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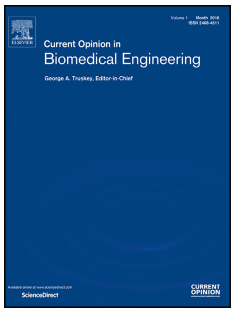


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# Increasing the precision of gene editing *in vitro*, *ex vivo*, and *in vivo*

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## ABSTRACT
New gene editing tools like CRISPR-Cas9 enable precision genome engineering within cell lines, primary cells, and model organisms, with some formulations now entering the clinic. "Precision" applies to various aspects of gene editing, and can be tailored for each application. Here we review recent advances in four types of precision in gene editing: 1) increased DNA cutting precision (e.g., on-target:off-target nuclease specificity), 2) increased on-target knock-in of sequence variants and transgenes (e.g., increased homology-directed repair), 3) increased transcriptional control of edited genes, and 4) increased specificity in delivery to a specific cell or tissue. Design of next-generation gene and cell therapies will likely exploit a combination of these advances.

## HIGHLIGHTS
- Precision can be defined with respect to targeting, sequence, expression, and/or delivery.
- Cas9 and other nucleases have been engineered to decrease off-target frequency.
- Precise sequence outcomes can be favored through various strategies.
- Insertion of transgenes at endogenous loci promotes well-regulated expression.
- New nanomaterials and vectors can help direct potential therapeutics to specific tissues.

## MAIN TEXT
The advent of precision genome engineering has permitted staggering advances in the past decade, both as a basic tool for biological research and as a potentially transformative therapeutic agent. As the field develops, it is vital to consider what precisely is meant by “precision” engineering, and to consider a holistic approach to this paradigm. Here, we define precise genome editing in four ways: 1) editing a specific location within the genome, 2) creating scarless, definable genomic changes, 3) deliberate promoter and editing locus selection for transcriptional control, and 4) spatiotemporal specificity with regard to which cell and tissue types receive editing machinery (**Figure 1**). These four considerations are vital across diverse applications to ensure maximal functionality of edited sequences, while minimizing the incidence of deleterious or unnecessary mutations. As the field of clinical genome editing

continues to evolve, researchers should consider each aspect of precision in their efforts to design the next generation of therapies.

***Precise on-target nuclease activity***

From the inception of genome editing, researchers have been concerned with the ability to edit the genome at the target site, while limiting edits elsewhere within the genome, commonly called "off-target effects." Shortly after CRISPR systems were identified as genome editing tools[1,2], several groups raised concerns that Cas9 may create excessive undesirable mutations[3–5]. Varying rates of off-target events were reported ranging from >1000 per sgRNA sequence[6] to negligible effects[7], resulting in calls to develop better off-target screening methods. Popular techniques to quantify off-target sites include Digenome-seq, Circle-Seq, Guide-Seq, BLISS and integrase-deficient lentiviral vectors[8–12].

Following these observations, many researchers adopted new methodologies to controllably introduce genome editing components, including tighter stoichiometric control that replaced plasmid-based systems with ribonucleoproteins[13] (RNPs), and strategies to regulate when and where Cas9 is expressed[14–16]. These controlled methods showed a concurrent decrease in the number of off-target effects[15], and it is likely that RNP-based editing systems will remain popular in the clinic. The timing and location of editing events can also be modulated by light and small molecules to control nuclease activity. Modified Cas9 nucleases can be selectively activated with small molecules to decrease the gene editing time window[14]. These ligand-dependent nucleases demonstrate 25-fold higher specificity with regards to on-target vs. off-target edits, and can also be used to induce Cas9 functionality *in vivo* at time-specific intervals during development. Combined, these technologies highlight the power of dynamic temporal control over genome engineering machinery.

Additional efforts to decrease off-target effects have emphasized further modifying the nuclease, including engineered "nickase" Cas9 proteins featuring only one active nuclease domain. When used alone, nickases cannot create a full double strand break (DSB). However, when two nickases are paired, the resultant break can be repaired via non-homologous end-joining (NHEJ)[17]. While this method lowers off-target effects, the efficiency of genome editing is greatly decreased, as two nickases and two sgRNAs need to be delivered to the nucleus to perform simultaneous cuts; furthermore, high efficiency repair of individual nicks can impede successful creation of a DSB. Thus, practical applications of nickase therapeutics may be limited. Others have engineered Cas9 RNP by mutating residues that interact with the protospacer-adjacent motif (PAM) (5`-NGG-3` in *S.Pyogenes*) region of the sgRNA[18]. These modifications expand the targeting capabilities of Cas9 to recognize PAM sites that occur less frequently throughout the genome, thereby decreasing off-target binding capability. Cas9 proteins from different species (*N. Menengitis*[19], *S. Aureus*[20]) have a similar potential.

