example is that of the *AAVS1* locus on the human chromosome 19 at 19q13.42). Solid boxes and continuous lines represent exons and introns, respectively. Abbreviations: *AAVS1*, adeno-associated virus integration site 1; DSB, double-stranded DNA break; DN, designer nuclease; GE, genome editing; HR, homologous recombination; NHEJ, non-homologous end-joining.

Delivering the goods: introducing GE tools into target cells

Introducing plasmids encoding DNs into target cell nuclei by electroporation or by transfection based on liposome or cationic polymer formulations are common and rapid procedures, applicable to complex somatic cell populations[6,7]. Frequently, however, these methods are either inefficient in primary cells, especially those that are quiescent or slowly dividing, or lead to substantial cytotoxicity. Therefore, approaches based on delivering DNs directly as proteins or as *in vitro*-transcribed mRNA are being investigated[8,9]. Advantages of protein and mRNA delivery include avoiding insertional mutagenesis risks and lowering off-target effects owing to their shorter half-lives relative to DNA. Related to this, DNs should ideally act in a hit-and-run fashion, in other words generate site-specific DSBs and decay (or cease being expressed) to minimize off-target activities.

Transfection reagent-free strategies based on direct DN delivery can capitalize on protein transduction domains (PTDs)[8]. Genetic fusion of recombinant proteins to these positively supercharged moieties favors their uptake by cellular internalization mechanisms (e.g., lipid raft-dependent macropinocytosis)[10]. There are, however, indications that it may be difficult to generate high yields of soluble and active PTD-containing ZFNs[11,12], transcription activator-like effector nucleases (TALENs)[13], or Cas9 (CRISPR-associated 9)[14] proteins in *Escherichia coli*. Alternative approaches are chemical conjugation of DNs to PTDs, for direct plasmalemma penetration, or to specific ligands, for receptor-mediated endocytosis. Indeed, gene knockouts in cell lines were detected when using TALENs whose exposed cysteine repeat residues were conjugated to a poly-arginine moiety[13]. In another study, ZFNs conjugated to transferrin were internalized via receptor-mediated endocytosis, and induced about 0.2% homology-directed gene repair of a defective reporter allele in 293 cells[15]. In other work, a cysteine-modified Cas9 nuclease conjugated to an artificial PTD was functional when used in combination with a guide RNA (gRNA) bound to another PTD via ionic interactions[14]. This methodology led to *CCR5* disruption in 8.4% of human fibroblasts and 2.3% of ESCs. Interestingly, owing to the net positive charge of their Cys_2-His_2 zinc-finger motifs, ZFNs display an intrinsic cell penetrating capacity, which can lead to targeted mutagenesis in a variety of cell types[11]. These

results are significant, considering the difficulties in producing particular PTD-containing DNs.

Other DN delivery options under investigation include protein transfection procedures. For instance, *in vitro*-assembled Cas9:gRNA ribonucleoprotein complexes induced about 20% targeted mutagenesis in human fibroblasts and ESCs following electroporation[16]. Of note, compared to DNA, protein electroporation yielded approximately twofold more alkaline phosphatase-positive colonies (a marker of pluripotency), presumably reflecting a lower cytotoxicity profile. In another study, enhanced GE was achieved by combining cell cycle synchronization of human cells with electroporation of ssODN donors and Cas9:gRNA ribonucleoprotein complexes[17]. Chemical transfection agents are also being investigated for direct DN transfer. These agents might protect protein cargos from serum inhibition or protease-mediated degradation and, possibly, aid in endosomal escape. In this regard, cationic lipids were successfully used to transfect Cas9. To mimic the highly anionic nature of nucleic acids, Cas9 was either fused to a poly-anionic GFP or simply coupled to its naturally negatively charged gRNA partner[18]. Albeit with some cytotoxicity, this approach achieved up to 80% targeted mutagenesis in an osteosarcoma reporter cell line. In further experiments, ssODN-mediated gene repair and *in vivo* gene disruption occurred at a frequency of approximately 8% in the reporter cell line and 20% in neonatal mice, respectively[18].

As with DNA, mRNA transfer can be accomplished by electroporation or by chemical transfection[19]. Electroporation is gaining momentum, especially owing to its efficiency in introducing synthetic mRNAs into specific hematopoietic cell types[19]. In fact, a protocol combining ZFN-encoding mRNA electroporation, integrase-defective lentiviral vector (IDLV) donor DNA delivery, and stem cell viability-maintaining drugs provided a proof-of-principle for gene targeting in $CD34^+$ hematopoietic stem cells[20]. Building upon these promising results, further studies are warranted to establish whether protein- and mRNA-based GE methodologies can become streamlined and broadly applicable, including in post-mitotic cells and in conjunction with different gene knock-in systems.

Although viral vectors are generally less straightforward to produce than most non-viral delivery systems (**Box 4**), they constitute instrumental gene, and in some cases, protein and mRNA transfer tools[21,22]. This is a consequence of their unsurpassed efficiency in entering into many cell types, both *in vitro* and *in vivo*. Therefore, the ongoing adaptation of viral vectors to GE paradigms is becoming natural and impactful. Indeed, they are suited for many GE applications both dependent and independent of targeting donor DNA. Because the ideal mode of operation for GE tools is via 'hit-and-run', episomal viral vectors are often preferable

over their chromosomally integrating counterparts. Thus, IDLVs[20,23-29], baculoviral vectors (BVs)[30-32], adenoviral vectors (AdVs)[5,28,33-43], and rAAVs[44-51] are all being adapted for transducing DNs and/or donor DNA into mammalian cells (**Box 5**).

