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Review article

Designed nucleases for targeted genome editing

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Summary

Targeted genome-editing technology using designed nucleases has been evolving rapidly, and its applications are widely expanding in research, medicine and biotechnology. Using this genome-modifying technology, researchers can precisely and efficiently insert, remove or change specific sequences in various cultured cells, micro-organisms, animals and plants. This genome editing is based on the generation of double-strand breaks (DSBs), repair of which modifies the genome through nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). In addition, designed nickase-induced generation of single-strand breaks can also lead to precise genome editing through HDR, albeit at relatively lower efficiencies than that induced by nucleases. Three kinds of designed nucleases have been used for targeted DSB formation: zinc-finger nucleases, transcription activator-like effector nucleases, and RNA-guided engineered nucleases derived from the bacterial clustered regularly interspaced short palindromic repeat (CRISPR)–Cas (CRISPR-associated) system. A growing number of researchers are using genome-editing technologies, which have become more accessible and affordable since the discovery and adaptation of CRISPR-Cas9. Here, the repair mechanism and outcomes of DSBs are reviewed and the three types of designed nucleases are discussed with the hope that such understanding will facilitate applications to genome editing.

Introduction

To understand how genotypes influence phenotypes, researchers have traditionally used targeted gene inactivation via homologous recombination (HR). However, this approach is time-consuming and challenging in plant cells mainly because the efficiency of such HR is extremely low (ranging from 1 in 10^4 to 10^5 of transformed cells) (Offringa *et al.*, 1990; Paszkowski *et al.*, 1988). Alternatively, targeted gene knockdown by RNA interference (RNAi) has become popular as a method for targeted inhibition of specific endogenous genes, because it is rapid, inexpensive and suited for high-throughput applications. However, knockdown of gene expression by RNAi is usually incomplete and only leads to temporary inhibition (Krueger *et al.*, 2007). Furthermore, RNAi-based knockdown is often complicated with unpredictable off-target effects (Jackson *et al.*, 2003).

A new genome-editing technology, based on designed nucleases that produce site-specific DNA double-strand breaks (DSBs), has emerged that enables precise and efficient targeted genetic modifications in various cells and organisms, including plants. In the absence of homologous templates, DSBs trigger error-prone nonhomologous end-joining (NHEJ), resulting in targeted mutagenesis (Bibikova *et al.*, 2002; Rouet *et al.*, 1994; Salomon and Puchta, 1998). In contrast, in the presence of an appropriate homologous template, DSBs can lead to precise homology-directed repair (HDR), which is at least two orders of magnitude more efficient than the conventional donor DNA-based gene inactivation method, which takes place in the absence of an appropriate DSB (Puchta *et al.*, 1993; Rouet *et al.*, 1994).

At the end of 2011, *Nature Methods* chose genome editing with designed nucleases, including zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), as the 'Method of the Year'. Soon after, in January 2013, several groups independently reported the use of a novel class of nucleases derived from the bacterial clustered regularly inter-spaced short palindromic repeat (CRISPR)-Cas9 system as a genome-editing tool (Cho *et al.*, 2013; Cong *et al.*, 2013; Hwang *et al.*, 2013; Jiang *et al.*, 2013; Jinek *et al.*, 2013; Mali *et al.*, 2013b). This latter class of nucleases, also referred to as RNA-guided engineered nucleases (RGENs), has been rapidly evolving since then.

Here, we review the three types of designed nucleases for inducing targeted DSBs. First, we will discuss the generation, repair and effects of DNA DSBs. Next, we will describe and compare the general features of ZFNs, TALENs and RGENs. Finally, we will speculate as to future directions and applications of designed nucleases for genome editing.

Double-strand breaks

The generation of DSBs is a key process in targeted genome editing. DSBs are a form of DNA damage that occurs when both DNA strands are cleaved. Genetically, DSBs result in discontinu-

ities of genetic information, leading to perturbation or inactivation of that information. Chemically, DSBs are discontinuities in the covalently linked carbon-phosphate backbones of both strands. Whereas some physiologic DSBs are generated in early-stage lymphocytes of the vertebrate immune system to produce antibody diversity, most DSBs are generated by pathologic causes that include ionizing radiation and oxidative free radicals (Lieber and Karanjawala, 2004; Lieber *et al.*, 2003). DSBs in mammalian cells can be repaired by homologous recombination (HR) and NHEJ. Traditionally, one of the most popular methods for gene modification was based on using HR, a technique that has been widely employed in mouse embryonic stem cells to generate germ-line knockout or knockin mice. However, the efficiency of HR events is extremely low (ranging from 1 in 10^6 to 1 in 10^7) in higher eukaryotes. In 1994, the discovery that the introduction of a DSB increases the frequency of HR by at least 2–3 orders of magnitude (Puchta *et al.*, 1993; Rouet *et al.*, 1994) led to efficient HR-based genome editing using programmable nucleases that generate DSBs at specific loci. Furthermore, in the absence of a homologous template, NHEJ repair of DSBs can lead to targeted gene disruption due to the error-prone nature of this process (Bibikova *et al.*, 2002; Rouet *et al.*, 1994). Such HR- or NHEJ-mediated repair of DSBs generated with programmable nucleases allows exquisitely precise genome modifications, such as gene disruptions (knockouts), insertions (knockins) and corrections (substitutions), as well as chromosomal rearrangements (Figure 1).

Repair of DNA double-strand breaks

Nonhomologous end-joining

Nonhomologous end-joining is a natural pathway for repairing DSBs through the ligation of two broken DNA ends. NHEJ often terminates the repair with errors and can lead to the introduction of small insertions and deletions (collectively called indels) at the site of the DSB (Figure 1a). Small indels often induce frameshifts, causing gene knockout by a combination of two mechanisms: premature truncation of the encoded protein and non-sense-mediated decay of the mRNA transcript (the latter is not always particularly efficient). NHEJ can occur during any phase of the cell cycle. In higher eukaryotes, NHEJ, rather than HDR, is the dominant DSB repair system (Lieber, 2010; Puchta, 2005).

