Review

DNA, RNA, and Protein Tools for Editing the Genetic Information in Human Cells

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Solving the structure of DNA in 1953 has unleashed a *tour de force* in molecular biology that has illuminated how the genetic information stored in DNA is copied and flows downstream into RNA and proteins. Currently, increasingly powerful technologies permit not only reading and writing DNA *in vitro* but also editing the genetic instructions in cells from virtually any organism. Editing specific genomic sequences in living cells has been particularly accelerated with the introduction of programmable RNA-guided nucleases (RGNs) based on prokaryotic CRISPR adaptive immune systems. The repair of chromosomal breaks made by RGNs with donor DNA patches results in targeted genome editing involving the introduction of specific genetic changes at predefined genomic positions. Hence, donor DNAs, guide RNAs, and nuclease proteins, each representing the molecular entities underlying the storage, transmission, and expression of genetic information, are, once delivered into cells, put to work as agents of change of that very same genetic text. Here, after providing an outline of the programmable nuclease-assisted genome editing field, we review the increasingly diverse range of DNA, RNA, and protein components (e.g., nucleases and "nickases") that, when brought together, underlie RGN-based genome editing in eukaryotic cells.

INTRODUCTION

Up until about 6 years ago, when speaking of genome editing using sequence-specific designer or programmable nucleases, scientists were mostly referring to transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) (Gaj et al., 2013; Maggio and Gonçalves, 2015). Currently, RNA-guided nucleases (RGNs) based on CRISPR systems are, undoubtedly, dominating the field (Doudna and Charpentier, 2014). Native type II CRISPR-Cas9 nucleases from *Streptococcus pyogenes* were the first to be adapted into genome editing tools (Cong et al., 2013; Cho et al. 2013; Jinek et al., 2013; Mali et al., 2013a), and similar to other prokaryotic CRISPR systems, they confer adaptive immunity by recording, detecting, and degrading foreign DNA from invading viruses and plasmids (Doudna and Charpentier, 2014). Soon after the discovery in 2012 that CRISPR-Cas9 nucleases cleave DNA in an RNA-programmable manner (Gasiunas et al., 2012; Jinek et al., 2012), researchers hijacked this prokaryotic defense system and turned it into a powerful tool to edit the genomes of eukaryotic cells in a targeted and permanent basis (Cong et al., 2013; Cho et al., 2013; Jinek et al., 2013; Mali et al., 2013a). As a result, RGN-based genetic engineering has been widely used in basic and applied research. Nevertheless, among other shortcomings, the tools underpinning RGN technologies suffer from suboptimal target cell delivery (Maggio and Gonçalves, 2015), off-target activities (Cho et al., 2014; Cradick et al., 2013; Fu et al., 2013; Hendel et al., 2015a; Kuscu et al., 2014; Lin et al., 2014), and hindrance by compact chromatin (Chen et al., 2016, 2017a; Daer et al., 2017). Toward overcoming these hurdles, and hence unleashing the full potential of RGN-based genome editing, researchers are devising improved delivery systems (Chen and Gonçalves, 2016; Glass et al., 2018; Maggio and Gonçalves, 2015), unearthing new tools buried in microorganism metagenome databases (Murugan et al., 2017), and engineering enhanced versions of established RGNs, most notably those based on CRISPR-Cas9 systems (Mitsunobu et al., 2017). Examples of the latter developments include *S. pyogenes* and *Staphylococcus aureus* Cas9 proteins with novel or higher target site specificities. Besides these efforts, researchers are also fine-tuning the structures and/or compositions of guide RNAs (gRNAs) and donor DNA molecules for bringing about efficient and accurate genome editing of cells from higher eukaryotes.

Genome Editing in a Nutshell

Efficient genome editing relies on programmable nucleases, which, by making site-specific chromosomal double-stranded DNA breaks (DSBs), trigger cellular DNA repair pathways that are exploited for establishing specific genome editing outcomes, e.g., gene knockouts or gene knockins (Doudna and Charpentier, 2014; Gaj et al., 2013; Krejci et al., 2012; Maggio and Gonçalves, 2015; Rouet et al., 1994). For instance,

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non-homologous end joining (NHEJ) can knockout *cis*- and *trans*-acting DNA elements after incorporating small insertions and deletions (indels) of varying length and composition at the programmable nuclease breakpoint; the homology-directed repair (HDR) can, in turn, accurately knock in new genetic information using exogenous DNA (donor DNA) as surrogate DSB-repairing templates (Doudna and Charpentier, 2014; Gaj et al., 2013; Krejci et al., 2012; Maggio and Gonçalves, 2015; Rouet et al., 1994). It is noteworthy mentioning that, in mammalian cells, DNA repair often favors NHEJ over HDR, with the HDR machinery largely restricted to the late G2 and S phases of the cell cycle (Kass and Jasin, 2010). As corollary, HDR-mediated genome editing is mostly restricted to cycling cells.

Among the different programmable nucleases currently in use, ZFNs and TALENs share a similar generic architecture in that a sequence-specific DNA-binding protein domain is fused to the nuclease domain of the type IIS FokI restriction enzyme (Bitinaite et al., 1998). Moreover, ZFNs and TALENs work in pairs, cleaving their target DNA after the *in situ* dimerization and ensuing catalytic activation of their FokI domains (Christian et al., 2010; Kim et al., 1996; Li et al., 2011; Mahfouz et al., 2011). The vertebrate Cys_2-His_2 zinc-finger motif is the basic unit of the DNA-binding domain of ZFNs, each of which typically recognizes a specific triplet on the DNA double helix (Wolfe et al., 2000). Thus, assembling 4–6 zinc-finger motifs per ZFN monomer generally provides enough target site specificity within expansive eukaryotic genomes. TALENs recognize DNA through an array of repeated domains from TALE proteins found in certain phytopathogenic bacteria, e.g., *Xanthomonas* sp. (Boch et al., 2009; Moscou and Bogdanove, 2009). Depending on the particular TALE, each repeat is normally composed of 33–35 conserved amino acids with polymorphic residues, dubbed repeat variable di-residues (RVDs), located at positions 12 and 13. Crucially, as each RVD recognizes a specific nucleotide, TALE repeat arrays targeting a predefined DNA sequence can be easily designed (Boch et al., 2009; Moscou and Bogdanove, 2009). Researchers are exploring ZFNs and TALENs not only for generating transgenic animal models (Geurts et al., 2009; Tesson et al., 2011) but also for treating human disorders, including acquired immunodeficiency syndrome (Tebas et al., 2014), hemophilia B (Sharma et al., 2015), and certain leukemias (Qasim et al., 2017). Nevertheless, many laboratories flinch from using ZFNs and TALENs as making these protein-guided nucleases requires particular expertise, longer timelines, and higher costs than those needed for assembling RGNs.

Indeed, RGNs from the prototypic *S. pyogenes* type II CRISPR-Cas9 system and more recently, those from other species as well, are offering unprecedented speed and versatility with which robust genome editing tools can be built (Figure 1) (Doudna and Charpentier, 2014; Gaj et al., 2013; Maggio and Gonçalves, 2015; Murugan et al., 2017; Ran et al., 2015; Zetsche et al., 2015). Native RGNs from *S. pyogenes* consist of a Cas9 nuclease (SpCas9) and two RNAs, i.e., a sequence-specific CRISPR RNA (crRNA) and a *trans*-activating crRNA (tracrRNA) (Deltcheva et al., 2011; Doudna and Charpentier, 2014). Together these two RNAs form a crRNA:tracrRNA duplex that binds the SpCas9 nuclease and directs it to a target site consisting of an NGG triplet called protospacer-adjacent motif (PAM) and a 20-nt-long sequence (protospacer) complementary to the 5' end of the crRNA (spacer) (Doudna and Charpentier, 2014; Gasiunas et al., 2012; Jinek et al., 2012). The recognition of the NGG triplet by the PAM-interacting domain of SpCas9 is followed by double helix melting, local RNA-DNA hybridization, R-loop elongation, and PAM-dependent allosteric activation of the HNH and RuvC nuclease domains (Anders et al., 2014; Nishimasu et al., 2014; Sternberg et al., 2014). This series of events results in the generation of a DSB mostly positioned 3 bp distal from the PAM (Anders et al., 2014; Gasiunas et al., 2012; Jinek et al., 2012; Sternberg et al., 2014).