So far, IDLVs have been mostly deployed for ZFN and/or donor template transfer. Albeit involving different DN delivery methods and target loci, the latter studies have established IDLV donor DNA as a prolific HR substrate in many cell types. For instance, gene knock-in frequencies of approximately 3.5% in ESCs[23], 5% in lymphocytes[23], and 9–40% in myoblasts[27,37] have been reported. Unfortunately, the optimal performance of IDLVs as a DN-expressing platform is curtailed[52] due to the susceptibility of IDLV genomes to epigenetic silencing phenomena[53,54]. In addition, *TALEN* open reading frames (ORFs) transduced by lentiviral systems suffer extensive deletions due to reverse transcriptase template switching within *TALE* repeats[34,55]. Importantly, this issue is not insurmountable because repetitive tract minimization via sequence recoding has permitted the transduction of not only intact TALEN monomers but also ZFN dimers in single lentiviral particles[29,55].

BVs have been tested in GE experiments involving the delivery of donor DNA, ZFNs, and TALENs[30,31]. The former experiments, deploying a *GFP* donor cassette, achieved up to 4.4% *CCR5*-targeted cells in ESC cultures[30]; the latter, using a 13.5 kb bicistronic donor cassette, required drug selection to enrich for glioma cell populations containing 95% *AAVS1*-targeted cells[31]. The very large packaging capacity of BV envelopes is being exploited for expressing DN dimers from single viral constructs[30,31]. As an instructive note, recent results have demonstrated that, under particular BV propagation conditions, *TALE* repeats suffer rearrangements, presumably due to the expression of recombination-enhancing factors in the producer insect cells[32].

AdVs provide for an efficient and general platform for the delivery of the main DN classes, namely, ZFNs[5,26,28,33,42,43], TALENs[34,36,37,40,41,43], and RNA-guided nucleases (RGNs)[35,37-40] both *in vitro*[5,26,28,33,34,36,37,39,40,42,43] and *in vivo*[35,38,41]. The former studies included targeted mutagenesis in $CD4^+$ T lymphocytes[5,33], mesenchymal stromal cells[39], and keratinocytes[28]; the latter encompassed modeling therapeutic and oncogenic loss-of-function phenotypes in the liver[35,41] and oncogenic chromosomal rearrangements in the lung[38]. The versatility and relatively high genetic stability of AdVs are also underscored by their capacity to co-transduce dimers of ZFNs[5,26,33,42,43] and, in their helper-dependent version (**Box 4**), TALENs[40,43]. The co-transduction of RGN elements in single AdV particles has also been demonstrated[35,38]. In addition, similarly to the aforementioned viral vectors, AdVs are also being explored for introducing donor HR substrates into DN-exposed target cells[28,37,40,42].

rAAVs have served for delivering ZFNs and donor DNA to target cells *in vitro* and

in vivo[44-49]. The former studies encompassed gene repair experiments at recombinant target alleles in human cell lines and ESCs[45,46,48,49]; the latter involved a proof-of-principle for in vivo therapeutic GE by using hemophilia B murine models[44,47]. More recently, rAAV technology was also deployed for introducing RGN components into mice. These experiments included loss-of-function studies to investigate gene function in the mammalian brain and to model lung cancer development[50,51]. Of note, the versatility of the rAAV-RGN system is set to profit from Cas9 variants (i.e., engineered or derived from orthogonal species) whose smaller sizes bypass the limited AAV capsid capacity issue.

Delivering predictability: improving DN specificity

DN specificity can vary greatly depending on the selected set of reagents and experimental conditions. For instance, there is evidence that short-term exposure of cells to DNs reduces off-target activity and cytotoxicity[11,14,16,18,56], with off-target activity correlating with cytotoxicity[57]. Acute cytotoxic effects may possibly result from ATM (ataxia telangiectasia mutated)- or ATR (ATM- and RAD3-related)-dependent protein kinase signaling pathway activation leading to cell cycle arrest and apoptosis. This, together with the potential involvement of off-target DSBs in generating unintended mutations, 'illegitimate' recombination events, and translocations, makes DN specificity a paramount parameter for advancing GE (**Box 6**). Therefore, the development of DNs is often associated with considerable efforts directed towards not only determining but also maximizing their specificity.

Improved ZFN specificity has been achieved through optimizing their DNA-binding domains (DBDs), nuclease motifs, and intervening linker sequences. Regardless of the DN platform, the DBD is clearly the major determinant conferring on-target selectivity. In the case of ZFNs, DBD assembly is complicated by the fact that interaction between each zinc-finger unit and its nucleotide triplet is often affected by the surrounding context. Although some of the various assembly platforms in use[58] take into account this context-dependency, ZFN construction remains difficult and time-consuming for non-experts. Despite this, zinc-finger assembly methods can be combined with refinements to the *Fok*I-derived nuclease domain. Through cooperative dimerization, the native *Fok*I enzyme forms a catalytically active site at the nuclease domain interface. In the context of ZFNs, dimerization involving the same ZFN subunit can generate DSBs at sequences recognized by such homodimers. To minimize this issue, the dimerization interface of *Fok*I cleavage domains has been redesigned by site-directed mutagenesis to generate obligate heterodimeric ZFN variants[59,60]. Rewardingly, these variant pairs lead to a significant reduction in the

number of off-target cleavage events. Furthermore, the inter-domain linker can also be optimized because its composition and length influences ZFN activity and target-site selectivity[61,62].