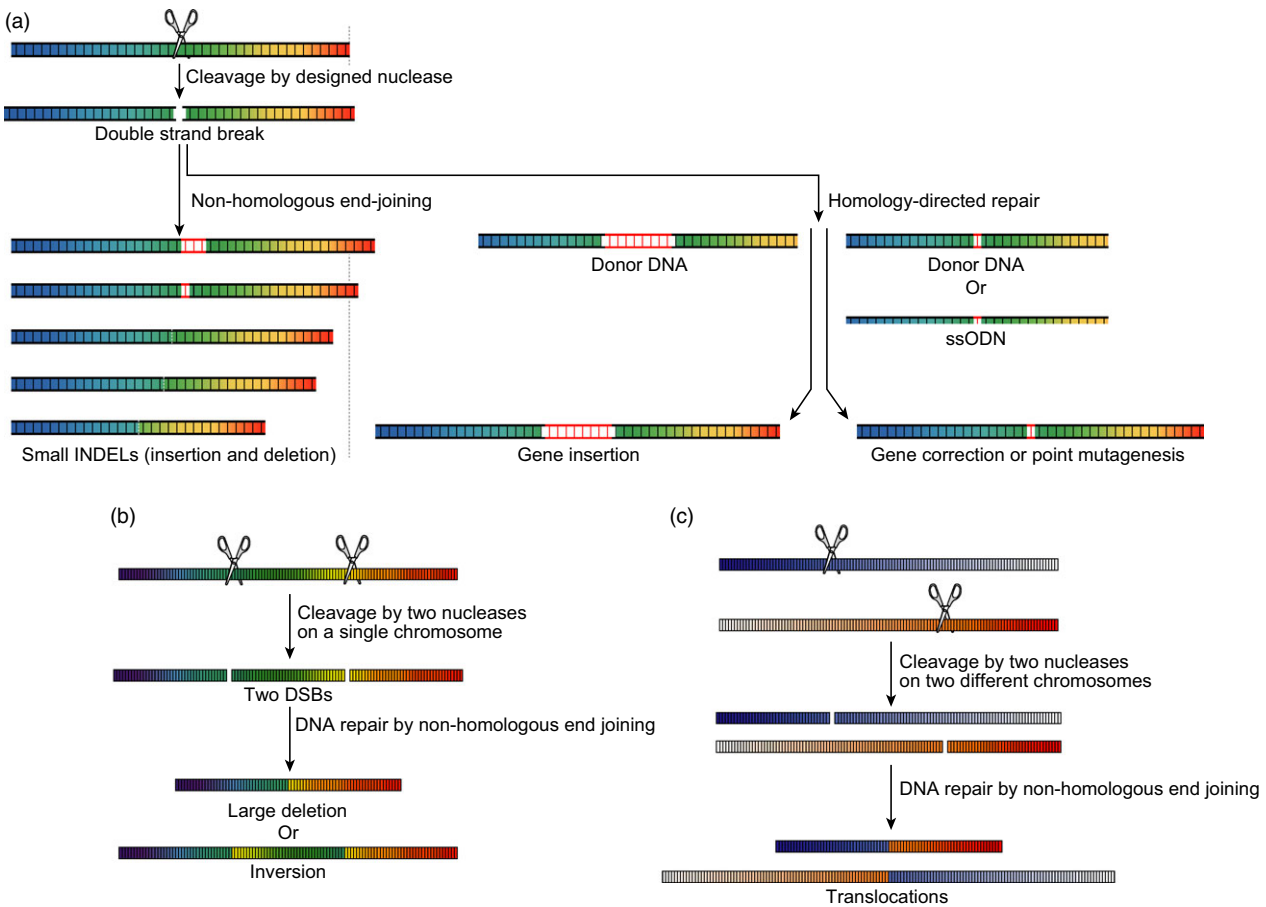


Figure 1 Outcome of genome editing through designed nuclease-based generation of double-strand breaks (DSBs). (a) In the absence of donor templates, nuclease-induced DSBs can be repaired by error-prone nonhomologous end-joining, which consequently often results in small insertions or deletions (indels). With appropriate donor DNA or single-strand oligodeoxynucleotide (ssODN), DSBs can be repaired by homology-directed repair, which can lead to sequence insertion and nucleotide substitution. (b) When designed nucleases generate two different DSBs on a single chromosome, the flanking region can be deleted or inverted. (c) When designed nucleases generate DSBs on two different chromosomes, interchromosomal translocations can be induced.

Ligation of two DNA ends through NHEJ requires various repair enzymes. Both Ku-dependent and Ku-independent NHEJ subpathways exist. In classical, Ku-dependent NHEJ, the DNA end protection factors (which form the Ku70/80 heterodimer) bind to the ends of the DNA strand at the break site and recruit the repair enzyme ligase IV and its cofactor. During NHEJ, annealing of exact complementary single-stranded ends can result in accurate repair. However, most breaks occurring in the cell do not have complementary ends, and NHEJ frequently proceeds through the annealing of short (1–4 bp) microhomologous sequences. Often, DNA end processing leads to the formation of small (1–4 bp) insertions and/or deletions (indels) at the DSB site (Lieber, 2010). The alternative end-joining (Ku-independent) pathway can repair DSBs without Ku-dependent pathway factors. Microhomology-mediated end-joining (MMEJ) is a major Ku-independent NHEJ pathway. MMEJ uses 5- to 25-bp microhomology sequences during the alignment of broken ends before joining. MMEJ proceeds by annealing the microhomology regions, removing overhanging nucleotides and filling in the missing base pairs. Thus, MMEJ frequently produces a longer deletion at the DSB site than does Ku-dependent NHEJ (McVey and Lee, 2008).

Homology-directed repair

Homology-directed repair is a template-dependent pathway for DSB repair (Figure 1a). In contrast to error-prone NHEJ pathways, HDR is precise. The defining step of HDR is the paring of a single-stranded DNA that is processed from a broken or damaged DNA site with its complement in a homologous region of undamaged double-stranded DNA (for example, the sister chromatid). This pairing is catalysed by the interaction of DNA strand exchange proteins such as RecA and Rad51 with a series of DNA substrates (Sarbajna and West, 2014). Unlike NHEJ, HDR is restricted to late S/G2 phases of the cell cycle.

Outcomes of DSB repair

Through the NHEJ mechanism

Small indels that are created at the target site through error-prone NHEJ can result in target gene knockout through the mechanisms discussed above. This process simply requires an appropriate designed nuclease; a homologous template is not needed. One of the standard methods for determining gene function is to observe the phenotype of knockout cells and organisms that lack functional copies of the gene of interest. NHEJ-mediated repair of DSBs that are generated by engineered nucleases has been widely used to produce various knockout cell and organism models (Kim and Kim, 2014; Segal and Meckler, 2013).

Two concurrent DSBs induced by two different designed nucleases, targeting regions far away from one another on a single chromosome, can give rise to chromosomal rearrangements or structural variations (Figure 1b,c). Deletions, inversions and translocations of large chromosomal segments (up to a few megabase pairs in length) have been achieved using three different types of designed nucleases (Carlson *et al.*, 2012; Cong *et al.*, 2013; Gupta *et al.*, 2013; Kim *et al.*, 2013a; Lee *et al.*, 2010, 2012; Petolino *et al.*, 2010; Qi *et al.*, 2013b; Xiao *et al.*, 2013). By inducing DSBs on two different chromosomes, inter-chromosomal translocations have also been made (Brunet *et al.*, 2009; Cho *et al.*, 2014) (Figure 1c). Recently, various cancer models containing chromosomal rearrangements have been generated using designed nucleases (Lagutina *et al.*, 2015; Maddalo *et al.*, 2014). This method has also been used to rescue a disease genotype caused by a chromosomal inversion (Lee *et al.*, 2012; Park *et al.*, 2014).

Through the HDR mechanism

In the vast majority of cases in plant and animal cells, transgene DNA integrates into nontargeted, random genomic locations. If the transgene integrates into undesired sites, it may inactivate essential genes or, in the case of mammalian cells, activate proto-oncogenes (Hacein-Bey-Abina *et al.*, 2003). Also, randomly integrated transgenes can be epigenetically silenced depending on the site of integration. In contrast, targeted gene knockin using designed nucleases has several advantages. Targeted DSB generation with programmable nucleases allows the insertion of desired genes into predetermined locations such as 'safe harbour' sites with enhanced efficiency (Doyon *et al.*, 2011; Hockemeyer *et al.*, 2011; Li *et al.*, 2011). 'Safe harbour' sites are locations in the genome where therapeutic transgenes can be integrated and expressed in a predictable manner without perturbing endogenous gene expression (Sadelain *et al.*, 2012). To insert genes of interest into specific loci including genomic safe harbours, the nuclease is delivered into cells together with a targeting vector (donor DNA) that comprises the transgene and flanking arms that are homologous to the sequences near the target region (Figure 1a).

Point mutations can be corrected or single-nucleotide variations can be introduced in the target site of the genome through codelivery of designed nucleases and targeting vectors (Bibikova *et al.*, 2001, 2003; Porteus and Baltimore, 2003) or single-stranded oligodeoxynucleotides (ssODNs) (Chen *et al.*, 2011) (Figure 1a). In the case of donor DNA, the preparation is often cumbersome and time-consuming. However, ssODNs can be easily designed and synthesized (Chen *et al.*, 2011). This ssODN-coupled point mutagenesis has been used in an easy, precise and efficient manner for the generation of disease models in animals (Cui *et al.*, 2011; Wang *et al.*, 2013; Wefers *et al.*, 2013) and human cells (Soldner *et al.*, 2011), for therapeutic purposes in an animal model of disease (Yin *et al.*, 2014) and for introducing point mutations in the plant genome (Shan *et al.*, 2013).

Three types of designed nucleases

Zinc-finger nucleases

Zinc-finger nucleases are composed of a zinc-finger protein (ZFP) domain, which is a designable, sequence-specific DNA-binding domain, and a nonspecific DNA cleavage domain derived from the type II restriction enzyme FokI (Kim *et al.*, 1996) (Figure 2). The FokI nuclease domain must be dimerized to cleave DNA (Bitinaite *et al.*, 1998); thus, two different ZFN monomers, each binding to a different strand, are required for an active nuclease. A ZFN is designed as a pair of monomers that recognizes two sequences, which flank the target site and are separated by a 5- to 7-bp spacer sequence (Figure 2a). One monomer binds to the forward strand and the other to the reverse strand.