Insights into Cas9 structural biology[21,22] yielded a rational design approach to create high-fidelity variants of Cas9: eSpCas9[23],Cas9-HF1[24], and xCas9[25] (**Figure 2A**). Cas9 variants function by decreasing the binding time of the sgRNA to the target sites within the genome,

resulting in a decrease in off-target binding and cutting. All variants also claim to only slightly decrease the frequency of on-target DSB formation. Recent work has shown that they actually decrease off-target cuts by modifying the kinetics of the change in the structural formation[26], but may also work poorly when introduced into human cells without modifications to the sgRNA[27]. At the moment, these high-fidelity Cas9 variants may represent a quick path to clinical relevance as they can greatly reduce off-target events.

***Precise scarless incorporation of new sequences***

While some desired outcomes can be accomplished via error-prone DSB repair (e.g. NHEJ in Figure 1), there are still challenges that can only be solved through precise point mutations or transgene insertion via homology-directed repair (HDR), herein termed "scarless editing". Researchers have thus attempted to increase both the overall efficiency of HDR as well as the ratio of precise to imprecise mutations.

Early work to increase scarless editing levels focused on modulating cellular DSB repair pathways. Multiple groups showed that small molecule mediators (e.g. SCR7, L755507) can increase the relative proportion of scarless editing events[28,29]. More recently, co-introduction of an i53 protein[30] was able to increase scarless editing. These methodologies are most applicable for *in vitro* cell culture applications where potential toxicity is less limiting.

Building on this idea, other research demonstrated that timed delivery of gene-editing particles to certain points in the cell cycle corresponding to DNA synthesis could also increase HDR rates[31]. This method has been further applied by synchronizing cell cycles and subsequent timed delivery during S-phase[32]. Others have combined these ideas with Cas9 protein engineering methods. This technique, called Cas9-hGem, retains Cas9 protein within the cell only when HDR is favored during the cell cycle[33]. These methodologies hold great promise for *in vitro* cell culture applications, but may be untenable for *in vivo* editing where the majority of cells are in a post-mitotic state.

For point mutations and short knock-ins, single-stranded oligodeoxynucleotide (ssODN) templates hold significant promise for treating disease variants due to their ease of synthesis. However, sequence changes encoded by the ssODN are infrequently incorporated after editing (<10%), and desired edits are typically outnumbered by other sequence outcomes (presumably NHEJ). Recent reports have shown that ssODN design can significantly alter how the DSB is repaired. By making ssODN homology arms asymmetrical around the cut site, HDR can be promoted up to 5-fold higher over symmetrical ssODNs[34,35]. However, these methods still require free-floating foreign DNA that is not necessarily available to create the desired edit at the cut site. To solve this problem, several groups have tried strategies to link the ssODN to Cas9. For example, the sgRNA and ssODN can be chemically tethered[36]. Other techniques have leveraged avidin/biotin binding capabilities to link the ssODN directly to the CRISPR protein[37] or the sgRNA through the use of accessory proteins and RNA aptamers[38] (**Figure 2B**). Each of these methods increase the ratio of precise edits:imprecise mutations and could potentially be delivered as a preassembled RNP for an all-in-one therapeutic.

Finally, other methods attempt to avoid the use of HDR altogether, and instead leverage other DNA repair pathways. One method that has been explored, especially in post-mitotic cells, is microhomology-mediated end-joining (MMEJ). This pathway is thought to use small regions of homology between single strands that form via exonuclease activity during resection after the DSB. This pathway may contribute to commonly occuring indel mutations[39] or small insertions[40], although recent work has shown that integration of novel DNA may be possible. Several groups have developed protocols using small homology arms that undergo resection to form regions that overlap with genomic DNA[41]. The most well-known technology using this pathway is homology-independent targeted integration[42], which requires only 8 bp of homology around the cut site to insert full transgenes in post-mitotic cells *in vivo*. These approaches avoid some of the challenges in assembling long homology arms into donor constructs.