The construction of functional TALENs is more straightforward and predictable than that of ZFNs owing to their direct repeat-to-nucleotide correspondence and lower context-dependency. Moreover, besides displaying wider genomic space coverage, TALENs present lower off-target activity and cytotoxicity in human cells, as revealed in side-by-side comparisons with ZFNs[63]. Because TALENs and ZFNs share a similar general architecture (**Box 3**), approaches to improve the already high specificity profile of TALENs are in some cases reminiscent of those applied to ZFNs. These strategies include coupling the DBDs of TALEN pairs to obligate heterodimeric *Fok*I motifs[64] and hybrid monomeric DNA-binding proteins consisting of TALE repeats fused to homing endonuclease domains[65,66]. The specificity of TALENs is also being improved via optimizing the TALEN terminal domains as well as the length and composition of TALE arrays[67-69].

Initial studies on the specificity profile of RGNs in target cell populations indicated substantial rates of off-target mutagenesis[70-73]. Of note, more recent experiments based on whole-genome sequencing of individual clones derived from RGN-modified pluripotent stem cells (PSCs) point towards rare RGN off-target activity at the single cell level[40,74,75]. Hitherto, the specificity of RGNs has been enhanced in three principal ways: (i) using paired Cas9 'nickases' to make targeted DSBs through cooperative offset nicks on opposite DNA strands[76,77], (ii) using truncated gRNAs for fine-tuning gRNA–DNA binding energies[78], and (iii) using catalytically inactive Cas9 fused to *Fok*I nuclease domains to induce DSBs through cooperative binding of such fusion products[79,80]. Furthermore, deploying orthogonal Cas9 variants[81] that recognize different protospacer adjacent motif (PAM) sequences is yet another promising strategy for enhancing the RGN specificity profile.

Regardless of the DN platform, readily available and plentiful bioinformatics tools can greatly aid target site selection in the context of complex genomic sequences to judiciously reduce off-target DNA cleavage. Of note, beyond primary target sequence selection, the impact epigenetically regulated chromatin conformations have on the overall specificity and activity of DNs remains poorly understood. Finally, the in-depth characterization of the specificity profile of the various DN platforms will require the implementation of unbiased genome-wide and sensitive methods for tracing off-target events in treated cells[24,82-84].

Delivering precision: improving GE fidelity

The depicted sought-after GE outcomes (**Fig. 1**) are often compounded by collateral adverse genome-modifying events (AGEs) (**Box 6**). In this regard, the DN-induced gene knock-in strategies are particularly multifaceted because their ultimate performance depends not only on DN efficiency and specificity but also on the frequency and fidelity with which the exogenous DNA is inserted at the target site. The deviant GE outcomes impacting the fidelity of DN-induced gene knock-in procedures are manifold. For instance, AGEs can result from the engagement of donor DNA not only with DN-induced off-target DSBs but also with breakage-prone fragile sites and DSBs created by DNA metabolic processes or environmental mutagens. Most notably, next to precise genome-modifying events, random insertions and targeted single-copy or concatemeric insertions generated through non-homologous recombination processes (e.g., NHEJ) can occur. Although targeted, the 'ugly' integrant fraction (**Box 6**) introduces disruptive delivery vehicle-derived backbone sequences (e.g., viral or bacterial DNA) into the cellular DNA. Moreover, in DN-exposed cell populations, a sizable fraction of target alleles will undergo gene disruption instead of homology-directed gene targeting. This stems from the fact that NHEJ occurs throughout the cell cycle and competes with HR for DSB repair[85]. Finally, targeted DSBs can lead to translocations involving not only ectopic but also allelic chromosomal sequences, as recently demonstrated[82].

The discovery that ssDNA breaks (nicks), albeit to a lesser extent than DSBs, trigger HR at recombinant and endogenous loci in mammalian cells[86-88] has provided a strong rationale for developing nicking versions of DNs. Indeed, the fact that nicks are normally not engaged by NHEJ makes such 'nickases' promising tools for increasing the overall precision of GE. Examples include developing mutant DN scaffolds[89-91] and engineered DBDs fused to heterologous sequence- and strand-specific cleaving motifs (e.g., Tev-I or MutH)[92,93].

The GE 'fidelity' parameter is expected to start receiving higher scrutiny and emphasis in the near future (**Box 6**). Indeed, high-fidelity GE will be instrumental for 'genome surgery' interventions and basic research (e.g., repairing defective genes and generating isogenic lines to unambiguously establish genotype–phenotype relationships, respectively). Therefore, the nature and structure of the donor DNA component is set to play a paramount role.