The initial adaptation of the native *S. pyogenes* CRISPR-Cas9 nuclease system into a powerful genome editing tool involved expressing human codon-optimized *Cas9* open reading frames (ORFs) and RNA Pol III-driven single guide RNAs (sgRNAs) formed by the fusion of crRNA and tracrRNA moieties mimicking the original crRNA:tracrRNA structure (Figure 1A) (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013a). Hence, by simply swapping the spacer sequence in the sgRNA, researchers can target virtually any genomic sequence of choice with unprecedented flexibility, as no protein engineering efforts are required. Depending on the type and location of target cells (e.g., transformed versus primary and cultured *in vitro* versus present in a living organism), RGNs can be delivered directly as ribonucleoproteins (RNPs) or instead encoded in mRNA, plasmid DNA, or viral vector genomes (Chen and Gonçalves, 2016; Glass et al., 2018; Maggio and Gonçalves, 2015). Depending on the goal(s), RGNs can be designed to induce (1) disruption of specific sequences (gene knockouts), (2) gene knockins, (3) targeted DNA deletions and inversions, (4) directed chromosomal translocations, or (5) single nucleotide substitutions (Maggio and Gonçalves, 2015).
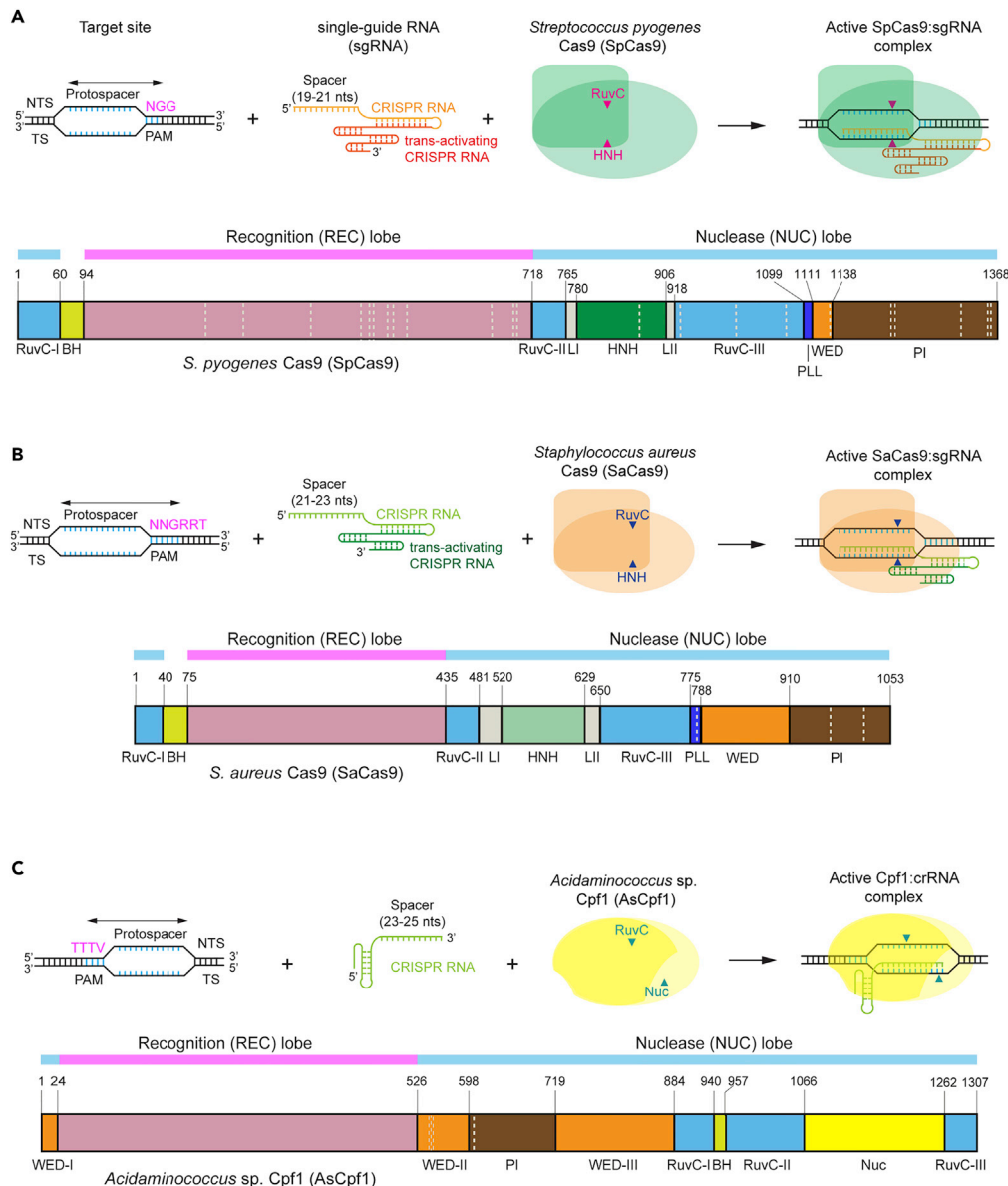
Figure 1. Schematics of Representative RNA-Programmable Nucleases Based on Type II and Type V CRISPR Systems and Domain Organization of Their Nucleases

(A and B) RGN components derived from type II CRISPR-Cas9 systems (*S. pyogenes* and *S. aureus*). HNH, histidine-asparagine-histidine nuclease domain; RuvC, RNase H-like fold nuclease domain formed by a tripartite assembly of RuvC I, II, and III. The HNH and RuvC domains form the nuclease lobe and cut the target and the non-target strands of DNA, respectively. LI and LII, linker region I and II, respectively; BH, bridge helix; WED, wedge domain, aids in recognizing the crRNA-DNA heteroduplex and the PAM; PLL, phosphate lock loop; PI, PAM-interacting domain responsible for the initial interaction between the Cas9 proteins and DNA; NTS and TS, RGN non-target strand and target strand, respectively.

(C) RGN components derived from a type V CRISPR-Cas9 system (*Acidaminococcus* sp.). WED, wedge domain formed by a tripartite assembly of WED I, II, and III. Nuc, atypical nuclease domain responsible for the cleavage of the target strand after its activation by the RuvC domain; BH, bridge helix. Vertical dashed lines indicate the locations of the various mutations present in the CRISPR nuclease variants (see Table 1). Numerals indicate the amino acid positions delimiting the domains and motifs of each protein.

Bottlenecks of CRISPR-Cas9 Technologies

Admittedly, there are no perfect programmable nucleases. Similar to ZFNs and TALENs, RGNs can cleave at unintended off-target sites, whose distribution can only be partially identified through *in silico*-guided and genome-wide assays (Hendel et al., 2015a; Martin et al., 2016). Given the complexity and large size of the human genome (~3.2 billion base pairs per haploid genome), the generation of off-target DSBs poses a non-negligible risk, which, for instance, might knock out essential or haploinsufficient genes or mutate cancer-associated genes (Cho et al., 2014; Cradick et al., 2013; Fu et al., 2013; Kuscu et al., 2014; Lin et al., 2014). These outcomes must be avoided especially when the goal is to develop genetic therapies that exploit the activation of the NHEJ or the HDR pathways (Cox et al., 2015). In the context of gene knockout approaches, the unpredictable indel footprints resulting from NHEJ-mediated DSB repair might produce aberrant products, e.g., truncated or misfolded proteins or proteins displaying immunogenic epitopes. In addition, whenever genome editing strategies involve the generation of multiple DSBs at different loci, chromosomal rearrangements might arise contributing to genomic instability. Regarding HDR-based genome editing, RGNs, apart from faithfully inducing the targeted integration of the exogenous DNA at one allele, often disrupt the other allele due to the aforementioned prevalence of NHEJ over HDR in mammalian cells (Chen et al., 2017b). Moreover, often, HDR-mediated chromosomal DNA insertion triggered by site-specific DSBs is not efficient enough (<1–5%), especially when donor templates encompass entire transcription units stretching several kilobases. Finally, the native chromatin environment in eukaryotic cells poses yet another hurdle compromising the efficiency and predictability of genome editing efforts (Chen et al., 2016, 2017a; Daer et al., 2017).

To address the aforementioned issues, many researchers are investigating a broad range of strategies as diverse as engineering or mining for new nucleases, redesigning sgRNA moieties, and testing new donor DNA structures aiming at efficient and accurate genetic manipulations in complex (epi)genomes.