In combination with donor DNA design, novel CRISPR systems have been discovered that may help precise gene correction. Cas12a (formerly Cpf1) functions similarly to Cas9 nickase but requires only one protein component to make a staggered DSB around the target site[43]. By creating a staggered DSB that is 17 nucleotides distal to the PAM, the PAM is less likely to be destroyed during cleavage, thus allowing for repeated DSBs. In bacteria, repeated DSBs increased the likelihood of HDR over NHEJ[44]. Further work has also shown that Cas12a has a lower rate of off-target effects, contributing to the precision of the nucleases[45]. However, following DNA cleavage, HDR can still be initiated. This increases the ratio of precise to imprecise mutations, and reduces the risk of undesired NHEJ products[46]. Most recently this has been shown in spinal muscular atrophy patient iPSCs[47], suggesting untapped potential for precision gene correction. While Cas12a has obvious benefits for precise gene editing, it has recently been suggested to possess ssDNA cleavage activity, even in the absence of a PAM site[48]; thus, it may be better suited for dsDNA templates used for large transgenes.

Base editors are particularly attractive for clinical translation, as they avoid DSBs entirely. They employ a catalytically dead version of Cas9 fused to a DNA deaminase to modify existing base pairs in the sgRNA protospacer region (**Figure 2C**). Base editors deaminate cytidine bases to form uridine. These modified bases are then recognized by the cell as mismatched and corrected to thymidine[49]. Current work in this area mostly focuses on C>T (or the analogous G>A) conversions, although future versions will aim to allow modifications of any single base[50].While this technology should avoid unwanted genomic instability through the breaking of DNA strands, imprecise editing can also occur, as all C nucleotides within the protospacer region are capable of being modified. Current work is addressing this shortcoming by shortening the available editing region[51].

***Precise transcriptional control***

Expression of edited transcripts can vary over time, as well as across cell differentiation and behavior patterns. Misregulation of the edited transcript can compromise therapeutic efficacy or lead to adverse events. Therefore, it is critical to consider strategies to maximize transcriptional control of any edited transcripts, especially knock-in constructs. Various

strategies target "safe harbor" loci, such as the well-characterized *AAVS1* locus in humans[52]. However, increasing efforts are focusing on selection of more specific editing loci, and emphasizing sophisticated transcriptional control of transgene expression beyond the use of constitutive promoters.

A striking discovery regarding the necessity for precise transgene expression recently emerged in the Chimeric Antigen Receptor (CAR) T cell therapy field. In the CAR T paradigm, a synthetic CAR transgene targeting a cancer-enriched antigen is knocked into the patient's T cells *ex vivo*, which are then expanded and reinfused, thereby engineering the immune system to recognize and target cells bearing the antigen[53]. Gene transfer traditionally employs retroviral or lentiviral vectors, which raises concerns ranging from insertional oncogenesis to unregulated CAR expression levels. One group recently used CRISPR-Cas9 to generate CAR T cells featuring a transgene at the T cell receptor alpha (TRAC) locus, which simultaneously knocked out the endogenous T cell receptor (TCR) and ensured that CAR expression was regulated by the endogenous TRAC promoter[54] (**Figure 2D**). These CAR T cells demonstrated striking results in a leukemic mouse model, and also displayed fewer biomarkers of dysfunctional CAR T cells, thus suggesting that precise transgene control may yield a more potent clinical product.

Additional recent work further underscored the importance of transcriptional considerations through a strategy to map protein binding sites for *BCL11A*, a regulator of fetal hemoglobin silencing which is aberrantly expressed in hemoglobinopathies such as sickle cell disease and ß-thalassemia[55]. This study elucidated promoter-mediated repression of *BCL11A* in adult cells driving the switch from fetal to adult globin, and indicated that some disease variants involve disruption to *cis*-regulatory elements of *BCL11A*. These elements of the genome are significant current targets for therapeutic development for diseases involving dysregulated protein expression.

***Precise editing within specific cells and tissues***

While the transcriptional regulation of gene editing outcomes is a critical consideration, delivery to appropriate tissues is equally if not more important for any somatic editing approach. Precise delivery of editing components remains an extant challenge within the field, as many delivery agents suffer from low efficiency, high toxicity, and immunogenicity. Both viral and nonviral delivery agents have been engineered to achieve cell and tissue specificity.