The required dimerization of ZFN monomers expands the length of recognition sites, which substantially increases ZFN specificity. Each zinc-finger domain usually recognizes a 3-bp DNA sequence (Wolfe *et al.*, 2000), and several domains arrayed in tandem can bind to proportionately longer nucleotide sequences (3–6 zinc-finger domains are used to generate a single ZFN subunit that binds to DNA sequences of 9–18 bp) (Figure 2a). Importantly, the specificity of a zinc-finger DNA-

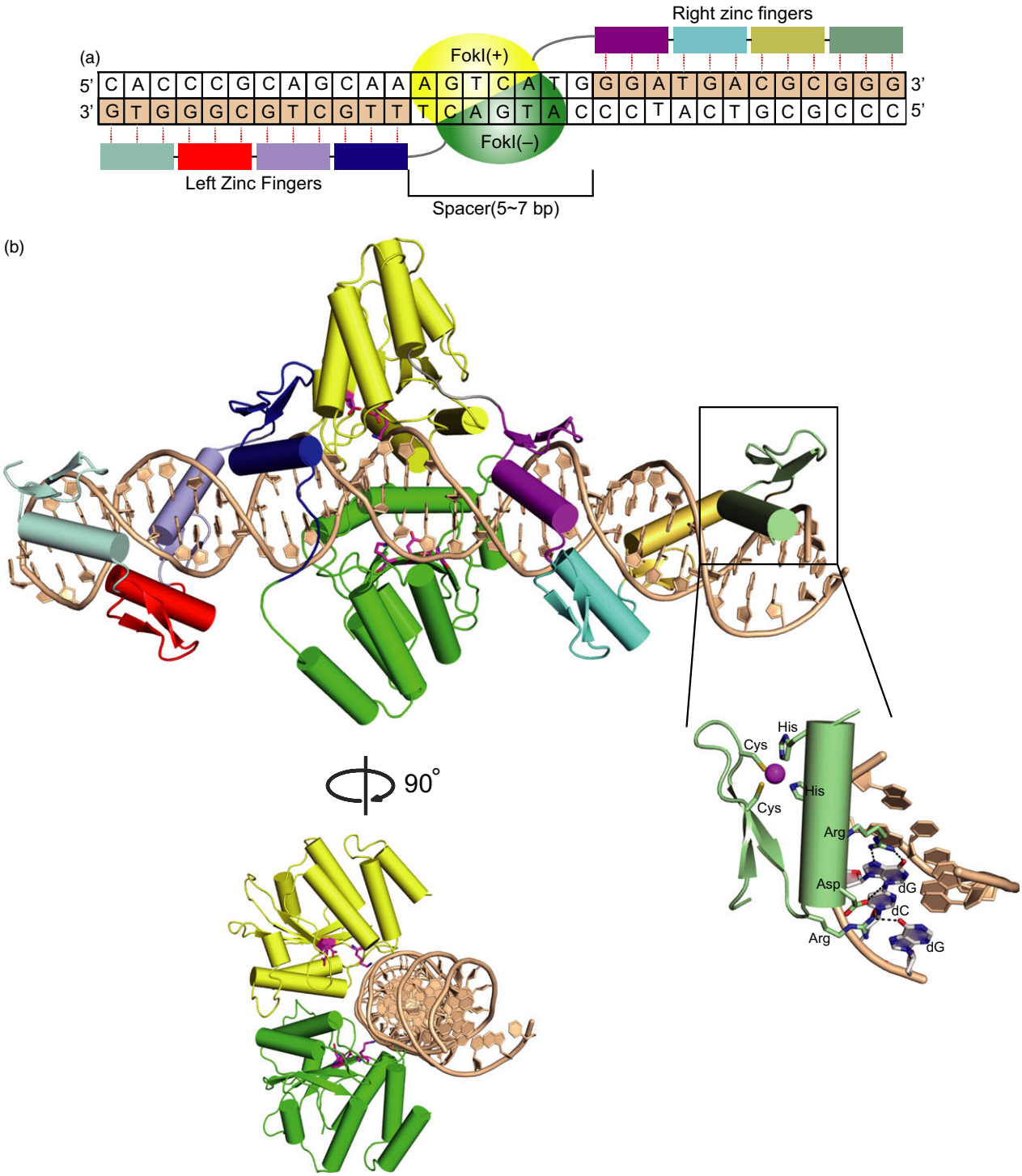


Figure 2 Structure of zinc-finger nucleases (ZFNs). Each ZFN domain is shown in the same colour in both (a) and (b). (a) A schematic representation of a ZFN dimer bound to DNA. Each ZFN is composed of a zinc-finger protein at the amino terminus and the FokI nuclease domain at the carboxyl terminus. The target sequence of a ZFN pair is typically 18–36 bp in length, excluding a 5- to 7-bp spacer. (b) A computer-generated model structure of a ZFN pair bound to DNA. Each zinc finger consists of approximately 30 amino acids in a ββα arrangement. The catalytic residues for FokI nuclease activity are presented as purple sticks. Residues in α-helix of zinc finger that contact 3 bp in the major groove of target DNA are shown as sticks (close-up view of inset). The side chains of the conserved two Cys and two His residues coordinating a Zn^{2+} ion (depicted as a purple ball) are shown from a different direction (close-up view of inset). This model was compiled from crystal structures of zinc fingers bound to DNA (Protein Database 1AAY) (Elrod Erickson *et al.*, 1996) and the FokI restriction endonuclease in the absence of DNA (2FOK) (Wah *et al.*, 1998).

binding domain can be altered by mutagenesis (Desjarlais and Berg, 1992; Rebar and Pabo, 1994). Such manipulation of ZFPs to alter their binding specificity is a key feature of constructing a designed nuclease. New ZFPs with desired specificities can be constructed by modularly assembling precharacterized zinc fingers (Bae *et al.*, 2003; Bibikova *et al.*, 2002, 2003; Kim *et al.*, 2010;

Segal *et al.*, 2003). Cell-based selection methods and modular assembly methods that consider context dependence between neighbouring zinc fingers have been developed to yield functional ZFNs (Bhakta *et al.*, 2013; Gupta *et al.*, 2012; Maeder *et al.*, 2008; Sander *et al.*, 2011). The use of obligatory heterodimeric Fok1 domain developed by modification of wild-type Fok1 domain significantly enhances specificity and reduces off-target effects (Miller *et al.*, 2007; Szczepek *et al.*, 2007). Nonetheless, it remains challenging to make efficient, specific ZFNs.

Zinc-finger nucleases have some disadvantages compared with newly developed programmable nucleases. First, compared with TALENs or RGENs, ZFNs have limited target availability. So far, there is no open-source collection of 64 (4 × 4 × 4) zinc fingers that covers all possible combinations of 3-bp subsites (Bae *et al.*, 2003; Segal *et al.*, 1999). Furthermore, not all engineered ZFNs create DSBs efficiently. Successful target sites are often in guanine-rich regions, consisting of 5′–GNN-3′ (where N represents any nucleotide) repeat sequences. Thus, a single functional ZFN pair can be obtained per ~100-bp DNA sequence on average (Kim *et al.*, 2009). This limitation is not too important for those intending to knock out a gene, because a frameshift introduced anywhere in the early coding sequence of the gene would suffice. However, generating a functional ZFN may be challenging if one particular target site is required, such as for creating a deletion, insertion or substitution at a particular site. Second, ZFNs often show low DNA-targeting activity (Ramirez *et al.*, 2008) or are cytotoxic owing to off-target effects (Cornu *et al.*, 2008). Third, it is difficult for nonspecialists to make ZFNs that target specific sites routinely. Although an academic consortium developed an open-source library of zinc-finger components and a screening protocol to identify ZFNs with high affinity and efficiency (Maeder *et al.*, 2008, 2009), the library has not yet been widely accepted among researchers. However, ZFNs also have advantages compared with TALENs and RGENs. ZFN-encoding sequences (~1 kb × 2) are smaller than TALEN- (~3 kb × 2) and RGEN-encoding sequences (~4.2 kb for the protein + 0.1 kb RNA), facilitating delivery with viral vectors that have limited cargo size, such as the adeno-associated viral (AAV) vector. In addition, ZFPs are derived from mammalian proteins, whereas TALENs and RGENs have a bacterial origin. Thus, we speculate that ZFN immunogenicity is lower than that of TALENs or RGENs, although a careful comparison awaits further investigation.

Transcription activator-like effector nucleases

Like ZFNs, a TALEN consists of a designable, sequence-specific DNA-binding domain and a nonspecific DNA cleavage domain derived from FokI (Miller *et al.*, 2011) (Figure 3). However, TALENs use a different type of DNA-binding domain known as transcription activator-like effectors (TALEs), which are derived from a species of plant pathogenic bacteria. Whereas each zinc-finger domain recognizes a 3-bp DNA sequence, there is a one-to-one correspondence between TALE domains and base pairs. TALEs are composed of tandem arrays of 33–35 amino acid repeats, each of which recognizes a single base pair in the major groove of DNA (Deng *et al.*, 2012; Mak *et al.*, 2012) (Figure 3b). The specificity of each repeat is conferred by the two amino acids at positions 12 and 13, known as repeat variable diresidues (RVDs) (Figure 3c). To recognize guanine, adenine, cytosine and thymine, RVD modules of Asn-Asn, Asn-Ile, His-Asp and Asn-Gly, respectively, are widely used. TALENs can be designed to target almost any given DNA sequence, which is a critical advantage over other types of nucleases.