Isolating and Engineering New Cas9 Proteins

Although the SpCas9 PAM sequence NGG occurs frequently in the human genome, it can, nonetheless, constitute a limitation, especially when the genomic sequences of interest are characterized by a high A•T content. Using a SpCas9 selection-based system in bacteria, Kleinstiver et al. isolated the VQR and VRER variants of SpCas9, which recognize the alternative PAM motifs NGAN and NGCG, respectively (Table 1) (Kleinstiver et al., 2015a). Importantly, their directed evolution system has also yielded the D1135E SpCas9 variant, which displays superior discrimination between canonical NGG and cryptic NGA PAMs than the wild-type SpCas9 protein (Table 1) (Kleinstiver et al., 2015a).

Multiple CRISPR-Cas systems isolated from different species, including those from *S. aureus* (Figure 1B) (Ran et al., 2015), *Acidaminococcus* sp. (Figure 1C) (Zetsche et al., 2015), and *Lachnospiraceae* sp. (Zetsche et al., 2015), have been equally adapted for genome editing in mammalian cells. Often, the RGNs based on these alternative CRISPR systems display an improved or complementary set of attributes when compared with the prototypic SpCas9-based system. For instance, in contrast to the 4.1-kb ORF of the SpCas9 nuclease (1,368 amino acids), the ORF coding for the *S. aureus* Cas9 (SaCas9) protein (1,053 amino acids) spans only ~3.2 kb, making it easily packaged within the limited confinements of commonly used viral vectors, e.g., adeno-associated viral vectors (Ran et al., 2015). In addition, Cas9 orthologs offer alternative DNA specificities and genomic targeting ranges owing to their different PAM sequences, e.g., SaCas9 has as PAM the NNGRRT motif instead of NGG (Table 1) (Ran et al., 2015). To further increase the versatility of SaCas9-based RGNs, Kleinstiver et al. generated through a directed evolution methodology the KKH SaCas9 variant, which recognizes as PAM the degenerate sequence NNNRRT and as a result, confers a broadened genomic coverage to these RGNs (Table 1) (Kleinstiver et al., 2015b). In contrast to the *S. pyogenes* and *S. aureus* type II CRISPR-Cas9 systems (Figures 1A and 1B, respectively), those based on the *Acidaminococcus* and *Lachnospiraceae* type V CRISPR-Cpf1 systems recognize T-rich PAM sequences, generate staggered DSBs with 5′ overhangs, intrinsically display high specificities, and do not have a tracrRNA component (Figure 1C) (Zetsche et al., 2015). Similar to the development of Cas9 proteins with altered PAMs, using structure-guided mutagenesis, Gao and colleagues engineered mutants of *Acidaminococcus* Cpf1 (AsCpf1) that recognize the PAM's TYCV and TATV instead of the native PAM sequence TTTV (Table 1) (Gao et al., 2017a).

In parallel to efforts on modifying the target range of RGNs, recent research is also leading to improvements in reducing RGN off-target activities. For instance, studies on the development of ZFNs and TALENs

Nuclease	PAM	Mutation(s)	Highlights	References
SpCas9	NGG	-	-	e.g. Mali et al. 2013a
Cas9-HF1	NGG	N497A/R661A/Q695A/Q926A	Off-target ↓	Kleinstiver et al. 2016
eSpCas9(1.1)	NGG	K848A/K1003A/R1060A	Off-target ↓	Slaymaker et al. 2016
HypaCas9	NGG	N692A/M694A/Q695A/H698A	Off-target ↓	Chen et al. 2017c
HeFm2SpCas9	NGG	N497A/R661A/Q695A/K848A/Q926A/R1060A	Off-target ↓ efficiency ↓	Kulcsár et al. 2017
D1135E variant	NGG	D1135E	PAM specificity ↑	Kleinstiver et al. 2015a
VQR variant	NGAN	D1135V/R1335Q/T1337R	Different PAM	Kleinstiver et al. 2015a
EQR variant	NGAG	D1135E/R1335Q/T1337R	Different PAM	Kleinstiver et al. 2015a
VRER variant	NGCG	D1135V/G1218R/R1335/T1337R	Different PAM	Kleinstiver et al. 2015a
EvoCas9	NGG	R661Q/K526E/Y515N/M495N	Off-target ↓	Casini et al. 2018
xCas9-3.7	NG/GAA/GAT	A262T/R324L/S409I/E480K/E543D/M694I/E1219V	Broad PAM	Hu et al. 2018
SaCas9	NNGRRT	-	-	Ran et al. 2015
KKH variant	NNNRRT	E782K/N968K/R1015H	Different PAM	Kleinstiver et al. 2015b
AsCpf1	TTTV	-	-	Zetsche et al. 2015
AsCpf1 variant	TYCV	S542R/K607R	Different PAM	Gao et al. 2017a
AsCpf1 variant	TATV	S542R/K548V/N552R	Different PAM	Gao et al. 2017a
LbCpf1	TTTV	-	-	Zetsche et al. 2015

Table 1. Summary of the Main Characteristics of RNA-Programmable Nucleases Based on Type II (SpCas9 and SaCas9) and on Type V (AsCpf1 and LbCpf1) Class 2 CRISPR Systems

have set the stage for optimizing the specificity of RGNs by mimicking the *modus operandi* of ZFNs and TALENs that, as mentioned earlier, encompass the dimerization of the FokI nuclease domain (Guilinger et al., 2014; Tsai et al., 2014). Tsai et al. and Guilinger et al. fused the FokI nuclease domain to the N terminus of a catalytically inactive SpCas9 protein to form FokI-dCas9 monomers (where "d" stands for "dead" Cas9) (Guilinger et al., 2014; Tsai et al., 2014). The binding of a pair of these monomers to their bipartite target sequences forms a dimeric RNA-guided FokI nuclease (RFN), offering a higher specificity profile when compared with conventional RGNs as the target sequences of RFNs span up to 44 bp (Figure 2). Despite their improved specificity, RFNs have limited genomic DNA coverage due to their strict gRNA design and spacing requirements. In particular, RFNs require a so-called PAM-out gRNA design with their activities peaking within a 14- to 17-bp spacing between the two hemi-nuclease target sites (Figure 2) (Guilinger et al., 2014; Tsai et al., 2014). These limitations, together with the fact that RFNs are larger than conventional RGNs, have been keeping this approach at the proof-of-principle stage.