Viral vectors are one of the most commonly used methods for delivering genetic payloads[56]. There is an increasing trend towards the use of adeno-associated viruses (AAV), which are capable of transducing non-mitotic cells while avoiding integration into the target genome. These vectors come in various serotypes with tropism specific to particular tissues, and have been used to edit the mammalian CNS[57,58] and retina, for which a first-in-kind AAV gene therapy, Luxterna™, has received FDA approval. AAV viruses can handle genetic payloads up to 5 kb, which limits their efficacy for some constructs; however, when used with smaller nucleases such as SaCas9, this issue is somewhat mitigated[20]. Viral constructs can also be

engineered to harbor cell and tissue-specific promoters driving expression of the gene editing system[20,59], such that editing machinery is not expressed in non-desired cell types.

In spite of the relative efficiency of AAV delivery vectors, capsid immunogenicity remains a barrier. Additionally, if used to deliver the nuclease sequence along with template DNA, there are significant concerns about the effects of long-term nuclease expression within the target cell that severely dampen the potential for clinical use. Thus, nonviral delivery methods, such as nanocarriers and other customized biomaterials, are being explored to circumvent these problems. In order for gene editing components to produce therapeutic effects, they must traffic to the desired tissue without producing an immune response, enter the target cell, escape the endosome, and enter the nucleus[60]. This is particularly challenging in the context of nuclease delivery, as Cas9 and other proteins are sizeable and sgRNAs carry a negative charge, two characteristics that limit cell penetration[61].

Several designs have demonstrated high gene-editing efficiencies when used with RNPs, ranging from 30-40% in cell lines, and up to 90% delivery efficiency[13,62–64]. These nanocarriers have demonstrated comparable efficiency to conventional electroporation or lipid-based reagents (e.g. Lipofectamine CRISPRMAX[65]), and have the potential to facilitate nuclease delivery *in vivo* at therapeutically active rates while remaining biocompatible in patients. To complement these nanoparticle designs, others have engineered gene-editing components themselves for improved tissue specificity. A recent paper described engineered Cas9 proteins featuring glycoprotein receptor ligands conferring specificity to liver cells[66]. These engineered nucleases were able to both penetrate liver cells *in vitro* and escape the endosome to confer organ-specific edits. While not yet validated *in vivo*, these findings raise the possibility for future precision editing designs featuring tissue-specific nucleases.

In addition to increasing the overall efficiency of delivery, custom biomaterials can be engineered to direct genetic payloads to specific tissue types to allow gene editing *in situ*, thereby bypassing many of the biomanufacturing challenges associated with *ex vivo* therapy design. Researchers recently developed DNA nanocarriers with the capacity to deliver CAR transgenes to T cells in a leukemic mouse model by coupling anti-CD3 ligands to polyglutamic acid (PGA)[67]. These nanocarriers demonstrated specificity to circulating T cells over other blood cell types shortly after delivery (34% and 6% respectively), demonstrated no immediately apparent toxicity, and caused tumor regression at rates comparable to adoptive T cell transfer. While further work is required to ensure the method's safety, this approach represents a tantalizing possibility for off-the-shelf CAR therapies.

**Complementary strategies**

It is likely that advances within each of these types of precision will be complementary, ultimately enabling more precise genomic surgery within patients' cells *in vitro*, *ex vivo* and *in vivo*. For *in vitro* applications, we envision precision drug discovery to be accelerated by enhanced tools for disease modeling, target validation and toxicological studies. Meanwhile, in *ex vivo* uses, we anticipate precision-engineered cell and tissue therapies that incorporate more

functionality from synthetic circuits[68]. Finally, for *in vivo* somatic gene editing applications, we envision injectable viral and nanoparticle strategies that specifically edit stem cells to regenerate tissues and correct disease-causing mutations. A key challenge for translation will be to demonstrate precision through the regulatory pathway, as new tools are required to assess off-target events, the full array of sequencing outcomes and genetic variants from gene editing, aberrant expression of the edited transcript, and potential immune response. Successful strategies to overcome this challenge will pave the way for an unprecedented class of therapeutics, with curative potential for many of the world's most pernicious and heterogeneous diseases.