Early studies showed that both linear and supercoiled plasmid conformations are prone to 'illegitimate' recombination processes resulting in random chromosomal insertion and concatemerization[94]. These events can further be compounded by the incorporation of immunogenic and silencing-prone prokaryotic sequences[37]. In line

with this, recent experiments targeting different loci in PSCs subjected to TALEN and donor plasmid transfections revealed that cellular fractions harboring random integrants were large, constituting in some cases the majority of the genome-modified population[95].

In settings compatible with small exogenous DNA insertions (e.g., point-mutation addition or removal), delivery of ssODNs offers a simpler and faster alternative to plasmid- and viral vector-borne donor DNA[17,18,55,96,97]. The first experiments combining DN and ssODNs resulted in 0.16% faithful repair of a defective *EGFP* allele in 293 cells following ZFN delivery. Similar levels were obtained with a control double-stranded plasmid[97]. Unfortunately, the majority of ssODN-modified cells harbored low-fidelity integrants represented by donor DNA capture and internal mutations. A subsequent study using panels of ZFNs and target cell lines demonstrated high-efficiency ssODN-based GE, with a ssODN yielding twofold higher GE levels than a corresponding plasmid substrate. Furthermore, this work also demonstrated that the single-stranded character of these templates reduces donor DNA capture at site-specific DSBs. Of note, however, DNA sequencing did reveal unintended mutations within ssODN-derived integrants[96]. Whether these mutations are the result of ssODN synthesis errors or are instead caused by intracellular DNA repairing processes requires further investigation. More recently, ssODN-based GE has been expanded to include the use of TALENs and RGNs[17,18,55].

Although IDLV genomes serve as efficient substrates for DN-assisted GE[23-25,27-29], a sizable proportion of the chromosomally integrated exogenous DNA represents inaccurate integrants which, among others, include off-targeted and concatemeric DNA forms. These fractions, which in some cases can be as high as 5–40% (ref. 24,37), are presumably generated by non-HR events involving end-to-end ligation of IDLV genomes to each other or to site-specific and sporadic chromosomal DSBs. This picture is generally recapitulated in DSB-induced gene targeting of rAAV donor DNA, which shares with IDLV free-ended linear genomes[47,49].

Conversely, although the absolute frequencies of AdV donor DNA-modified cells are significantly lower than those obtained with IDLV, detailed side-by-side clonal analyses revealed that the vast majority of AdV-modified cells undergo proper GE[37] – that is, they harbor targeted and single-copy integrants whose chromosomal junctions result from *bona fide* HR events at both ends (**Box 6**). This finding of scarless DN-induced AdV DNA editing ('Ad.iting', in short) could be attributed to the capping of linear AdV genomes by covalently attached terminal proteins[37]. These protein-DNA structures presumably hinder, either in a steric or biochemical manner, 'illegitimate' recombination-mediated joining of vector genomes with each other and with spontaneous or off-target chromosomal DSBs. It is enticing to

Figure 2. Grading of GE precision resulting from DNs and donor DNA templates with different specificity- and fidelity-imparting features.

'Sloppy' nucleases inducing high rates of off-target cleavage and donor DNA templates with free-ended termini yield the highest frequencies of adverse genome-modifying events (AGEs) (Level I). AGEs include mutagenesis and translocations caused by 'illegitimate' recombination-mediated repair of induced chromosomal breaks, concatemeric integrant forms, off-target integrants, and on-target integrants with undefined structures (e.g., formed by NHEJ) and/or with unwarranted composition (e.g., delivery vehicle-derived sequences). Conversely, highly-specific nucleases generating low rates of off-target cleavage and end-protected donor DNA templates yield the lowest frequencies of AGEs (Level IV). Intermediate levels of AGEs follow from using free-ended donor DNA and highly-specific nucleases (Level II), and from deploying end-protected donor DNA together with 'sloppy' nucleases (Level III). Shaded DNA forms indicate their relative lower frequencies in genome-modified cell populations. Upward and downward vertical arrows represent high and low rates, respectively, of mutations and translocations involving off-target DSBs. Abbreviations: DSB, double-stranded DNA break; GE, genome editing; NHEJ, non-homologous end-joining.

speculate a parallel operational role between the sheltered telomeric ends of linear eukaryotic chromosomes[98] and the end-protected linear AdV genomes in preventing their DNA termini from degradation and inappropriate recombination. Hence, DNs and donor DNA structures both contribute decisively to the ultimate precision of the GE process (**Fig. 2**). Whether protein–DNA structures other than those of AdV genomes will also yield high-fidelity GE awaits further investigation.

Concluding remarks and future perspectives

The relative emphases given to the efficiency, specificity, and fidelity of GE are changing. The earlier focus on achieving meaningful levels of GE tool delivery and ensuing GE efficiency is being complemented by the relatively more recent efforts in improving the specificity of DNs and the precision with which exogenous DNA becomes incorporated at genomic target sequences. The prolific and rapidly emerging research lines reviewed in this work are systematically addressing these complementary parameters, which together underlie robust and accurate GE. This trend is expected to continue gathering momentum and, considering the multifaceted nature of GE, be grounded on a wide range of disciplines and scientific insights.