The nuclease domains of SpCas9, HNH and RuvC, cut the target and non-target strands, respectively (Figure 1A). Mutating to alanine the catalytic residues D10 and H840 in each of these nuclease domains yields the RuvC mutant SpCas9[D10A] and the HNH mutant SpCas9[H840A] proteins (Figure 2), which nick the target and non-target strands, respectively (Doudna and Charpentier, 2014; Jinek et al., 2012). Delivering into cells such a Cas9 "nickase" together with two different gRNAs targeting opposite DNA strands of a bipartite recognition sequence induces a DSB owing to the local coordinated action of both nicking RGN complexes (Figure 2) (Mali et al., 2013b; Ran et al., 2013). Should either of the nicking complexes cleave at off-target sites, the resulting single-strand breaks (SSBs) are, for the most part, faithfully repaired.
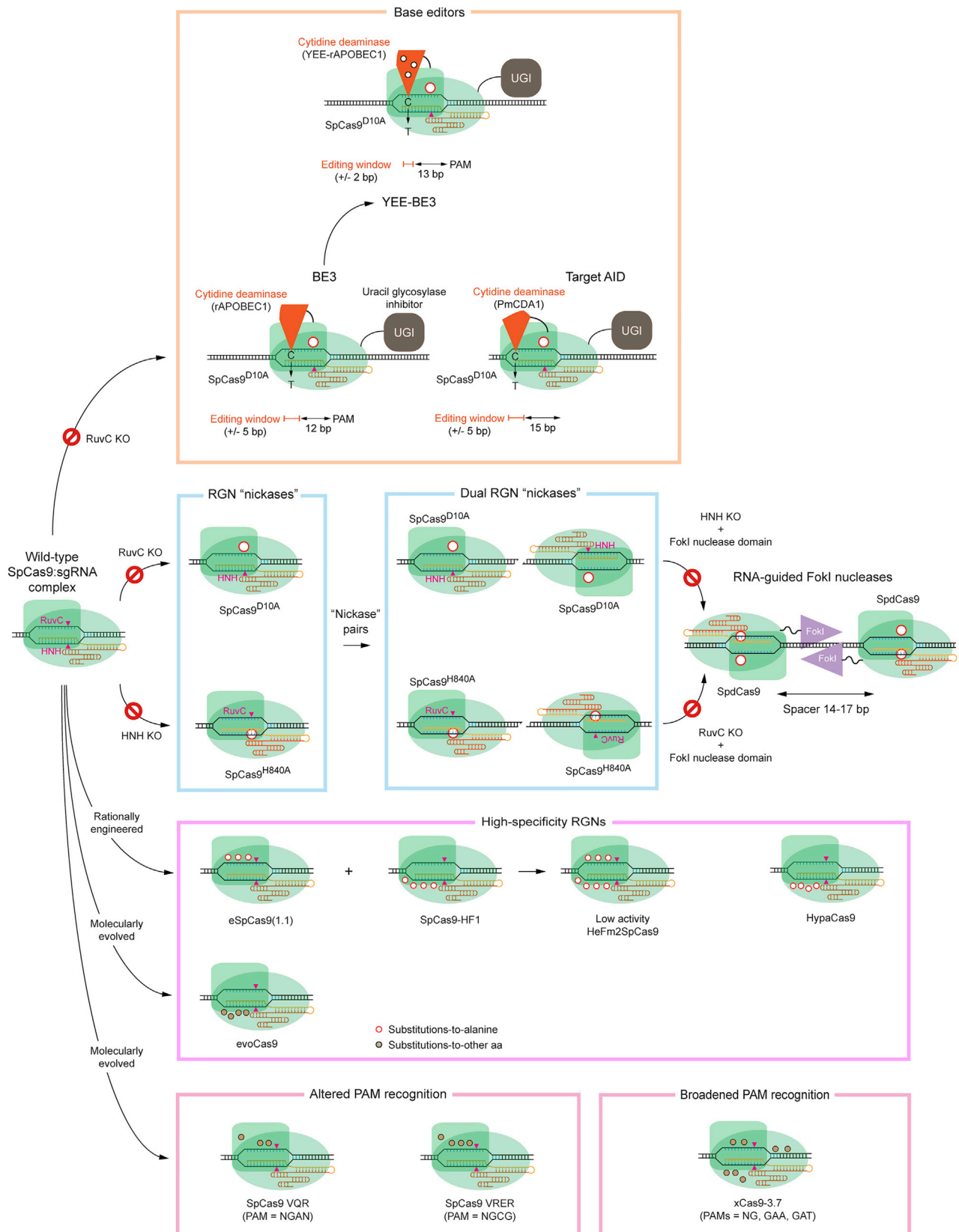
Figure 2. Representative Examples in the Growing Family Tree of RNA-Guided DNA Editing Tools Based on Type II CRISPR Systems

SpCas9^{D10A} and SpCas9^{H840A}: operate as sequence- and strand-specific DNA endonucleases ("nickases") owing to D10A and H840A mutations in their catalytic RuvC and HNH domains, respectively.

Dual RGN "nickases": entail a tandem pair of SpCas9 "nickases" (SpCas9^{D10A} or SpCas9^{H840A}), in which each member cleaves one of the two DNA strands at a bipartite target sequence resulting in a staggered DSB. RNA-guided FokI nucleases: entail a tandem pair of fusion proteins in which a catalytically inert ("dead") SpCas9 protein (dSpCas9) is linked to the nuclease domain of the type IIS restriction enzyme FokI. Binding to a bipartite target sequence leads to *in situ* FokI domain dimerization and ensuing targeted DSB formation. Base editors: fusion proteins that operate through DNA repair mechanisms other than NHEJ and HDR involving chemical modification of specific bases, i.e., C•G to T•A (shown) or A•T to G•C (not shown). AID, activation-induced deaminase ortholog PmCDA1 from sea lamprey; rAPOBEC, rat APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 1). High-specificity RGNs: structure-guided rational design and directed evolution methodologies are yielding SpCas9 variants with reduced off-target activities, e.g., eSpCas9(1.1), SpCas9-HF1, HypaCas9, and evoCas9. Cas9 proteins with altered and broadened PAM recognition: directed evolution approaches are leading to the isolation of Cas9 variants recognizing different specific PAMs or multiple PAMs. Of note, gene editing tools and strategies are also being combined to further expand the range of genome editing options. Examples include fusing base editing effector moieties (e.g., APOBEC1 and UGI) to different scaffolds based on Cas9 orthologs (e.g., SaCas9) or variants (e.g., xCas9-3.7).

Thus, this confers an overall high specificity to the dual nicking approach. Indeed, when compared with conventional RGNs, the dual nicking strategy can reduce off-target activities by up to 1,500-fold (Ran et al., 2013). Notably, reminiscent of RFNs, the relative positions and spacing of the two gRNAs are important for efficient target DNA cleavage. Usually, dual RGN "nickases" yielding 5′ overhangs and a spacing between the 5′ ends of the two gRNAs from −4 to 20 bp and from −4 to 100 bp results in high and moderate DSB formation activities, respectively (Ran et al., 2013). Of note, SaCas9 "nickases" bearing D10A or N580A mutations were also shown to be compatible with the dual nicking strategy, although in this case, the optimal distance between the gRNAs seems to lie between 0 and 125 bp (Friedland et al., 2015). The relatively broad spacing afforded by dual RGN "nickases" gives them a higher genomic coverage than that of conventional RGNs. However, often, there is a trade-off in which the gains in specificity are accompanied by a decrease in efficiency (Friedland et al., 2015; Mali et al., 2013b; Ran et al., 2013).

To obviate finding a suitable pair of gRNAs for dual nicking to increase the specificity of RGNs, researchers have generated high-specificity SpCas9 variants through structure-guided protein engineering involving mutations-to-alanine of specific DNA-interacting residues (Figure 2) (Kleinstiver et al., 2016; Slaymaker et al., 2016). They reasoned that the resulting reduction in the RGN-DNA binding energies would be adequate to cleave the target site but insufficient to cleave at off-target sites (Kleinstiver et al., 2016; Slaymaker et al., 2016). Slaymaker et al. reported enhanced specificity of the SpCas9 variants eSpCas9(1.0) and eSpCas9(1.1) containing triple mutations (i.e., K810A/K1003A/R1060A and K848A/K1003A/R1060A, respectively) at the positively charged nt-groove of SpCas9, which plays a role in stabilizing the non-target DNA strand (Figure 2) (Slaymaker et al., 2016). Kleinstiver et al. generated in turn the quadruple mutant SpCas9-HF1 (i.e., N497A/R661A/Q695A/Q926A). The SpCas9-HF1 mutations are thought to attenuate hydrogen bonding between SpCas9 and the phosphate backbone of target DNA strands (Figure 2) (Kleinstiver et al., 2016). Both strategies significantly reduce off-target chromosomal DNA cleavage while retaining on-target activities similar to those of wild-type SpCas9 for the majority of target sequences tested (Kleinstiver et al., 2016; Slaymaker et al., 2016). Interestingly, the SpCas9 variant HeFm2SpCas9, harboring combinatorial mutations from eSpCas9(1.1) and SpCas9-HF1 (Figure 2), albeit more specific than their parental proteins, suffers from low activity at most target sites tested (Table 1) (Kulcsár et al., 2017). More recently, Chen et al. showed that the increase in specificity of eSpCas9(1.1) and SpCas9-HF1 were most likely due to a failure in the conformational change of the REC3 domain necessary for HNH catalytic activation at off-target sites (Chen et al., 2017c). Based on this information, they engineered a SpCas9 protein containing the REC3 mutations N692A/M694A/Q695A/H698A and showed that this so-called HypaCas9 has comparable to or higher specificity than SpCas9-HF1 and eSpCas9(1.1) (Figure 2, Table 1) (Chen et al., 2017c). Besides rationally engineering high-specificity RGNs, researchers are also starting to devise powerful directed evolution approaches for isolating nucleases with improved specificities and/or with new attributes, e.g., widened genomic space coverage. Indeed, by screening a SpCas9 library containing REC3 mutations established by error-prone PCR, Casini and coworkers succeeded in building the highly specific evoCas9 protein (M495V/Y515N/K526E/R661Q) (Figure 2, Table 1) (Casini et al., 2018). At the target sequences tested, the specificity profiles of evoCas9 were shown to be as high as or superior to those of eSpCas9(1.1) and SpCas9-HF1. Importantly, the on-target activity of evoCas9 was not significantly compromised (Casini et al., 2018). However, it is noteworthy mentioning that the eSpCas9(1.1), SpCas9-HF1, and evoCas9 variants are less efficient than wild-type SpCas9 when the gRNAs contain a 5′ mismatched nucleotide (e.g., 21-mer spacer with a 5′ end "G" extension) or are truncated (i.e., < 20-mer
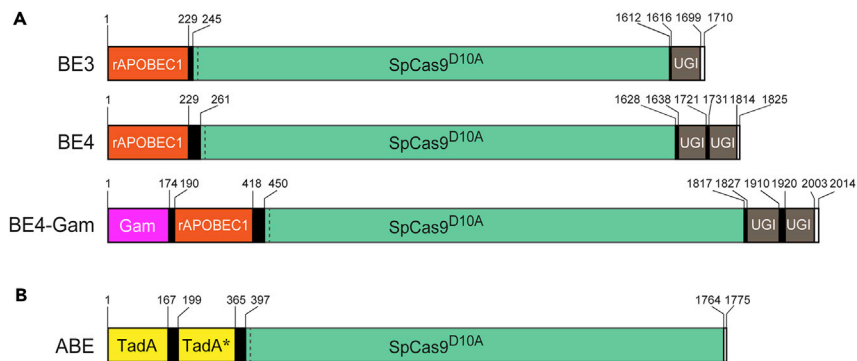
Figure 3. Structure of Representative Base Editors