**Table 1. Four types of "precision" in genome editing.**

| Type of Precision | Description | Potential Tools to Improve Precision |
|---|---|---|
| Targeting | The nuclease cuts precisely at the desired genomic locus, without producing off-target double strand breaks. | RNP vs. plasmid[13]<br>Ligand-dependent nucleases[14]<br>Light-dependent nucleases[16,69]<br>Nickases[17]<br>Engineered PAM recognition[18]<br>High fidelity nucleases[23,24] |
| Sequence | Sequence outcomes are precisely defined (typically, increased homology-directed repair vs. non-homologous end joining). | Small molecule HDR mediators[28,29]<br>Cell cycle-timed gene editing[31,32]<br>Cas9-hGem[33]<br>Asymmetrical donor templates[34,35]<br>Tethering ssODN to Cas9 or sgRNA[36–38]<br>Cas12a/Cpf1[43,45,46]<br>Base editors[49–51] |
| Expression | The edited gene is expressed in a definable manner mimicking endogenous expression levels. | Selection of precise knockin loci/promoters[54]<br>CUT&RUN mapping[55] |
| Delivery | Gene edits occur precisely within the target cell or tissue. | Targeted AAV vectors[57,58]<br>Nanocarriers[13,62,64,67,70]<br>Tissue-specific nucleases[66] |

**Acronyms:**
CRISPR, Clustered Regularly-Interspaced Short Palindromic Repeats; BLISS, Breaks Labeling *In Situ* and Sequencing; RNP, Ribonucleoprotein; NHEJ, Non-Homologous End Joining; HDR, Homology-Directed Repair; PAM, Protospacer-Adjacent Motif; ssODN, Single-Stranded Oligodeoxynucleotide; DSB, Double Strand Break; sgRNA, Single-Guide RNA; MMEJ, Microhomology-Mediated End-Joining; CAR, Chimeric Antigen Receptor; CUT&RUN, Cleavage Under Targets and Release Using Nuclease; TRAC, T Cell Receptor Alpha Constant; TCR: T Cell Receptor; AAV: Adeno-Associated Virus; PGA, Polyglutamic acid; CNS, Central Nervous System; iPSC, Induced Pluripotent Stem Cell; BE, Base Editor; RV, Retrovirus

**Figure 1: Four types of "precision" in genome editing.** Schematic illustrates four ways in which precision genome editing can be achieved: (1) the binding of genome editing machinery to the desired target genomic locus, (2) the incorporation of the correct sequence into the edited locus following DSB formation or after base editing (not shown), (3) precise regulation of integrated transgenes by endogenous promoters and distal elements in comparison to random integration, and (4) delivery to specific cell types by engineered nanomaterials or viral capsids.

**Figure 2: Examples of increased precision with genome editing.** a) Decreasing off-target mutation through Cas9 protein engineering. xCas9 has engineered catalytic sites to recognize different PAM sites. This development led to decreased levels of off-target effects at the human *EMX1* locus[25]. b) Increasing precise gene editing through localization of donor DNA templates. *Left:* Ratio of precise to imprecise editing using S1mplex. *Right:* S1mplex technology tethers donor ssODN to Cas9 RNP through aptamers in the sgRNA[38]. c) *Top:* Precise editing of genomic loci without DSB formation. Schematic of base editor (BE) technology, deaminase attached to Cas9 RNP is capable of creating a G>A mutation without the formation of genomic instability. *Bottom:* Efficiency of base editor system at three genomic loci[49]. d) Insertion of a Chimeric Antigen Receptor (CAR) transgene into the human T cell receptor (TCR) alpha constant (*TRAC*) locus. Insertion into this locus regulated the gene via the endogenous *TRAC* promoter, yielding more potent CAR T cells that prolonged the survival of a leukemic mouse model[54]. RV: retroviral. All data pending reprint permission.

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**Conflict of Interest**
J.C-S. and K.S. have filed a patent on the S1mplex technology. K.M. declares no conflict of interest.