Compared with ZFNs, TALENs are much easier to design and construct. TALENs are often built to bind 18- to 20-bp sequences. In fact, larger TALENs may result in lower specificity (Guilinger *et al.*, 2014a). It is also tricky to construct longer TALE arrays because of the recombination that can occur due to the highly homologous TALE sequences (Holkers *et al.*, 2013). Several methods have been developed for the assembly of custom-designed TALE arrays (Briggs *et al.*, 2012; Cermak *et al.*, 2011; Reyon *et al.*, 2012; Schmid-Burgk *et al.*, 2013). The target site binding affinity of an engineered TALE repeat array has been reported to be as high as 96% (Hockemeyer *et al.*, 2011; Miller *et al.*, 2011; Reyon *et al.*, 2012). Additionally, genomewide libraries of TALENs that target protein-coding genes (Kim *et al.*, 2013a) and microRNA-coding sequences (Kim *et al.*, 2013b) have been constructed.

As mentioned above, the relatively large size of TALEN-encoding sequences can limit TALEN delivery and expression. This limitation is especially restrictive in mammalian cells, where viral vectors such as AAV are often used. Because of their low immunogenic potential and the low oncogenic risk from host-genome integration, AAV vectors are attractive as delivery vehicles for programmable nucleases. However, the cargo size of AAV is ~4.5 kb excluding the inverted terminal repeats, preventing delivery of a TALEN pair using this method. Furthermore, the highly repetitive nature of TALEN sequences may hinder their ability to be packaged and delivered by some viral vectors (Holkers *et al.*, 2013). Although it is not a major issue for plant transformation, which is mostly performed using Agrobacterium T-DNA or plasmid DNA, the size of TALEN sequences would pose challenges for DNA assembly in multiplexing or multilocus targeting. The strategy of diversifying TALEN repeat coding sequences may be helpful for overcoming this problem (Yang *et al.*, 2013).

CRISPR-Cas9 (RNA-guided engineered nucleases)

Zinc-finger nucleases and TALENs are relatively expensive due to the difficulty of synthesis. Genome editing became more accessible with the discovery and adaptation of the CRISPR-Cas9 system. This system, owing to its efficiency and ease of use, has now become the most popular genome-editing tool.

CRISPR-Cas9 as an adaptive immune system in bacteria and archaea. The RNA-guided DNA cleavage system naturally exists as an adaptive form of immunity against invading phages or plasmids in bacteria and archaea (Barrangou *et al.*, 2007; Makarova *et al.*, 2006). These organisms 'remember' the sequences of previously invading viral genomes and protect themselves by recognizing and cutting those sequences when they are encountered again. This type of acquired immunity proceeds via the capture of foreign DNA fragments (~20 bp) from invading phages or plasmids and the incorporation of these sequences (termed protospacers) into the bacterial or archaeal genome to form CRISPR. In type II CRISPR systems, these CRISPR regions (memory elements) are transcribed as pre-CRISPR RNA (pre-crRNA) and processed to form the target-specific CRISPR RNA (crRNA). Trans-activating crRNA (tracrRNA), a target-independent component, is also transcribed from the CRISPR region and is involved in the processing of pre-crRNA (Deltcheva *et al.*, 2011). Both RNAs complexed with CRISPR-associated protein 9 (Cas9) form an active DNA endonuclease system, and destroy any DNA sequences that match the protospacer. In the case of the system from *Streptococcus pyogenes*, which is the

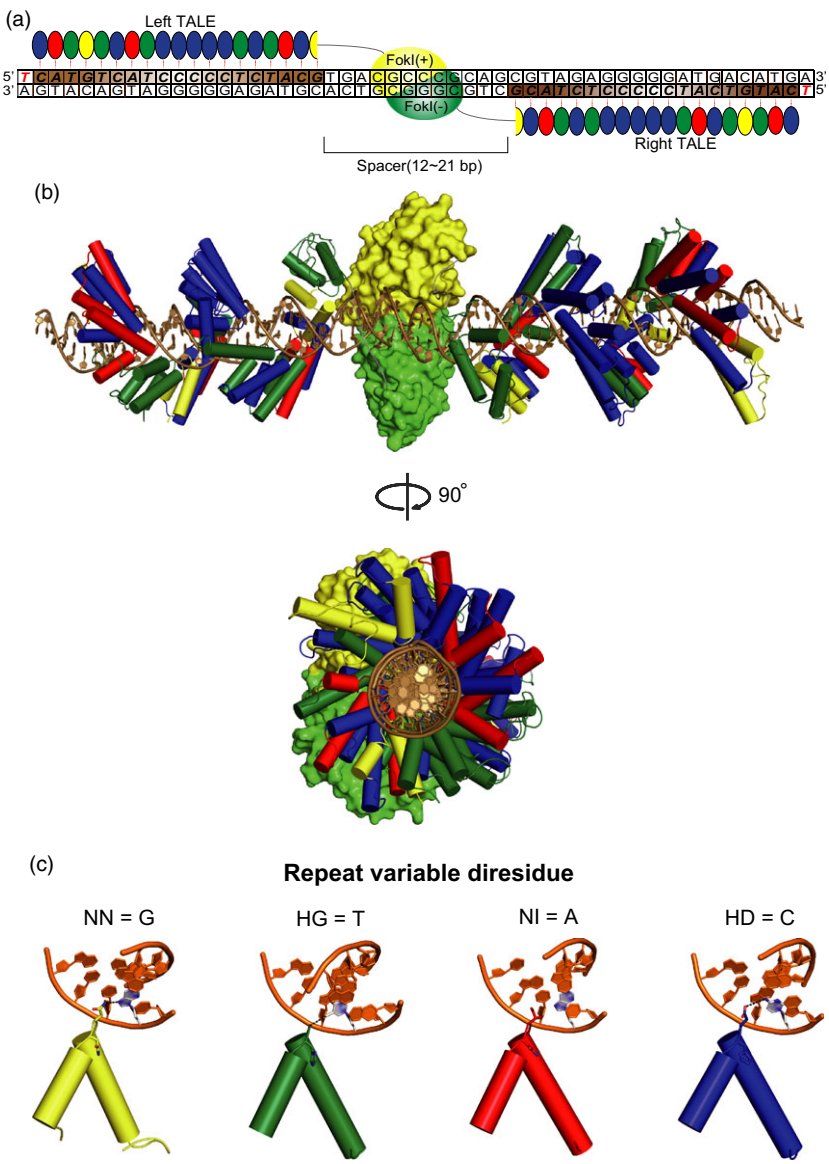


Figure 3 Structure of transcription activator-like effector nucleases (TALENs). Each TALEN domain and module is shown in the same colour in both (a), (b) and (c). (a) A schematic representation of a TALEN pair is shown. Each TALEN is composed of transcription activator-like effectors (TALEs) at the amino terminus and the FokI nuclease domain at the carboxyl terminus. Target sequences of TALEN pairs are typically 30–40 bp in length, excluding a 12- to 21-bp spacer. (b) A TALE protein in complex with target DNA. Each TALE repeat comprises 33–35 amino acids and recognizes a single base pair at the major groove through the hypervariable residues at positions 12 and 13, which are called a repeat variable diresidue. This model was prepared from crystal structures of TALE bound to DNA (Protein Database 3UGM) (Mak *et al.*, 2012) and the FokI restriction endonuclease in the absence of DNA (Protein Database 2FOK) (Wah *et al.*, 1998) based on a previous analysis. (c) Recognition of bases by corresponding repeat variable diresidues.

origin of the first engineered CRISPR-Cas9 system, the endonuclease can cleave a 23-bp target DNA sequence that is composed of a 20-bp guide sequence identical to the crRNA (protospacer) and a 5′-NGG-3′ (or, to a lesser extent, 5′-NAG-3′ (Hsu *et al.*, 2013; Jiang *et al.*, 2013; Mali *et al.*, 2013a) sequence known as protospacer adjacent motif (PAM), which is recognized by Cas9 itself (Mojica *et al.*, 2009). This PAM sequence can distinguish between 'self' (protospacers) and 'nonself' (invader) DNA sequences, priming the nonself sequences for a DSB at a site 3 bases before the PAM. Cas9 proteins derived from species other than *S. pyogenes* recognize different PAM sequences (Cong *et al.*, 2013; Fonfara *et al.*, 2014; Hou *et al.*, 2013; Mojica *et al.*, 2009; Shah *et al.*, 2013).

CRISPR-Cas9 for genome engineering. Here, we use the term 'RNA-guided engineered nuclease (RGEN)' to represent a new type of genome-editing nuclease to avoid confusion with the natural type II CRISPR-associated adaptive immune system in bacteria.