Acknowledgments

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Box 1

Classical genome modification technologies

The genetic manipulation of mammalian cells can generically be achieved by non-targeted and targeted chromosomal integration of exogenously added recombinant DNA. The latter genetic engineering procedures are preferable over the former because they result in uniform transgene expression, owing to reduced chromosomal positional effects and predictable phenotypes, owing to decreased risk of endogenous gene disruption (i.e., insertional mutagenesis). However, until the late 1990s the deployment of such precise genome manipulations was restricted to particular HR-based experimental systems, most notably to those involving the generation of knock-in and knockout transgenic mice[99]. In these systems, the very low HR rates and the high frequencies of random non-homologous chromosomal DNA insertions are circumvented by positive/negative selection regimens based on a combination of genetic tools and cytotoxic drugs. These strategies are, however, often difficult to apply in other biotechnological settings. Hence, early approaches aiming at genetic modification of mammalian somatic cells exploited instead the efficient, albeit non-targeted, chromosomal DNA integration capacity of γ-retroviral vectors (γ-RVs)[100]. The γ-RV-mediated genetic modification of hematopoietic stem cells from boys afflicted by X-linked severe combined immunodeficiency provided the first proof-of-concept for gene therapy and, at the same time, materialized genotoxicity risks in the form of leukemogenesis in some of the treated patients[101]. These severe adverse events (SAEs) were linked to the insertion of γ-RV genomes carrying strong promoter/enhancer elements in the vicinity of proto-oncogenes[100,101]. These insertional mutagenesis findings initiated a trend towards HIV-1-based lentiviral vectors (LVs)[102] and the use of self-inactivating retroviral backbones in which viral regulatory sequences are replaced by more physiological cellular promoters[100-102]. Of note, although LVs also display a semi-random integration profile, their proviral insertions are less biased towards the transcription start-sites of host cell genes[103,104]. Furthermore, in contrast to γ-RVs, LVs possess active nuclear import mechanisms leading to efficient transduction of non-cycling cells[105]. Although genotoxic risks associated with LV-induced insertional mutagenesis remain[106], the aforementioned tangible improvements led to therapeutic outcomes in Wiskott–Aldrich syndrome and metachromatic leukodystrophy patients[107,108]. An alternative DNA modification approach consists of adapting transposon/transposase elements from vertebrate genomes. In contrast to retroviral vectors, some of these genetic mobile elements display a truly random chromosomal insertion profile – in other words, do not show a preference for gene bodies and associated regulatory sequences[109].

Box 2

Recombinase- and homing endonuclease-assisted genome engineering

The high demand for controlled chromosomal DNA insertion in both scientific and technological settings has been spurring the development of different genome manipulation technologies. In addition to artificial DNs, preeminent examples include the deployment of site-specific recombinases and integrases (e.g., CRE, FLP, and ΦC31)[110], adeno-associated virus (AAV) replicase/integrase complexes (i.e., Rep78/68)[111,112], and intron-encoded homing endonucleases (HEs), also known as ▶

◀ meganucleases (e.g., I-*Sce*I)[113]. Although these native proteins are limited to targeting fixed chromosomal positions (e.g., ΦC31 and Rep78/68), and/or require the engineering of their cognate recognition sequences into the target cell DNA in the first place (e.g., CRE, FLP, and I-*Sce*I), they have proved to be very useful tools in particular biotechnology platforms and experimental models. For instance, site-specific recombinases have been thoroughly used for setting up conditional gene activation/deletion systems[110,114], whereas the I-*Sce*I endonuclease has been instrumental in DSB repair studies[3,4]. In fact, the latter studies based on the generation of DSBs at specific model alleles and ensuing activation of DNA repair pathways provided a strong rationale for the development of sequence-tailored designer nucleases. This research, initiated in the 1990s with the introduction of ZFNs[115], heralded the beginning of the DN-assisted GE field. More recently, the tailoring of site-specific recombinases and rare-cutting HEs to new predefined target sequences is also underway. These technologies consist of designing chimeric proteins formed by recombinase or HE domains fused to DNA-binding motifs based on zinc-finger arrays or TALE repeats[116]. In addition, strategies based on complex protein engineering endeavors aiming at altering HE target-site preference have equally been pursued[117].

Box 3

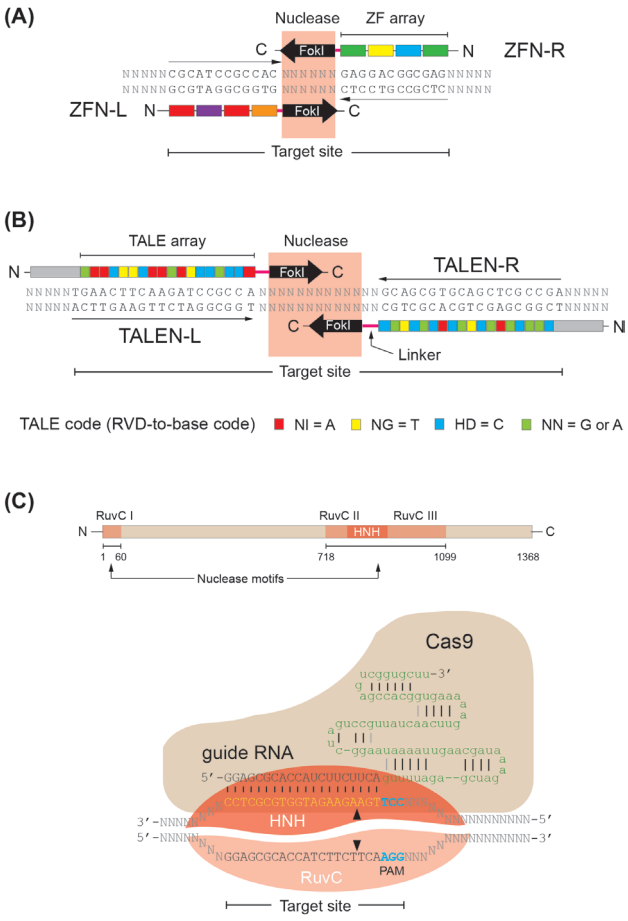
Characteristics and *modus operandi* of the main classes of DNs