(A) C•G to T•A base editors built on the SpCas9^D10A "nickase." rAPOBEC, rat APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 1), cytidine deaminase, converts cytosine to uracil; UGI, *Bacillus subtilis* bacteriophage PBS1, blocks the activity of uracil glycosylases; Gam, bacteriophage protein Mu binds to DNA termini, thereby reducing indel formation caused by residual nick-derived DSBs.

(B) A•T to G•C-based editor built on the SpCas9^D10A "nickase." TadA, mammalian codon-optimized *Escherichia coli* tRNA adenine deaminase responsible for converting adenine to inosine in the anticodon loop of tRNA^Arg. In bacteria, TadA forms a homodimer with one monomer responsible for deamination and the other for docking the tRNA substrate. TadA-TadA*: fusion between TadA coding sequences. The evolved TadA* sequence has a set of mutations (W23R/H36L/P48A/R51L/L84F/A106V/D108N/H123Y/S146C/D147Y/R152P/E155V/I156F/K157N) conferring a change in the substrate from RNA to DNA and a higher deamination activity. White boxes, nuclear localization signals; vertical dashed line, marks the D10A position.

spacers) (Casini et al., 2018; Chen et al., 2017a; Kim et al., 2017a). Aiming at broadening the genomic coverage of SpCas9, Hu and colleagues have developed the phage-assisted continuous evolution (PACE) procedure. PACE includes an *in vivo* mutagenesis system, which, after selecting from a phage library of catalytically inert dSpCas9 variants exhibiting expanded PAM affinities (NNN), enriches for dSpCas9 variants recognizing non-NGG motifs. Finally, after restoring the D10 and H840 catalytic residues into several evolved variants, the authors identified xCas9-3.7 as having the widest PAM range, recognizing the NGA, NGC, NGT, GAA, and GAT motifs better than SpCas9 by 1.6-, 2.1-, 4.5-, 7.5-, and 7.5-folds, respectively. Remarkably, although the specificity trait was not selected for, xCas9-3.7 can present >100-fold lower levels of genome-wide cleavage activity at off-target sites harboring the canonical NGG PAM (Figure 2) (Hu et al., 2018).

To circumvent DSB formation and undesirable NHEJ-derived mutations, Komor et al. and Nishida et al. introduced the base editing concept, which ultimately replaces one base pair for another (Komor et al., 2016; Nishida et al., 2016). The rationale behind base editors is that the cytidine in a C•G pair can be converted to a U•G pair by cytidine deaminases, namely, APOBEC1 (Komor et al., 2016) or AID (Nishida et al., 2016). After mismatch repair the U•G becomes a U•A pair, which finally turns into a T•A pair after DNA replication or repair. The first generation of base editors made by Komor et al. has the rat cytidine deaminase APOBEC1 (rAPOBEC1) fused to the dSpCas9 (BE1). To avoid the removal of uracil by uracil glycosylases, a uracil glycosylase inhibitor (UGI) was added to their second-generation base editor (BE2). To increase the base editing efficiency, the dSpCas9 was replaced by a SpCas9 "nickase," yielding a third-generation base editor (BE3), which is a fusion between the rAPOBEC1, a UGI, and the SpCas9^D10A "nickase" scaffold (Figure 3). BE3 generates an SSB in the G-containing strand converting any C•G base pair to a T•A (C→T) within a 5-bp window about 12 bp away from the PAM (Figure 2) (Komor et al., 2016). Of note, a BE3 mutant named YEE-BE3 displaying a weaker cytidine deaminase activity has narrowed the peak of C→T transitions from a 5- to a 2-bp window (Figure 2) (Kim et al., 2017b). More recently, base editors containing as nicking scaffolds SpCas9-HF1^D10A or SaCas9^D10A have improved the specificity of base editors or have expanded their PAM requirements, respectively (Komor et al., 2017; Rees et al., 2017). With a fine-tuned design, the fourth-generation base editor, BE4, has larger linkers between SpCas9^D10A, rAPOBEC1, and UGI and incorporates a second copy of the UGI (Figure 3) (Komor et al., 2017). This construction enriches for the intended C→T transitions and reduces unwanted conversions likely mediated by uracil glycosylases. Other BE3 and BE4 base editors, e.g., BE3-Gam, SaBE3-Gam, BE4-Gam (Figure 3), and SaBE4-Gam, incorporate the bacteriophage Mu Gam protein, which purportedly, by binding to DSBs, reduces

Base editor	Main outcome	Composition	Highlights
BE1	C→T	rAPOBEC1-dCas9	Efficiency ↓
BE2	C→T	rAPOBEC1-dCas9-UGI	Efficiency ↓/ C→T enrichment ↑
BE3	C→T	rAPOBEC1-Cas9n-UGI	Efficiency ↑/ C→T enrichment ↑
YEE-BE3	C→T	rAPOBEC1(W90Y/R126E/R132E)-Cas9n-UGI	Deaminase activity ↓ (editing window: 5→2 bp) / Efficiency ↑/ C→T enrichment ↑
BE4	C→T	rAPOBEC1-Cas9n-UGI-UGI	Efficiency ↑/ C→T enrichment ↑↑
BE3-Gam	C→T	Gam-rAPOBEC1-Cas9n-UGI	Nick-derived DSB ↓ / Efficiency ↑/ C→T enrichment ↑
BE4-Gam	C→T	Gam-rAPOBEC1-Cas9n-UGI-UGI	Nick-derived DSB ↓ / Efficiency ↑/ C→T enrichment ↑↑
SaBE4	C→T	rAPOBEC1-SaCas9n-UGI-UGI	Efficiency at SaCas9 target sites ↑/ C→T enrichement ↑↑
SaBE3-Gam	C→T	Gam-rAPOBEC1-SaCas9n-UGI	Nick-derived DSB ↓ / Efficiency at SaCas9 target sites ↑/ C→T enrichment
ABE7.10	A→G	TadA-Tad*(7.10)-Cas9n	Efficiency within editing window 4-7 bps from PAM ↑
ABE6.3	A→G	TadA-Tad*(6.3)-Cas9n	Efficiency within editing window 4-9 bps from PAM ↑

Table 2. Summary of the Main Characteristics of Representative Base Editors Built on "Dead" SpCas9 (dCas9), Nicking SpCas9^{D10A} (Cas9n), and Nicking SaCas9^{D10A} (SaCas9n) Scaffolds

rAPOBEC1, rat cytidine deaminase (cytosine to uracil) apoliprotein B mRNA editing enzyme, catalytic polypeptide-like1; UGI, *Bacillus subtilis* bacteriophage PBS1 uracil glycosilase inhibitor; Gam, bacteriophage Mu binds to DNA termini; TadA, RNA adenine deaminase, converts adenine to inosine in the anticodon loop of tRNA-Arg; TadA-TadA*, fused homodimer of the *Escherichia coli* RNA adenine deaminase TadA enzyme with mutations conferring a change of substrate from DNA to RNA and higher deamination activity. BE1, BE2, and BE3 are described in Komor et al. (2016); YEE-BE is described in Kim et al. (2017b); BE4 through SaBE3-Gam are described in Komor et al. (2017); ABE7.10 and ABE6.3 are presented in Gaudelli et al. (2017).

the residual levels of nick-derived chromosomal breaks (Table 2) (Komor et al., 2017). Finally, the recent development of adenine base editors nicely complements their base editor (BE) counterparts as these new base editors can convert A·T to G·C pairs (A→G) (Figure 3 and Table 2) (Gaudelli et al., 2017). Theoretically, with current base editors, a large proportion of pathogenic single base mutations can be corrected and, in addition, ATG start codons can be disabled for targeted and precise gene knockouts. The combination of xCas9-3.7 and BE proteins should further expand the genomic coverage of base editors (Hu et al., 2018). An issue that needs to be addressed, however, is that of delivering in an efficient manner these rather large multifunctional proteins into cells.