In 2012, it was reported that guide RNA and purified Cas9 protein can cleave target DNA *in vitro* (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012). In January 2013, several groups independently reported a new class of genome-editing nucleases (Cho *et al.*, 2013; Cong *et al.*, 2013; Hwang *et al.*, 2013; Jiang *et al.*, 2013; Jinek *et al.*, 2013; Mali *et al.*, 2013b), soon followed by their application in plants (Li *et al.*, 2013a; Shan *et al.*, 2013). The specificity of this system is determined by small guide RNAs rather

Figure 4 Structure of RNA-guided engineered nucleases (RGENs). Each domain of Cas9 is shown using the same colours in (a) to (h). (a) Domain organization of *S. pyogenes* Cas9. HNH and RuvC domains are nuclease domains of Cas9. Topo and CTD are protospacer adjacent motif (PAM)-interacting domains. Arg, Arg-rich bridge helix; REC, recognition lobe; Topo, topoisomerase-homology domain; CTD, C-terminal domain. (b, c) Schematic representations (b) and three-dimensional models (c) of Cas9, target DNA and single guide RNA. Guide RNA loading induces conformational rearrangements in Cas9, leading to the formation of a central channel that may accommodate target DNA. Regions marked with orange, blue and pink boxes represent the HNH catalytic site, the RuvC catalytic site and the PAM recognition site, respectively, and are depicted in greater detail in (d), (e) and (f), respectively. This model was prepared from Protein Database 4UN3, 4CMP based on previous analyses (Anders *et al.*, 2014; Jinek *et al.*, 2014). (d) A three-dimensional model of the HNH domain, which cleaves the complementary DNA strand. Here, the nuclease activity of the HNH domain is inactivated by the introduction of a H840A mutation, leading to preservation of the target strand. (e) A three-dimensional model of the RuvC domain, which cleaves the noncomplementary DNA strand. Here, the target DNA strand is cleaved. (f) A three-dimensional model and schematic representation of PAM recognition by Cas9. Cas9–RNA recognizes the PAM GG dinucleotide using Arg 1333 and Arg 1335, and positions the target DNA duplex such that the +1 phosphate (orange circle) interacts with the topo-homology domain. This interaction leads to local strand separation immediately upstream of the PAM, which promotes heteroduplex formation between the guide RNA and the complementary target DNA strand. This model was prepared from Protein Database 4UN3, 4CMP based on previous analyses (Anders *et al.*, 2014; Jinek *et al.*, 2014).

than by DNA-binding proteins such as ZFP or the TALE (Figure 4). A single chimeric guide RNA (sgRNA), which is a fused form of crRNA and tracrRNA (Jinek *et al.*, 2012), simplifies RGEN components further. Using the CRISPR-Cas system derived from *S. pyogenes* [comprising the Cas9 protein along with guide RNA (s)], in mammalian cells results in DSBs at target sites with a 20-bp sequence matching the protospacer of the guide RNA and an adjacent downstream NGG nucleotide sequence (PAM) (Figure 4f). Site selection for RGENs is limited by the requirement for the PAM sequence, which is recognized by Cas9. Thus, the targetable sequences are 5′-X20NGG-3′ (or 5′-X_{20}NAG-3′ to a lesser extent), with X_{20} corresponding to the 20-bp crRNA sequence and NGG or NAG corresponding to the PAM sequence, which theoretically occurs on average once every 8 bp (including NAG, once every 4 bp). When guide RNAs are transcribed by RNA polymerase III under the control of the U6 promoter in cells, a guanine at the 5′ end is required (Cho *et al.*, 2013, 2014). If the first nucleotide in a guide RNA is not guanine, the addition of at least one additional guanine base at the guide RNA 5′ end is required. Finally, unlike ZFNs and TALENs, RGENs can cleave methylated DNA (Hsu *et al.*, 2013).

Advantages of RGENs. A crucial advantage of RGENs over ZFNs and TALENs is their simple and feasible preparation. The complicated protein engineering required for constructing ZFN- and TALEN-encoding sequences is not necessary for preparing new RGENs. Because the Cas9 protein component remains unchanged, new RGEN plasmids can be easily prepared by cloning short guide DNA sequences into the guide RNA plasmid backbone (Cong *et al.*, 2013; Ding *et al.*, 2013). This feasibility facilitates the generation of large sets of vectors targeting various genes, including genomewide libraries (Findlay *et al.*, 2014; Gilbert *et al.*, 2014; Koike-Yusa *et al.*, 2014; Konermann *et al.*, 2015; Shalem *et al.*, 2014).

Multiplex genome editing is relatively easy using Cas9 nucleases. In the case of ZFNs and TALENs, multiple pairs of nucleases are needed for this process (Sollu *et al.*, 2010). In contrast, because Cas9 remains unchanged in all RGENs, one Cas9 and multiple guide RNAs can disrupt multiple genes simultaneously. This approach has been used for making multigene knockout animals (Li *et al.*, 2013a,b; Niu *et al.*, 2014; Wang *et al.*, 2013) and plants (Xie *et al.*, 2015).

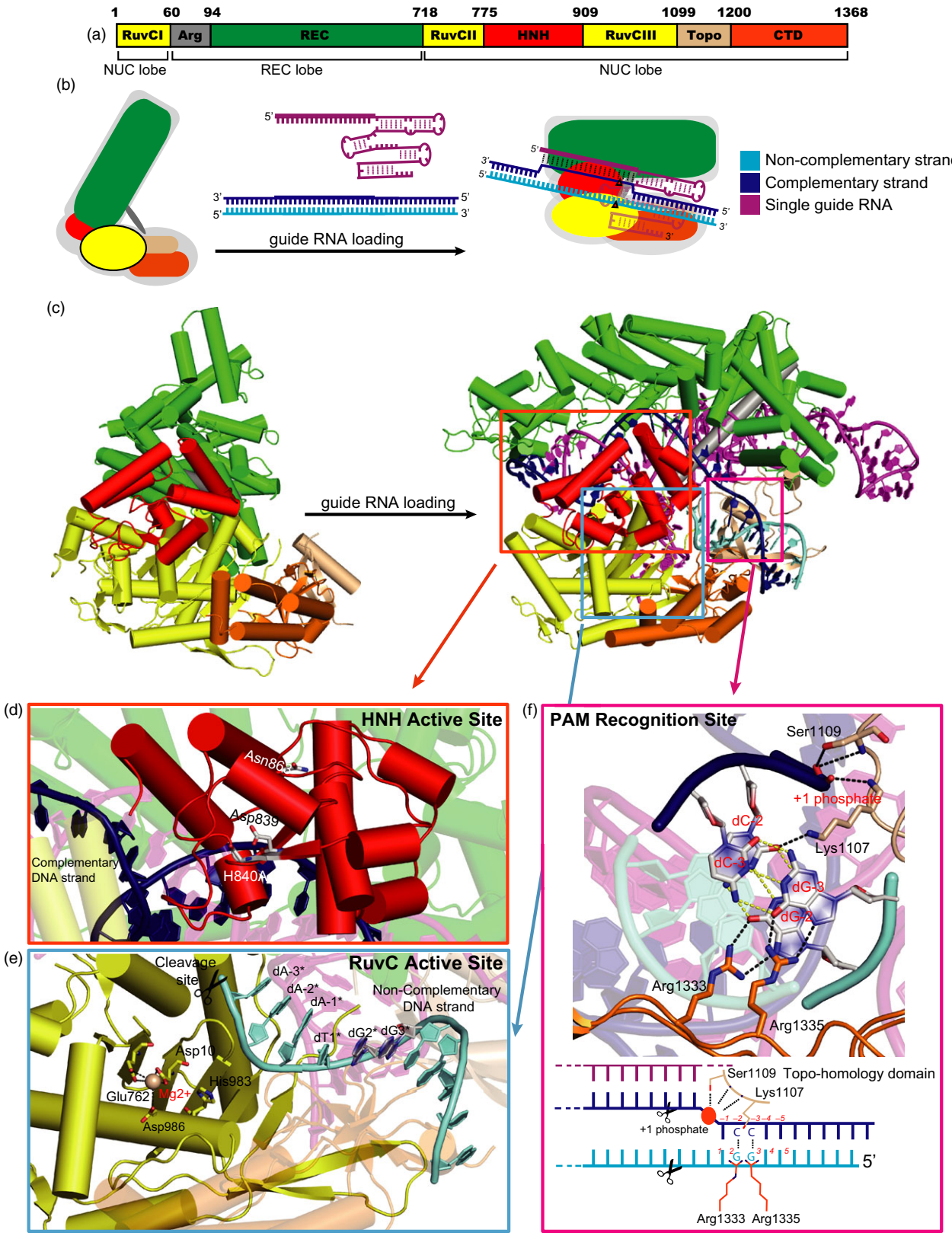
Furthermore, new RGENs can be made without plasmids. The Cas9 protein and *in vitro* transcribed guide RNA can be easily prepared. The plasmid-free approach also has the advantage of being safer for therapeutic applications (Kim *et al.*, 2014; Ramakrishna *et al.*, 2014; Zuris *et al.*, 2015).