ZFNs (**Fig. IA**) are modular artificial proteins consisting of an array of typically 4–6 synthetic Cys2-His2 zinc-finger motifs fused through a linker to the catalytic domain of a restriction enzyme, traditionally that of the type IIS endonuclease *Fok*I (the recognition and cleavage DNA sequences of type IIS restriction enzymes are non-overlapping). Functional ZFNs consist of two monomers assembled in a tail-to-tail orientation at the target site on opposite DNA strands. The local dimerization of the *Fok*I nuclease domains catalyzes upper and lower strand DNA cleavage at a spacer sequence located between the ZFN half-target sites. As a result, a site-specific DSB is formed.

TALENs (**Fig. IB**) display an architecture generically similar to that of ZFNs because the DNA-binding domain (DBD) is also fused via a linker to the non-specific *Fok*I nuclease domain that becomes catalytically active upon in situ dimerization. For TALENs, however, the DBD is derived from TALE proteins found in specific phytopathogenic bacteria (e.g., genus *Xanthomomas*) and comprises an array of typically 15.5–19.5 repeat units of approximately 34 residues each. The repeat residues at positions 12 and 13, called repeat-variable di-residues (RVDs), dictate nucleotide recognition (e.g., RVDs NI, NG, and HD recognize preferentially A, T, and C, respectively). Commonly used TALEN scaffolds comprise 17.5 repeats per monomer and are encoded by ORFs of approximately 3 kb.

RGNs (**Fig. IC**) are RNA-dependent nucleases built on components from clustered, regularly interspaced, short palindromic repeat (CRISPR)-associated Cas systems evolved in bacteria and archaea as immune mechanisms against foreign nucleic acids. The most commonly used RGNs are based on the type II CRISPR-Cas9 nuclease system from *Streptococcus pyogenes.* Cas9 is a large protein (~160 kDa) encoded by a 4.1 kb ORF and contains two nuclease domains (RuvC and HNH). This nuclease is ▶

◀ addressed to the target site via its association with a single guide RNA (gRNA) molecule. The gRNA component is a bipartite molecule engineered by fusing a sequence-tailored CRISPR RNA (crRNA) to a scaffolding trans-activating crRNA (tracrRNA). The target site consists of a nucleotide stretch matching the 5′ terminal gRNA sequence (usually 20 bp in length) followed by a short nucleotide sequence called protospacer adjacent motif (PAM; NGG, in the case of *S. pyogenes* Cas9). The fact that target sequence specificity of RGNs is governed by RNA–DNA hybridization, as opposed to protein–DNA interactions, confers versatility and multiplexing capabilities to RGNs.



Box 3. Figure I. The main classes of DNs.

(**A**) Zinc-finger nucleases (ZFNs). ZF, zinc-finger; ZFN-L and ZFN-R, 'left' and 'right' ZFN monomers, respectively, bound to their cognate half-target sites. (**B**) Transcription activator-like effector nucleases (TALENs). TALEN-L and TALEN-R, 'left' and 'right' TALEN monomers, respectively, bound to their cognate half target sites. (**C**) RNA-guided nucleases (RGNs). The distribution of the nuclease motifs along the primary *S. pyogenes* Cas9 protein sequence (upper panel) and schematics of a RGN ribonucleoprotein complex bound to its target site (lower panel). Vertical arrowheads mark the position at which the blunt-ended DSB is formed. PAM, protospacer adjacent motif. Guide RNA, fusion product between crRNA and tracrRNA moieties.

Box 4

The structure of the main gene-editing viral vectors

LVs are made via transient transfection of producer cells (e.g., 293T) with a mixture of recombinant lentiviral, packaging, and pseudotyping plasmids. The recombinant vector contains, in addition to the transgenic sequences, HIV *cis*-acting elements (i.e., 5' and 3' long terminal repeats, packaging signal, *Rev*-responsive element, and a central polypurine tract). The packaging and pseudotyping plasmids encode a minimal set of primary HIV products (i.e., Gag, Pol, and Rev) and a heterologous envelope protein (e.g., VSV-G), respectively. The latter moiety directs vector particle–target cell interactions. The full-length genomic RNAs are packaged in the nucleocapsids, which in turn become enveloped upon budding from the producer cell membrane. The resulting vector particles are subsequently collected from the producer cell supernatants and purified. Crucially, for generating episomal IDLVs, the packaging construct harbors specific point mutations in the *pol* region that yield non-functional integrase moieties[118].