Redesigning and Modifying gRNAs

sgRNAs have secondary structures forming hairpins and stem loops that mimic the architecture of native gRNAs consisting of the sequence-specific crRNA and the scaffolding tracrRNA (Figure 4A). As mentioned earlier, the 5' terminal 20 nt of the sgRNA (spacer) and the SpCas9-interacting scaffold are the two crucial elements in sgRNA molecules. Truncated gRNAs (tru-gRNAs) displaying 17- to 19-mer spacers instead of the typical 20-mer spacers, have a weaker DNA-binding energy, which, presumably, biases the cleaving activity to fully complementary target sites in detriment of mismatched (off-target) sequences (Figure 4B) (Fu et al., 2014). Indeed, Fu et al. showed that RGNs bearing tru-gRNAs and wild-type SpCas9 can present reduced off-target effects (up to 5,000-fold) while maintaining their on-target activity (Fu et al., 2014). Of note, however, at certain off-target sites, tru-gRNAs can lead to higher frequencies of indels than their full-length counterparts (Slaymaker et al., 2016).

The most commonly used sgRNA structure has a shorter crRNA:tracrRNA duplex region when compared with the native crRNA:tracrRNA duplex, potentially impairing stable Cas9:gRNA complex assembly. Moreover, this common sgRNA structure has a continuous stretch of four uridines, which, in the context of the corresponding DNA template, might serve as a premature RNA polymerase III (Pol III) termination signal (Figure 4A). On the basis of this information, Chen et al. and Dang et al. optimized the sgRNA scaffold by extending the duplex region next to the 5' spacer by 5 bp and showed significantly enhanced dSpCas9-based chromosomal imaging and SpCas9-induced DNA cleaving, respectively (Chen et al., 2013; Dang et al., 2015). By combining the sgRNA duplex extension with the disruption of the uridine stretch, Dang and coworkers demonstrated higher *CCR5* and *CD4* gene knockout frequencies when compared with those achieved by the original sgRNA scaffold (Figure 4C) (Dang et al., 2015). Considering that these optimized scaffolds seem to yield more stable and full-length sgRNAs, they might also be compatible with different (epi)genome editing tools, e.g., Cas9 variants, dSpCas9-based transcriptional regulators, and base editors.

Currently, Pol III promoters are the most widely used elements for sgRNA synthesis as they evolved for expressing short, unprocessed, transcripts in eukaryotic cells. Although highly active, Pol III promoters require a G (e.g., U6 and 7SK promoters) or an A or a G (e.g., H1 promoter) to efficiently initiate
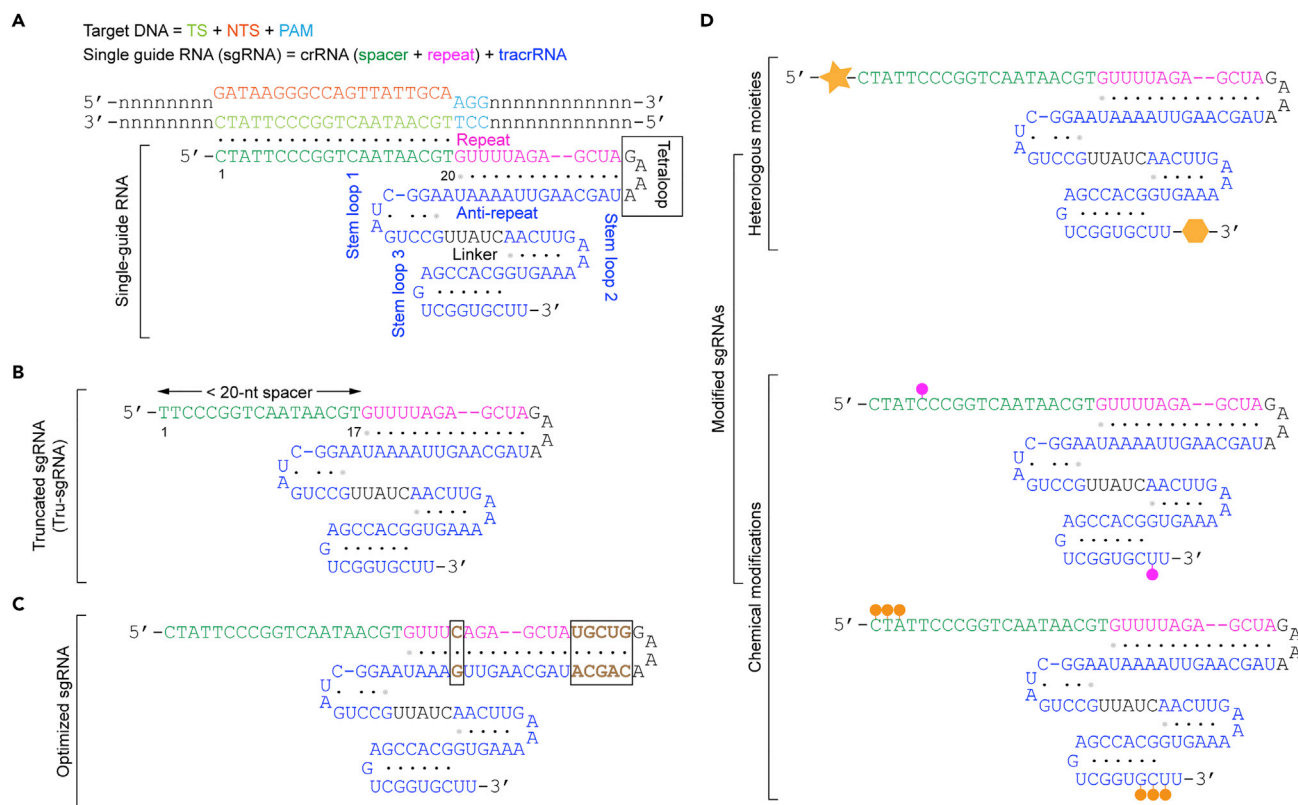
Figure 4. Illustration of Representative sgRNA Structures

(A) A canonical sgRNA interacting with a DNA target sequence. NTS, non-target strand; TS, target strand; PAM, protospacer-adjacent motif; boxed nucleotides, artificial tetraloop covalently linking crRNA and tracrRNA moieties.

(B) Truncated sgRNA (tru-sgRNA) structure. Tru-sgRNAs have sequence-tailored spacer sequences shortened at their 5′ end for enhancing RGN specificity.

(C) Optimized sgRNA structure. The extension of the first loop together with the reduction of the U•A tract from 4 to 3 bp by U•A to C•G substitution (boxed nucleotides) increases RGN activity, presumably by augmenting the yields of full-length sgRNA expressed from RNA Pol III promoters.