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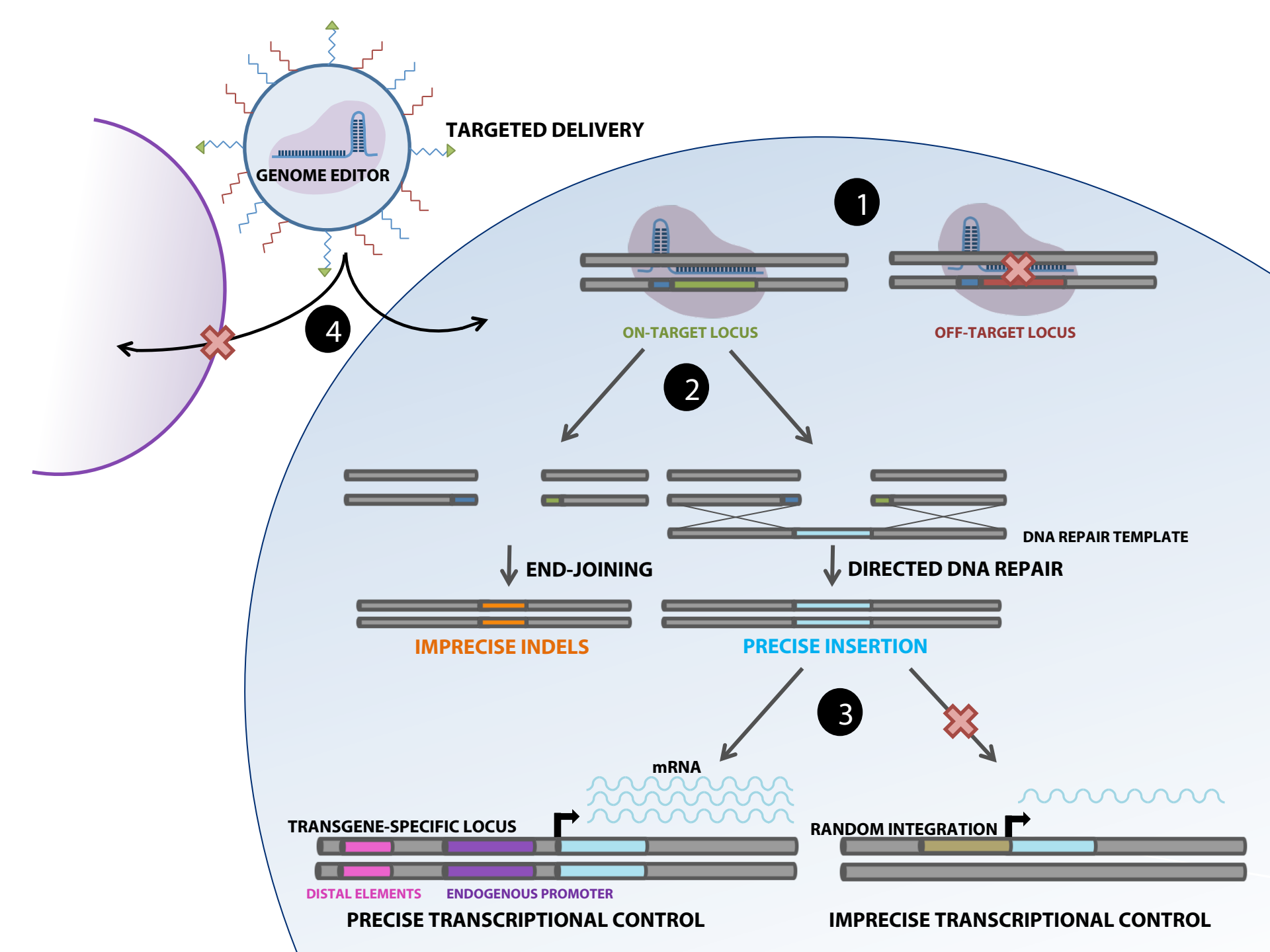
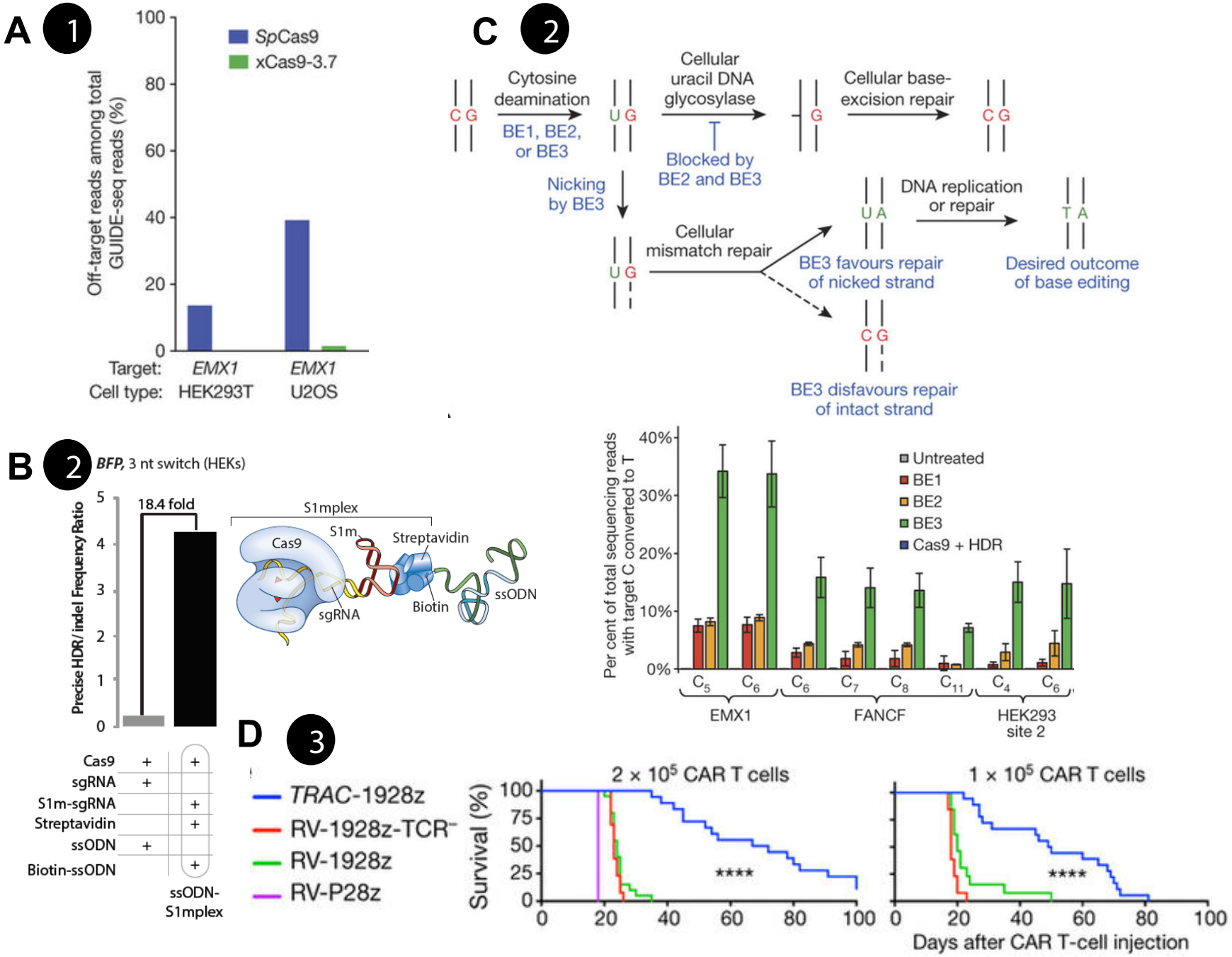