AdVs can be divided into helper-independent and helper-dependent systems based on whether their production occurs in the absence or presence, respectively, of a *trans*-complementing helper AdV vector[119]. The former are deleted in one (first-generation) or more (second-generation) early genes essential for *in vitro* replication (i.e., *E1, E2A, E2B,* and *E4*); the latter wholly lack viral genes, and thus contain from the parental virus genome exclusively the non-coding *cis*-acting sequences involved in DNA replication and packaging (also known as 'gutless' AdVs). The foreign DNA packaging capacities of helper-independent and helper-dependent platforms range from 5–8 kb to 37 kb, respectively. The generation of AdVs starts by transfecting complementing packaging cell lines (e.g., 293, 911, or PER.C6) with recombinant AdV DNA. The rescued AdV particles are subsequently amplified through serial propagation in producer cells. The resulting AdV preparations routinely reach high titers and display high infectious/non-infectious particle ratios.

Normally, rAAV particles are assembled by transient transfection of producer cells (e.g., 293T) with a rAAV plasmid mixed with constructs expressing *rep* and *cap* (replication and packaging functions, respectively) and specific adenoviral genes (helper functions)[120]. Once in the cotransfected producer cells, the rAAV genomes, whose structure consists of inverted terminal repeat (ITR)-flanked transgene sequences, are excised from the plasmid backbone, replicated, and packaged into pre-formed empty viral capsids. The resulting rAAV particles are subsequently harvested and purified. High-titer rAAV preparations can be made despite the fact that scale-up of transient transfection procedures remains challenging with the resulting stocks often containing low infectious/non-infectious particle ratios.

Box 5

The viral vector concept and its main scaffolds

Viral vectors are built to exploit the refined cellular infection mechanisms evolved by viruses for transferring their genomes into host cell nuclei. In general, these mechanisms involve the binding of virions to specific cell surface receptors, internalization, cytoplasmic trafficking, and nuclear entry of the genetic material. Viral vectors are in fact engineered viral particles whose genomes contain no or only a subset of viral genes. This renders them replication-incompetent and, simultaneously, creates space in their capsids and envelopes for the packaging of exogenous nucleic acids. The retention of *cis*-acting replication and encapsidation signals in viral vector genomes allows their assembly in so-called packaging cells. These complementing cell lines express in *trans*, either constitutively or transiently, the viral functions for which the recombinant viral genomes are deleted. Often, viral vectors are re-targeted to new cell types through the modification (pseudotyping) of their capsids or envelopes. Importantly, viral vectors have been under preclinical and clinical development for many years as 'classical' gene therapy, oncolytic, or recombinant vaccine agents. These efforts encompass vigorous testing and regulatory history build-up. Such track-records bode well for the implementation of viral vectors in GE-based translational research.

Lentiviral vectors (LVs), integrating or otherwise, are mostly based on HIV-1 (*Retroviridae* family). This virus contains two ssRNA molecules (~9.5 kb) packaged in a nucleocapsid shell surrounded by a phospholipid envelope (~120 nm). After host cell entry, triggered by envelope glycoprotein–cell surface receptor interactions, the RNA genome is converted *en route* to the nucleus into linear free-ended double-stranded complementary DNA (cDNA) via virion-associated reverse transcriptases. Next, the cDNA actively enters the nucleus via its association with a karyophylic pre-integration complex. Finally, proviruses are formed through integrase-dependent semi-random host chromosomal DNA insertion[102].

Adenoviral vectors (AdVs) are derived from *Adenoviridae* family members. Adenoviruses contain a linear, protein-capped, double-stranded DNA genome (~35–40 kb) packaged in an icosahedral nonenveloped capsid (~90–100 nm) that displays protruding receptor-interacting fiber proteins[119].

Recombinant adeno-associated viral vectors (rAAVs) are derived from *Parvoviridae* family members. Because AAVs depend on an unrelated virus for replication (e.g., adenovirus), they are assigned to the *Dependovirus* genus. These viruses have a linear ssDNA genome (~4.7 kb) with two ORFs (*rep* and *cap*) packaged in a fiberless icosahedral non-enveloped capsid (~20 nm). The coding sequences are flanked by *cis*-acting inverted terminal repeats (ITRs), which can form T-shaped secondary structures[120].