(D) Modified sgRNA structures. Modifications to sgRNAs for enhancing RGN performance in terms of activity and/or specificity. Modifications can also be exploited for tracing sgRNA delivery, isolating RGN-exposed cells, or endowing RGN complexes with new functionalities, such as RNA aptamers (Nowak et al., 2016).

transcription. Therefore, the most suited sgRNAs have spacers starting with a G or an A at their 5′ terminus (Gao et al., 2017b). Of note, as mentioned earlier, there seems to be an impairment in the activity of the high-specificity variants eSpCas9(1.1), SpCas9-HF1, and evoCas9 in cases in which their sgRNA partner is truncated or has a 5′ terminal G that does not hybridize to the target site (Casini et al., 2018; Chen et al., 2017a; Kim et al., 2017a). Although RNA polymerase II (Pol II)-driven primary transcripts are subjected to complex processing events and nucleus-to-cytoplasm export, they offer the possibility for conditional or tissue-specific sgRNA expression or for the expression of multiple sgRNAs from a single template. In this regard, Nissim and coworkers designed Pol II expression units containing sgRNA coding sequences flanked by recognition sites for the RNA endonuclease Csy4 (Csy4-gRNA-Csy4), so that single or multiple sgRNAs can be released after Csy4 cutting (Nissim et al., 2014). To avoid the need for the delivery of an RNA endonuclease, e.g., Csy4, the sgRNA sequence can be inserted after a 5′ hammerhead (HH) ribozyme or in between a 5′ HH and a 3′ hepatitis delta virus (HDV) ribozyme (HH-gRNA-HDV). These HH-gRNA-HDV constructs generate mature sgRNAs through self-cleaving (Gao and Zhao, 2014; Nissim et al., 2014; Yoshioka et al., 2015). Of note, however, Yoshioka et al. showed that ribozyme-flanked sgRNAs expressed from the CAG RNA Pol II promoter (CAG-HH-gRNA-HDV) induced less genome editing events than sgRNAs arising from the commonly used U6 RNA Pol III promoter (Yoshioka et al., 2015). Elegantly, Xie et al. hijacked the endogenous tRNA maturation mechanism instead. These authors constructed a Pol III promoter-driven tRNA-sgRNA tandem array whose transcripts yielded functional sgRNAs after their cleavage by endogenous RNases in rice (Xie et al., 2015). Follow-up studies confirmed that sgRNAs produced from the tRNA-gRNA module could achieve efficient

genome editing in *Yarrowia lipolytica,* maize, and, more recently, *Drosophila* (Port and Bullock, 2016; Qi et al., 2016; Schwartz et al., 2016).

Pre-assembling Cas9 and gRNA RNP complexes *in vitro* before their delivery into target cells avoids chromosomal integration of foreign DNA encoding these gene editing components and ensures a strictly transient nuclease activity window (Glass et al., 2018; Maggio and Gonçalves, 2015). The gRNAs destined for RNP assembly are usually made from *in vitro* transcription or by solid-phase chemical synthesis (DeWitt et al., 2017; Kelley et al., 2016). Unlike *in vitro* transcribed gRNAs, chemically synthesized gRNAs bypass the need for difficult-to-upscale enzymatic reactions. Lately, several groups have started testing gRNAs chemically modified at different nucleotide moieties (i.e., base, ribose sugar, and phosphate group) aiming at increasing their stability and activity (Hendel et al., 2015b; Lee et al., 2017; Rahdar et al., 2015; Ryan et al., 2018). For example, Hendal et al. showed that sgRNAs modified with 2'-*O*-methyl, 2'-*O*-methyl-3'-phosphorothioate, or 2'-*O*-methyl-3'-thioPACE at both ends of the sgRNA induced robust genome editing at the *CCR5* and *HBB* loci in CD34^{+} hematopoietic stem and progenitor cells (Figure 4D) (Hendel et al., 2015a, 2015b). In addition, Rahdar and coworkers carried out a systematic screen of crRNAs modified at different positions with chemical substitutions in their phosphate and/or sugar groups. This endeavor resulted in the identification of a shortened, yet equivalently functional, 29-nt-long crRNA containing 2'-fluoro and 2'-S-constrained ethyl at the 5' and 3' ends, respectively (Rahdar et al., 2015). Interestingly, in addition to improving the stability and reducing the size of gRNA components, chemical modifications are also paying off in terms of generating reagents that confer enhanced RGN specificity. Indeed, a recent study demonstrated that 2'-*O*-methyl-3'-phosphonoacetate modifications at positions 5 or 11 of sgRNAs can remarkably reduce off-target activity while maintaining on-target cleavage (Figure 4D) (Ryan et al., 2018). Finally, instead of substituting chemical groups in the ribose phosphate backbone, Lee and coworkers have added large groups at the termini of crRNAs (e.g., 5'-rhodamine and 5'-amine) to endow them with a new functionality, i.e., fluorescence to aid enriching for gene-edited cells (Lee et al., 2017).

Manipulating Donor DNA Templates

As mentioned previously, when partnered with programmable nucleases, donor DNA molecules can act as DSB-repairing HDR templates to precisely incorporate customized DNA changes into specific genomic positions (Maggio and Gonçalves, 2015). Regardless of their foreign nucleic acid composition, conventional donor HDR templates have sequences identical to those framing a programmable nuclease target site ("homology arms"). These "homology arms" serve as regions for sequence pairing and strand invasion that, ultimately, allow for HDR-mediated insertion of foreign DNA whose sizes can vary from single base pairs to whole transgenes (Maggio and Gonçalves, 2015). For the targeted insertion of large transgenes, the donor DNA is traditionally provided in recombinant plasmids or viral vector genomes (Chen and Gonçalves, 2016; Maggio and Gonçalves, 2015). Yet, regardless of the delivery method, as mentioned earlier, HDR occurs mainly during the late G2 and S phases of the cell cycle (Kass and Jasin, 2010). Moreover, the competing and constantly available NHEJ pathway leads to a large fraction of heterozygous alleles due to the concurrent action of HDR and NHEJ, or NHEJ alone (Chen et al., 2017b).

Studies on the design and testing of different types of donor DNA molecules unveiled attractive research avenues to increase the efficiency, specificity, and/or accuracy of HDR-based genome editing (Maggio and Gonçalves, 2015). In this regard, Holkers and coworkers demonstrated that the donor templates delivered in protein-capped adenoviral vector genomes greatly enhance the specificity and fidelity of HDR-based genome editing (Holkers et al., 2014). By using a "double-cut" donor DNA design in which the "homology arms" are flanked by sgRNA target sites (Figure 5; paired breaking strategy), Zhang et al. achieved 8% and 12% transgene knock-in in human induced pluripotent stem cells at the *CTNNB1* locus when the flanking "homology arms" were 300 and 600 bp, respectively (Zhang et al., 2017). These targeted DNA insertion frequencies were 2- to 5-fold higher than those achieved by using the corresponding covalently closed plasmid templates (Zhang et al., 2017). Chen et al. observed a similar effect after comparing "double-cut" with standard donor plasmids at the *DMD* and *AAVS1* loci in human cells (Chen et al., 2017b).

To circumvent the dependency on the HDR pathway, researchers are also testing "double-cut" DNA donors containing no or short "homology arms" (e.g., 10–40 bp) for exploiting the NHEJ or the microhomology-mediated end joining (MMEJ) pathways instead (He et al., 2016; Hisano et al., 2015; Maresca et al., 2013; Suzuki et al., 2016; Yao et al., 2017). For instance, by using RGNs together with homology-free
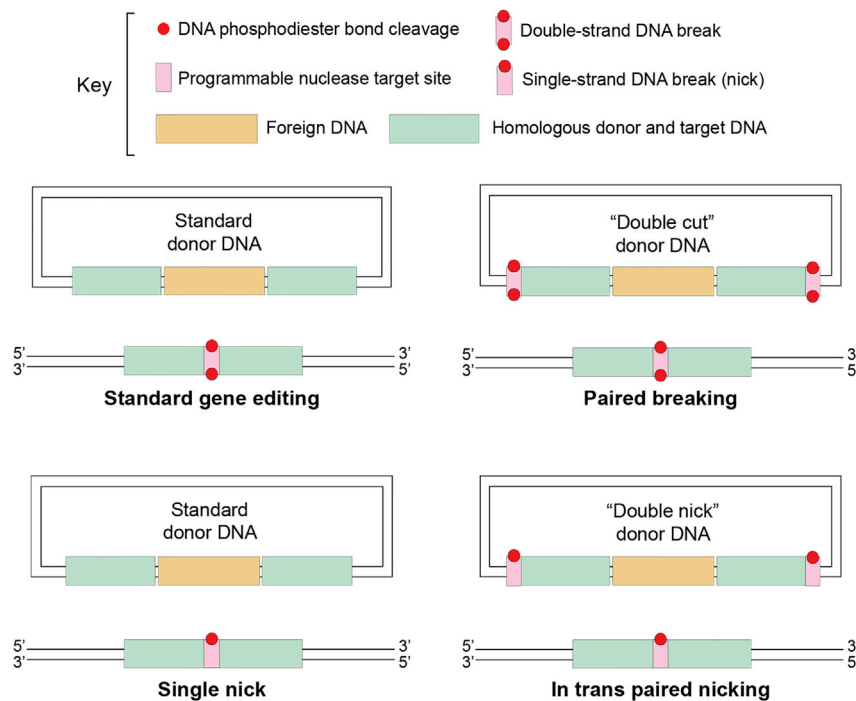
Figure 5. Schematics of Double-Stranded DNA Recombination Substrates Engaged in Different Genome Editing Strategies

The standard and single nick strategies comprise unmodified double-stranded DNA donors for repairing targeted DSBs and SSBs, respectively. The paired breaking and in *trans* paired nicking approaches encompass double-stranded DNA donors containing RGN target sites for concomitant DSB and SSB formation at target and donor sequences, respectively. In contrast to DSBs, nicks are generally not processed through the error-prone NHEJ pathway, yielding, as a result, seamless gene editing outcomes (Chen et al., 2017b).