Disadvantages of RGENs. The coding sequence of *S. pyogenes* Cas9 is ~4.2 kb. Even though the other designed nucleases act as dimers, this Cas9 sequence is longer than that encoding a TALEN monomer (~3 kb) or a ZFN monomer (~1 kb). Therefore, delivery of RGENs via viral systems is somewhat challenging. For proper transcription, a promoter and a polyadenylation sequence are required in addition to the Cas9 sequence. The sgRNA is approximately 100 bp, which must be delivered in parallel with the Cas9 sequence to produce an active RGEN. Inclusion of an RNA III polymerase promoter such as the U6 promoter for sgRNA transcription means that ~500 bp is needed for sgRNA. RGENs have been delivered to various types of plants using polyethylene glycol (PEG)–protoplast transfection, Agrobacterium-mediated transformation and microparticle bombardment, all of which have relatively large cargo capacities that are sufficient for RGEN accommodation (Bortesi and Fischer, 2015). The efficiency of RGEN-mediated genome editing clearly is affected by the delivery method in plant as well as animal cells. For example, PEG-mediated protoplast transfection resulted in a 10-fold higher mutation rate than that by Agrobacterium-mediated transformation in *N. benthamiana* (Li *et al.*, 2013a,b). Although it is less of an issue for plant biotechnology, efficient application of RGENs in mammalian cells sometimes requires the use of a specific DNA delivery system such as lentivirus that can accommodate the *S. pyogenes* RGEN system; AAV, with its cargo size limited to less than 4.8 kb, cannot easily do so. Efforts have been made to reduce the size of RGEN-encoding sequences (using a short promoter and polyadenylation sequence) for use in AAV (Swiech *et al.*, 2015). In addition, CRISPR-Cas systems from other species, some of which involve smaller Cas9 proteins, should be helpful in this regard. Recently, RGEN AAV, which contains the sequence encoding the smaller *Staphylococcus aureus* Cas9 (more than 1 kb shorter than that encoding *S. pyogenes* Cas9) and guide RNA in one shuttle vector, has been reported (Ran *et al.*, 2015). These developments allow for the delivery of RGENs via AAV, which may be important for therapeutic applications that often require high delivery efficiency.

Modification of designed nucleases

Designed nickases

Nonhomologous end-joining-mediated repair of DSBs induced by designed nucleases inevitably causes the formation of uncontrolled and undesirable indels at the target site and, potentially, at off-target sites, even in the presence of a homologous donor template for HDR. In higher vertebrates and plants, DSBs are



primarily repaired by NHEJ. Altered (or 'resistant') sequences with indels at the cleavage site cannot be retargeted with the original designed nuclease. To correct these resistant sequences, they must first be identified; then, programmable nucleases that target each resistant sequence must be newly designed. Given that NHEJ-mediated indel formation is hard to predict, a variety of resistant sequences can be generated, making it difficult to design nucleases that target all of the resistant sequences. DSBs generated in the resistant sequences can then lead to the creation of a second set of resistant sequences, again through NHEJ-

mediated repair of the DSBs, preventing precise genome editing. To avoid the generation of unwanted mutations by designed nucleases, nickases that produce single-strand breaks (SSBs) rather than DSBs have been proposed as an alternative. Chemically, SSBs are discontinuities in the covalently linked carbon-phosphate backbone of one strand in the DNA double helix. A SSB can result in a discontinuity of the genetic information in the affected strand. However, SSBs perturb genetic information much less frequently than do DSBs because the other, intact strand can be used as a template to guide the correction of the damaged strand. Naturally, one of the most common sources of SSBs is oxidative attack by endogenous reactive oxygen species. A SSB can enhance HDR, although the efficiency is lower than that of the nucleases (Davis and Maizels, 2011; McConnell Smith *et al.*, 2009). SSBs are repaired via the high-fidelity base excision repair (BER) pathway (Dianov and Hübscher, 2013) and do not activate the NHEJ pathway, preventing generation of unwanted indels. Thus, nickases can lead to precise genome editing.

The first designed nickases were modified ZFNs that consisted of one intact and one mutant FokI subunit with a mutation at the active catalytic site (Kim *et al.*, 2012; Ramirez *et al.*, 2012; Wang *et al.*, 2012). ZF nickase heterodimers generate a SSB at the target site and do not cause undesirable DSBs at either the target site or off-target sites (Kim *et al.*, 2012; Wang *et al.*, 2012). ZF nickases induced precise genome editing via HDR with a lower efficiency than the corresponding ZF nucleases (Kim *et al.*, 2012; Ramirez *et al.*, 2012; Wang *et al.*, 2012). Other designed nickases derived from TALENs have also been reported and tested *in vitro* (Gabsalilow *et al.*, 2013).

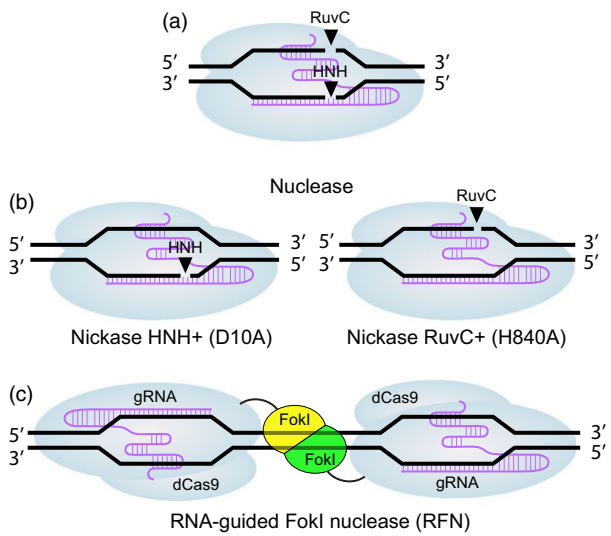


Figure 5 Modifications of Cas9 as a genome engineering platform. (a) Cas9 nuclease. The Cas9 nuclease cleaves both strands of DNA via its RuvC and HNH nuclease domains, each of which makes a nick in a DNA strand, leading to the generation of blunt-end DSBs. (b) Cas9 nickases. Either catalytic domain can be inactivated to generate nickase mutants that each make single-strand DNA breaks. (c) RNA-guided FokI nuclease. Here, both of the Cas9 catalytic domains are inactivated to generate dead Cas9 (dCas9). Two FokI–dCas9 fusion proteins are recruited to adjacent target sites by two different guide RNAs to facilitate FokI dimerization, leading to a double-strand DNA cleavage between the two target sites by the activated FokI dimer.

Cas9 has two active catalytic domains, RuvC and HNH, which each cleave one strand and together generate a blunt-ended DSB (Figures 4d,e and 5a). Two types of Cas9 nickases have been constructed via the introduction of point mutations in RuvC (D10A) and HNH (H840A) (Figure 5b); both nickases have been shown to form SSBs (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012; Sapranauskas *et al.*, 2011). RNA-guided engineered nickases (RGENickases) that contain the *S. pyogenes* Cas9 HNH+/RuvC- nickase mutant (D10A), which has better efficiency than the HNH-/RuvC+(H840A) mutant, lead to high-fidelity HDR with negligible NHEJ-driven mutations (Cong *et al.*, 2013; Fauser *et al.*, 2014; Mali *et al.*, 2013b).

To improve DSB specificity, paired nickases, like dimeric ZFNs and TALENs, can be used to increase the number of bases that are recognized. Because individual nicks in the genome are repaired with high fidelity through the BER pathway, off-target SSBs would be precisely repaired. Furthermore, because the probability that two nickases would make off-target SSBs that are close to each other in the genome is extremely low, the off-target mutation rate would be dramatically reduced. Paired nickases designed to make two SSBs, one on each of the two DNA strands, collectively generate a composite DSB, which will lead to indel formation through NHEJ. Properly spaced 'paired nickases' showed efficiency comparable to that of the corresponding nuclease with up to 500-fold reduced off-target activity in human and mouse cells (Cho *et al.*, 2014; Kim *et al.*, 2012; Mali *et al.*, 2013a; Ran *et al.*, 2013a). Comparable with the results from experiments using animal cells, a recent study on Arabidopsis found that the on-target mutagenic rate of paired nickases was the same as that of the Cas9 nuclease (Schiml *et al.*, 2014).

RNA-guided FokI nucleases

An RNA-guided FokI nuclease, analogous to dimeric ZFNs or TALENs, is a fusion of a dimerization-dependent FokI nuclease domain as the cleavage domain and a catalytically inactive Cas9 (termed dead Cas9; dCas9) as the DNA-binding domain (Guilinger *et al.*, 2014b; Tsai *et al.*, 2014) (Figure 5c). As with paired nickases, highly specific gene targeting is feasible using RNA-guided FokI nucleases (RFNs) because of the increased number of bases that are recognized at a given site.

Transcriptional regulation using dead Cas9

Cas9 coupled with guide RNA has two key properties. One is the ability to bind to DNA at a targeted site, and the other is its catalytic function. A catalytically inactivated Cas9 (dCas9) has been repurposed to allow controlled transcriptional regulation of genes. Whereas transcriptional regulation using dCas9 has only transient effects, similar to RNAi, active Cas9 elicits permanent changes in the genome.