GENOME EDITOR
TARGETED DELIVERY
1
ON-TARGET LOCUS
OFF-TARGET LOCUS
2
DNA REPAIR TEMPLATE
END-JOINING
DIRECTED DNA REPAIR
IMPRECISE INDELS
PRECISE INSERTION
3
mRNA
TRANSGENE-SPECIFIC LOCUS
DISTAL ELEMENTS
ENDOGENOUS PROMOTER
RANDOM INTEGRATION
4
PRECISE TRANSCRIPTIONAL CONTROL
IMPRECISE TRANSCRIPTIONAL CONTROL


A 1
SpCas9
xCas9-3.7
Off-target reads among total GUIDE-seq reads (%)
100
80
60
40
20
0
Target: EMX1 EMX1
Cell type: HEK293T U2OS
C 2
Cytosine deamination
BE1, BE2, or BE3
Cellular uracil DNA glycosylase
Blocked by BE2 and BE3
Cellular base-excision repair
Nicking by BE3
Cellular mismatch repair
DNA replication or repair
BE3 favours repair of nicked strand
Desired outcome of base editing
BE3 disfavours repair of intact strand
B 2
BFP, 3 nt switch (HEKs)
Precise HDR/indel Frequency Ratio
18.4 fold
S1mplex
Cas9
S1m
Streptavidin
sgRNA
Biotin
ssODN
Cas9
sgRNA
S1m-sgRNA
Streptavidin
ssODN
Biotin-ssODN
ssODN-S1mplex
Per cent of total sequencing reads with target C converted to T
Untreated
BE1
BE2
BE3
Cas9 + HDR
EMX1
FANCF
HEK293 site 2
D 3
TRAC-1928z
RV-1928z-TCR−
RV-1928z
RV-P28z
Survival (%)
2 × 10^5 CAR T cells
1 × 10^5 CAR T cells
****
Days after CAR T-cell injection