"double-cut" donors encompassing a 4.6-kb transgene, He et al. reached up to 20% and 1.7% transgene knock-in at the *GAPDH* locus in LO2 cells and embryonic stem cells, respectively (He et al., 2016). Suzuki et al. have, in turn, applied the delivery of RGNs and homology-free "double-cut" donors for achieving NHEJ-mediated targeted chromosomal insertion of different expression units in post-mitotic mouse neurons, both in *vitro* and in *vivo* (Suzuki et al., 2016). Alternatively, "double-cut" donors with short "homology arms" were shown to be efficient substrates for MMEJ-mediated transgene insertion in zebrafish (Hisano et al., 2015) and, more recently, in mice (Yao et al., 2017). However, it is to be noted that free DNA termini generated from "double-cut" donors increase the risk for random insertions and/or imprecise insertion of the foreign DNA due to illegitimate recombination processes (Chen et al., 2017b; Holkers et al., 2014; Zhang et al., 2017).

To avoid inducing DSBs and hence minimizing NHEJ-derived mutations, there are efforts focusing on strategies that resort to SSBs instead of DSBs to facilitate genome editing (Figure 5; single nick strategy) (Gao et al., 2017c; Metzger et al., 2011; McConnell Smith et al., 2009; Ramirez et al., 2012; van Nierop et al., 2009; Wang et al., 2012). Albeit at low levels, SSB-induced HDR using programmable "nickases" can yield targeted and accurate chromosomal insertion of foreign DNA in mammalian cells without the attendant catalytic induction of DSBs (Gao et al., 2017c; Metzger et al., 2011; McConnell Smith et al., 2009; Ramirez et al., 2012; Wang et al., 2012). Recently, building on earlier experiments using the adeno-associated virus nicking Rep78/68 proteins (Gonçalves et al., 2012), Chen and coworkers have developed an efficient SSB-induced HDR approach that can place large DNA segments into specific genomic sites without provoking the mutagenic NHEJ (Chen et al., 2017b). The key aspect to this approach, named in *trans* paired nicking, consists of combining SpCas9 "nickases" with plasmid donors whose "homology arms" are framed by sgRNA target sites (Figure 5) (Chen et al., 2017b). This arrangement assures concomitant SSB formation at target and donor sequences, generating homologous recombination substrates as initially postulated by Holliday in 1964 (Holliday, 1964). The in *trans* paired nicking strategy was shown to increase HDR at different human loci (i.e., *AAVS1*, *CCR5,* and *DMD*) and facilitate multiplexed gene targeting

(Chen et al., 2017b). More recently, Nakajima and coworkers have confirmed that coordinated SSB formation at target and donor DNA by nicking RGNs can enhance HDR-based gene editing without the attendant generation of mutant alleles (Nakajima et al., 2018).

Single-stranded oligodeoxyribonucleotides (ssODNs) are commonly used donors whenever the intended genetic modifications are limited in length, e.g., the introduction of point mutations, polymorphisms, or short protein-tagging motifs. Typical ssODN donors are symmetrical in that they harbor similar sized "homology arms" at both ends of targeting modules. However, recent studies have shown that, when compared with symmetric ssODNs, asymmetric ssODNs can achieve higher RGN-assisted genome editing frequencies (Richardson et al., 2016). Specifically, optimal ssODN donors should be complementary to the sgRNA non-target strand and encompass shorter homology at the PAM distal end (Richardson et al., 2016). Biophysical data support the view that this asymmetric configuration favors donor-target hybridization owing to the generation, after DNA cutting, of a 3'-ended flap corresponding to the non-target strand (Richardson et al., 2016). Liang et al. confirmed the superiority of asymmetric ssODNs and, in addition, tested short double-stranded ODNs (dsODNs) consisting of annealed ssODNs displaying either 3' or 5' overhangs of 30 nt (Liang et al., 2017). They showed that dsODN donors with 3' overhangs lead to higher frequencies of RGN-induced genome editing than those achieved with asymmetric ssODNs or with dsODNs containing 5' overhangs (Liang et al., 2017).

Similar to sgRNA components, donor DNA templates are also substrates for chemical modifications to improve the performance of genome editing. For instance, chemically modifying the terminal nucleotides of ssODNs with phosphorothioate increased the efficiency of genome editing by 2- to 3-fold at the *AAVS1* safe harbor locus in osteosarcoma U2OS cells (Renaud et al., 2016). In another study, Ma et al. introduced the CAB system, short for Cas9-avidin-biotin ssDNA, in which avidin is fused to Cas9 and binds biotin-conjugated ssODNs inside cells (Ma et al., 2017). Equally relying on high-affinity biotin-avidin interactions, Carlson-Stevermer et al. developed the S1mplex system, where Biotin-conjugated ssODNs are tethered *in vitro* to RGNs containing sgRNAs displaying a streptavidin-binding S1m aptamer (Carlson-Stevermer et al., 2017). Both the CAB and S1mplex systems increased HDR frequencies and the ratio of HDR to NHEJ in mammalian cells (Carlson-Stevermer et al., 2017; Ma et al., 2017). Future research should help determining whether multicomponent systems such as CAB and S1mplex will become broadly adopted and applicable.

Conclusions and Outlook

The ongoing discovery of RGN systems scattering prokaryotic genomes, combined with structure-guided and directed evolution-based protein screens are yielding an increasing number of programmable nucleases with new properties. These enzymes have, for instance, sequence- and strand-specific cleaving activities (i.e., nicking), alternative PAM requirements, and higher target DNA specificities. In addition, base editors built on nicking RGN scaffolds, albeit not suited for large genomic DNA changes, constitute promising tools for precise, DSB-free genome editing. Besides isolating and engineering new RNA-guided proteins, various efforts involving reshaping the native gRNA architecture and chemically modifying the gRNA composition are improving the activity and specificity of customized RGNs. By the same token, manipulating the structure and composition of donor DNA molecules is paying off in terms of enhancing the efficiency, specificity, and/or fidelity of genome editing comprising the targeted chromosomal addition of new genetic information (Maggio and Gonçalves, 2015). Concerning the specificity and fidelity parameters, much more research is needed for assessing the role of donor DNA structures (e.g., ssODNs versus dsDNAs) and modifications thereof in the ultimate genome editing precision. This stems from the fact that donor DNA molecules, depending on their type and composition, are differently produced (e.g., bulk ssODN synthesis versus clonal plasmid amplification) as well as differently processed by cellular enzymes and DNA repair pathways (e.g., exonucleases and HDR, respectively). Another aspect warranting further investigation is the impact of higher order chromatin conformations on gene editing outcomes, both wanted and unwanted. This includes investigating (1) the proportion between accurate HDR and mutagenic NHEJ events at alternative chromatin states using different types of donor substrates and (2) the extent to which off-target RGN activities change locally and genome-wide due to specific epigenetic contexts, such as those underpinning chromosome domain architecture, cell-type specification, and organismal development. Besides improving the DNA, RNA, and protein reagents themselves, it will become increasingly important to develop efficient delivery methods for these tools as well. Indeed, reminiscent of "classical" gene therapies based on gene supplementation, tackling the "delivery issue" will be particularly crucial for translating genome-editing technologies into clinical practice. In this regard, non-viral and viral vector systems are

being co-opted for genome editing purposes (Chen and Gonçalves, 2016; Maggio and Gonçalves, 2015). Importantly, regardless of the delivery platform, target cells should be exposed to RGNs for the shortest time frame possible to minimize off-target activities. To this end, genome editing procedures might include the direct delivery of RNP complexes or the controlled activity of RGNs *in vivo* via devices responsive to exogenous stimuli (e.g., small-molecule drugs or light) (Richter et al., 2017). Finally, it is expected that in keeping with these developments, sensitive and unbiased genome-wide assays will become ever more important to thoroughly access the global mutation levels resulting from the application of continuously improved genome editing tools such as those reviewed herein and those yet to come.

AUTHOR CONTRIBUTIONS

X.C. and M.A.F.V.G. wrote the paper.

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