Although RNAi is a popular tool for knockdown of target gene expression, RNAi-based experiments are often complicated by inefficiency or unpredictable off-target effects. Transcriptional regulation using dCas9 represents a good alternative, which can elicit both up- and down-regulation of the expression of multiple genes simultaneously (Bikard *et al.*, 2013; Gilbert *et al.*, 2013; Qi *et al.*, 2013a,b). Binding of a dCas9–guide RNA ribonuclear protein complex to an appropriate DNA element can repress transcription by blocking transcriptional elongation, RNA polymerase binding or transcription factor binding (Qi *et al.*, 2013a, b). CRISPR-based interference using dCas9–guide RNA complexes themselves is less efficient in eukaryotes compared to prokaryotes

(Gilbert *et al.*, 2013). The fusion of a transcriptional activator or repressor such as VP64 or KRAB, respectively, leads to more efficient transcriptional activation or suppression in human cells (Gilbert *et al.*, 2013; Konermann *et al.*, 2013). The efficiency of transcriptional regulation by dCas9 has also been improved by modifying the structure of the guide RNA to recruit additional activators or cofactors (Konermann *et al.*, 2015; Zalatan *et al.*, 2015).

Comparison of the three types of engineered nucleases

Efficacy

Not all newly designed nucleases are functional and equally efficient (Table 1), and it is difficult to predict the efficiency of newly designed nucleases. ZFNs usually exhibit relatively low efficiency for generating DSBs in cultured cells or organisms compared to TALENs or RGENs. Gene knockout efficiency with functional TALENs is difficult to predict and has been reported to range from 1% to ~60% in mammalian cells (Kim *et al.*, 2013a; Reyon *et al.*, 2012). RGENs also have shown a wide range of genome-editing activities (2.3–79%) in cultured cells (Cho *et al.*, 2013; Cong *et al.*, 2013; Ding *et al.*, 2013; Jinek *et al.*, 2013; Mali *et al.*, 2013b). Both the target cell type and the delivery method seem to significantly affect the activity of all three classes of nucleases. Recently, a program was designed to enable approximate prediction of the activity of designed guide RNAs based on high-throughput efficiency data from 1841 guide RNAs (Doench *et al.*, 2014). Although the standard, most accurate method for determining the activity of individual guide RNAs is cell-based analysis, this *in silico* analysis can be useful for selecting several highly active guide RNA candidates that could then be subjected to actual evaluation in cells, which requires much more labour, time and cost than *in silico* analysis. This *in silico* program-assisted approach can be useful for obtaining highly active guide RNAs for gene knockout, for which a large number of guide RNA can be designed. However, the users of this program should be aware of the possibility that sgRNA activity predicted by this program can be different from the actual sgRNA activity measured by cell-based analysis. Similar programs for plant genome editing are also available (Xie *et al.*, 2014).

Table 1 Comparison of three classes of designed nucleases

	ZFN	TALEN	RGEN (CRISPR/Cas9)
Recognition site	18–36 bp per ZFN pair	30–40 bp per TALEN pair	22 bp (20-bp guide sequence + 2-bp protospacer adjacent motif (PAM) from *Streptococcus pyogenes*)
Restriction in target site	G-rich	Start with T	End with an NGG (NAG: lower activity) sequence
Success rate	Low	High	High
Off-target effects	High	Low	Variable
Cytotoxicity	Variable to high	Low	Low
Size	~1 kb × 2	~3 kb × 2	4.2 kb (Cas9 from *Streptococcus pyogenes*) + 0.1 kb (sgRNA)
Ease of engineering	Difficult	Moderate	Easy
Ease of multiplexing	Low	Low	High

ZFN, zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; RGEN, RNA-guided engineered nuclease; CRISPR, clustered regularly interspaced short palindromic repeat; Cas9, CRISPR-associated protein 9; sg RNA, single-chain guide RNA.

Safety

The specificity of ZFNs and TALENs can be determined by the number of zinc fingers and TALE modules the nucleases contain. More modules are generally thought to signify a higher specificity. However, too many modules can elevate the possibility of partial binding at many unwanted sites. Theoretically, nucleases should recognize DNA sequences of at least 16 bp to eliminate potential off-target effects in the human genome because the complexity of 16-bp sequences ($4^{16} = 4.3 \times 10^9$) is greater than the size of the human haploid genome (3.2×10^9). However, modifying crop plant genomes that are larger than the human genome may require a longer target site sequence to minimize off-target effects. In reality, however, all three nucleases with target site sizes greater than 16 bp have shown some off-target effects (Fu *et al.*, 2013; Gabriel *et al.*, 2011; Hsu *et al.*, 2013; Mussolino *et al.*, 2011; Pattanayak *et al.*, 2011). In the case of ZFNs, too many off-target cleavages are thought to cause cytotoxicity (Cornu *et al.*, 2008).

Compared to dimeric ZFNs and TALENs, RGENs theoretically have lower specificity because of functioning as monomers. Several studies have examined off-target effects of RGENs (Cho *et al.*, 2014; Cradick *et al.*, 2013; Fu *et al.*, 2013; Hsu *et al.*, 2013; Mali *et al.*, 2013a; Pattanayak *et al.*, 2013). One study showed that RGENs can induce off-target mutations at sites that differ by up to five nucleotides from on-target sites, which implies that thousands of potential off-target cleavages can occur in the human genome for every RGEN (Fu *et al.*, 2013), whereas some studies reported that off-target mutations were below the detection range when analysed by unbiased whole-genome (Veres *et al.*, 2014) or exome sequencing (Cho *et al.*, 2014). Recent genomewide off-target analysis based on deep sequencing also revealed a broad spectrum of RGEN specificities (Frock *et al.*, 2015; Kim *et al.*, 2015; Tsai *et al.*, 2015; Wang *et al.*, 2015).

Several strategies have been suggested and found to minimize or prevent off-target effects (Koo *et al.*, 2015). First, when designing a nuclease, choosing unique target sites that lack highly homologous sequences elsewhere in the genome is recommended. Many web-based programs have been developed for searching for potential TALEN or RGEN off-target sites (Bae *et al.*, 2014; Heigwer *et al.*, 2013, 2014; Hsu *et al.*, 2013). Second, RGEN off-target effects can be modulated by controlling the level or duration of nuclease expression (Hsu *et al.*, 2013). Third, the use of recombinant proteins and *in vitro* transcribed RNA, rather than plasmids encoding these components, can further reduce the frequency of off-target mutations due to the rapid degradation of the protein and RNA in cells (Gaj *et al.*, 2012; Kim *et al.*, 2014; Ramakrishna *et al.*, 2014). Jin-Soo Kim's group and we have recently reported that plasmid-free delivery of Cas9 protein and guide RNA can dramatically reduce off-target mutations without reducing efficiency (Kim *et al.*, 2014; Ramakrishna *et al.*, 2014). Additionally, sgRNAs truncated at the 5′ end (length less

than 20 bp) (Fu *et al.*, 2014) or those with two extra guanine nucleotides at the 5′ end (Cho *et al.*, 2014; Kim *et al.*, 2015) are reported to reduce off-target mutations up to 5000-fold (truncated sgRNA) or 660-fold (sgRNA with two extra guanine nucleotides) without alteration of mutation efficiencies at target sites. Paired nickases (Ran *et al.*, 2013a) or RNA-guided FokI nucleases (Guilinger *et al.*, 2014b; Tsai *et al.*, 2014) for knockout of genes can be good alternatives to nucleases with minimum off-target effects (Ran *et al.*, 2013a; Tsai *et al.*, 2014).

Conclusion

Generation of DSBs in a targeted manner using designed nucleases greatly facilitates genome editing. Recent breakthroughs in programmable nucleases have made genome editing an efficient and affordable process. Furthermore, technologies for 'reading' and 'writing' (that is, sequencing and synthesizing, respectively) genomes are currently being developed in parallel with genome editing.

Nonetheless, several aspects of programmable nuclease technology, including activity, off-target effects, ease of engineering and delivery, can be improved. Recently published analyses based on deep sequencing showed Cas9 nuclease-induced hard-to-predict off-target cleavages across the whole genome (Frock *et al.*, 2015; Kim *et al.*, 2015; Tsai *et al.*, 2015; Wang *et al.*, 2015), raising a safety issue. To increase the efficiency of precise genome editing, DSB repair pathways can be controlled either genetically or pharmacologically. Recently, several methods that improve HDR efficiency by reducing NHEJ have been developed (Chu *et al.*, 2015; Maruyama *et al.*, 2015; Yu *et al.*, 2015). If more precise genome editing can be performed, breeders will be able to manipulate the genomes of plants and animals with reduced adverse effects. In addition, genome-scale libraries of designed nucleases or transcriptional regulators (Findlay *et al.*, 2014; Gilbert *et al.*, 2014; Koike-Yusa *et al.*, 2014; Konermann *et al.*, 2015; Shalem *et al.*, 2014) can be used for deciphering new biological findings by enabling high-throughput loss- and gain-of-function studies. In the future, designed nucleases with improved efficiency and precision are expected to open a new era of biological research, medicine and biotechnology.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Anders, C., Niewoehner, O., Duerst, A. and Jinek, M. (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature*, **513**, 569–573.