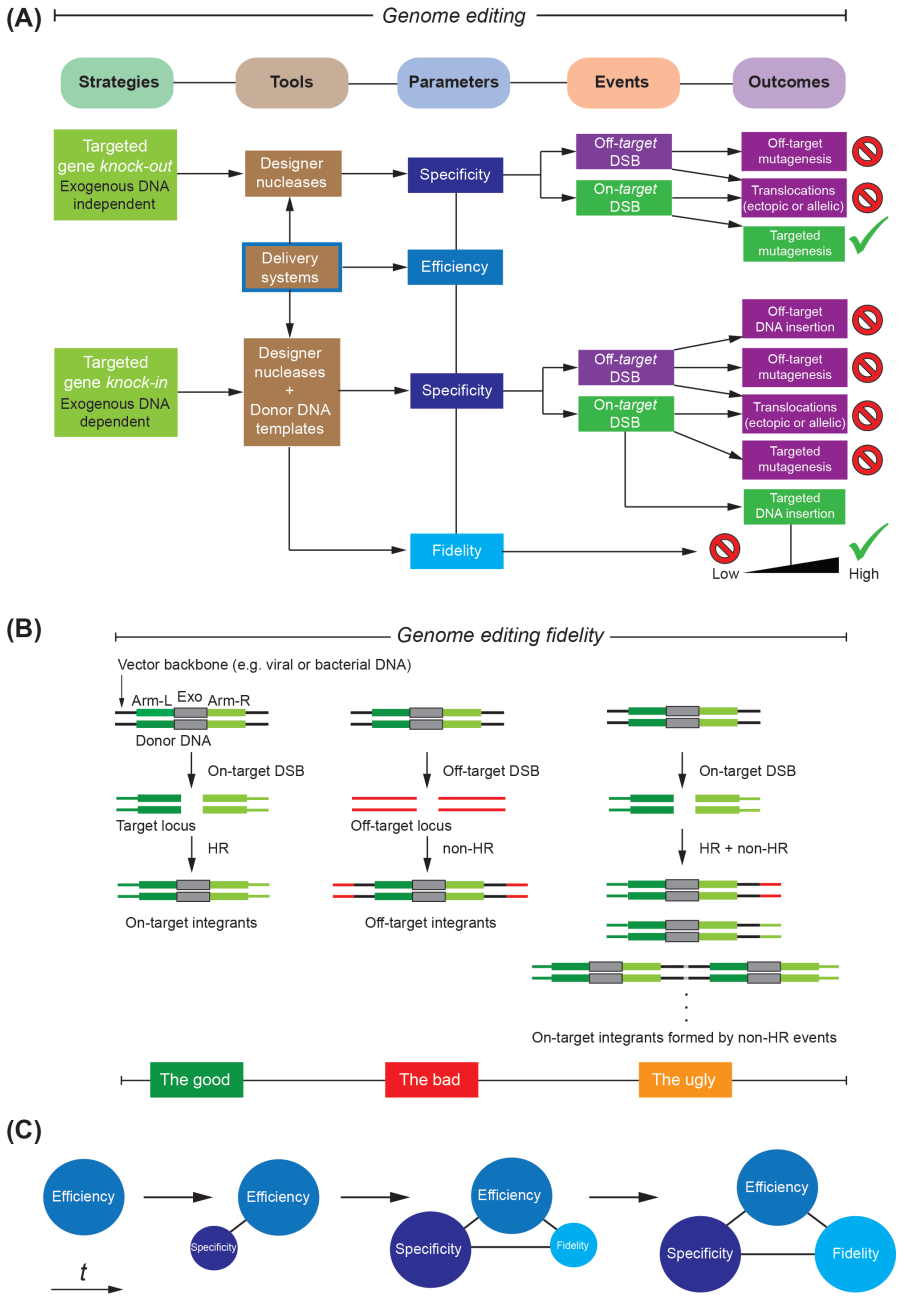
Box 6

The GE landscape: a relationship among multiple factors

Exogenous template-independent targeted gene knockout (mutagenesis) strategies are based on the repair of site-specific DSBs by the NHEJ pathway. The specificity of gene knockout strategies depends on the ratio between on-target versus off-target DSB-forming events resulting from DN activity. Off-target DSBs are undesirable in that they induce unpredictable mutagenesis and can serve as deleterious translocation-initiating substrates. On-target DSBs lead to the intended targeted mutagenesis but can also serve as translocation-initiating lesions. Exogenous template-dependent gene knock-in (targeting) strategies are based on the repair of site-specific DSBs by the HR pathway. The exogenously added donor DNA templates bear sequence identity with the target site (HR substrates) and are used as the source of new genetic information. The fidelity of gene knock-in strategies depends on the generation of integrants whose endogenous–exogenous DNA junctions are the result of HR events at the target site. Both of these strategies rely on delivery systems for introducing the different sets of GE tools (i.e., DNs or DNs plus donor DNA) into the target cells. Regardless of the GE strategy, there are sometimes, in addition to the intended gene-modifying event, stochastic AGEs either in the same cell or in other cells of the population (**Fig. IA**).

The 'good' integrants are on-target and contain exogenous DNA-target site junctions formed by HR events; the 'bad' correspond to off-target integrants; the 'ugly' contain one junction derived from homology-directed gene targeting and another junction formed by non-HR or 'illegitimate' recombination processes (e.g., NHEJ) involving on-target or off-target DSBs. The 'bad' and the 'ugly' integrant forms can also include undefined tandem repeats formed by exogenous DNA end-to-end ligations (concatemers) and delivery vehicle-derived sequences (e.g., prokaryotic DNA and/or viral *cis*-acting elements). These integrant forms contribute to reducing the uniformity of transgene expression in genome-modified cell populations and hindering the restoration of endogenous reading frames following gene targeting and gene repair approaches (**Fig. IB**).

The relative weight given to the 'efficiency', 'specificity', and 'fidelity' of GE procedures is in a trend towards equilibrium (**Fig. IC**).



Box 6. Figure I. The GE landscape.

(**A**) From strategies to outcomes. (**B**) Intended and deviant GE-derived integrants. (**C**) The evolving *Zeitgeist* regarding the three principal GE parameters.

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