Bae, K., Kwon, Y.D., Shin, H., Hwang, M., Ryu, E., Park, K., Yang, H., Lee, D., Lee, Y., Park, J., Kwon, H.S., Kim, H., Yeh, B., Lee, H., Sohn, S.H., Yoon, J., Seol, W. and Kim, J. (2003) Human zinc fingers as building blocks in the construction of artificial transcription factors. *Nat. Biotechnol.* **21**, 275–280.

Bae, S., Park, J. and Kim, J.S. (2014) Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics*, **30**, 1473–1475.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A. and Horvath, P. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, **315**, 1709–1712.

Bhakta, M.S., Henry, I.M., Ousterout, D.G., Das, K.T., Lockwood, S.H., Meckler, J.F., Wallen, M.C., Zykovich, A., Yu, Y., Leo, H., Xu, L., Gersbach, C.A. and Segal, D.J. (2013) Highly active zinc-finger nucleases by extended modular assembly. *Genome Res.* **23**, 530–538.

Bibikova, M., Carroll, D., Segal, D.J., Trautman, J.K., Smith, J., Kim, Y.G. and Chandrasegaran, S. (2001) Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol. Cell. Biol.* **21**, 289–297.

Bibikova, M., Golic, M., Golic, K.G. and Carroll, D. (2002) Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases. *Genetics*, **161**, 1169–1175.

Bibikova, M., Beumer, K., Trautman, J.K. and Carroll, D. (2003) Enhancing gene targeting with designed zinc finger nucleases. *Science*, **300**, 764.

Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F. and Marraffini, L.A. (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res.* **41**, 7429–7437.

Bitinaite, J., Wah, D.A., Aggarwal, A.K. and Schildkraut, I. (1998) FokI dimerization is required for DNA cleavage. *Proc. Natl Acad. Sci. USA*, **95**, 10570–10575.

Bortesi, L. and Fischer, R. (2015) The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv.* **33**, 41–52.

Briggs, A.W., Rios, X., Chari, R., Yang, L., Zhang, F., Mali, P. and Church, G.M. (2012) Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. *Nucleic Acids Res.* **40**, e117.

Brunet, E., Simsek, D., Tomishima, M., DeKelver, R., Choi, V.M., Gregory, P., Urnov, F., Weinstock, D.M. and Jasin, M. (2009) Chromosomal translocations induced at specified loci in human stem cells. *Proc. Natl Acad. Sci. USA*, **106**, 10620–10625.

Carlson, D.F., Tan, W., Lillico, S.G., Stverakova, D., Proudfoot, C., Christian, M., Voytas, D.F., Long, C.R., Whitelaw, C.B. and Fahrenkrug, S.C. (2012) Efficient TALEN-mediated gene knockout in livestock. *Proc. Natl Acad. Sci. USA*, **109**, 17382–17387.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J. and Voytas, D.F. (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* **39**, e82.

Chen, F., Pruett-Miller, S.M., Huang, Y., Gjoka, M., Duda, K., Taunton, J., Collingwood, T.N., Frodin, M. and Davis, G.D. (2011) High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nat. Methods*, **8**, 753–755.

Cho, S.W., Kim, S., Kim, J.M. and Kim, J.S. (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* **31**, 230–232.

Cho, S.W., Kim, S., Kim, Y., Kweon, J., Kim, H.S., Bae, S. and Kim, J. (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res.* **24**, 132–141.

Chu, V.T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K. and Kuhn, R. (2015) Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat. Biotechnol.* **33**, 543–548.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. and Zhang, F. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**, 819–823.

Cornu, T.I., Thibodeau-Beganny, S., Guhl, E., Alwin, S., Eichtinger, M., Joung, J.K. and Cathomen, T. (2008) DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol. Ther.* **16**, 352–358.

Cradick, T.J., Fine, E.J., Antico, C.J. and Bao, G. (2013) CRISPR/Cas9 systems targeting beta-globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Res.* **41**, 9584–9592.

Cui, X., Ji, D., Fisher, D.A., Wu, Y., Briner, D.M. and Weinstein, E.J. (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat. Biotechnol.* **29**, 64–67.

Davis, L. and Maizels, N. (2011) DNA nicks promote efficient and safe targeted gene correction. *PLoS ONE*, **6**, e23981.

Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y., Pirzada, Z.A., Eckert, M.R., Vogel, J. and Charpentier, E. (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, **471**, 602–607.

Deng, D., Yan, C., Pan, X., Mahfouz, M., Wang, J., Zhu, J.K., Shi, Y. and Yan, N. (2012) Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science*, **335**, 720–723.

Desjarlais, J.R. and Berg, J.M. (1992) Redesigning the DNA-binding specificity of a zinc finger protein: a data base-guided approach. *Proteins*, **12**, 101–104.

Dianov, G.L. and Hübscher, U. (2013) Mammalian base excision repair: the forgotten archangel. *Nucleic Acids Res.* **41**, 3483–3490.

Ding, Q., Regan, S.N., Xia, Y., Oostrom, L.A., Cowan, C.A. and Musunuru, K. (2013) Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. *Cell Stem Cell*, **12**, 393–394.

Doench, J.G., Hartenian, E., Graham, D.B., Tothova, Z., Hegde, M., Smith, I., Sullender, M., Ebert, B.L., Xavier, R.J. and Root, D.E. (2014) Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat. Biotechnol.* **32**, 1262–1267.

Doyon, J.B., Zeitler, B., Cheng, J., Cheng, A.T., Cherone, J.M., Santiago, Y., Lee, A.H., Vo, T.D., Doyon, Y., Miller, J.C., Paschon, D.E., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D. and Drubin, D.G. (2011) Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited mammalian cells. *Nat. Cell Biol.* **13**, 331–337.

Elrod Erickson, M., Rould, M.A., Nekludova, L. and Pabo, C.O. (1996) Zif268 protein-DNA complex refined at 1.6 A: a model system for understanding zinc finger-DNA interactions. *Structure*, **4**, 1171–1180.

Fauser, F., Schiml, S. and Puchta, H. (2014) Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant J.* **79**, 348–359.

Findlay, G.M., Boyle, E.A., Hause, R.J., Klein, J.C. and Shendure, J. (2014) Saturation editing of genomic regions by multiplex homology-directed repair. *Nature*, **513**, 120–123.

Fonfara, I., Le Rhun, A., Chylinski, K., Makarova, K.S., Lecrivain, A.L., Bzdrenga, J., Koonin, E.V. and Charpentier, E. (2014) Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. *Nucleic Acids Res.* **42**, 2577–2590.

Frock, R.L., Hu, J., Meyers, R.M., Ho, Y.J., Kii, E. and Alt, F.W. (2015) Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat. Biotechnol.* **33**, 179–186.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K. and Sander, J.D. (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822–826.

Fu, Y., Sander, J.D., Reyon, D., Cascio, V.M. and Joung, J.K. (2014) Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* **32**, 279–284.

Gabriel, R., Lombardo, A., Arens, A., Miller, J.C., Genovese, P., Kaeppel, C., Nowrouzi, A., Bartholomae, C.C., Wang, J., Friedman, G., Holmes, M.C., Gregory, P.D., Glimm, H., Schmidt, M., Naldini, L. and von Kalle, C. (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat. Biotechnol.* **29**, 816–823.

Gabsalilow, L., Schierling, B., Friedhoff, P., Pingoud, A. and Wende, W. (2013) Site- and strand-specific nicking of DNA by fusion proteins derived from MutH and I-SceI or TALE repeats. *Nucleic Acids Res.* **41**, e83.

Gaj, T., Guo, J., Kato, Y., Sirk, S.J. and Barbas, C.F. 3rd (2012) Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. *Nat. Methods*, **9**, 805–807.

Gasiunas, G., Barrangou, R., Horvath, P. and Siksnys, V. (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl Acad. Sci. USA*, **109**, E2579–E2586.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., Lim, W.A., Weissman, J.S. and Qi, L.S. (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*, **154**, 442–451.

Gilbert, L.A., Horlbeck, M.A., Adamson, B., Villalta, J.E., Chen, Y., Whitehead, E.H., Guimaraes, C., Panning, B., Ploegh, H.L., Bassik, M.C., Qi, L.S., Kampmann, M. and Weissman, J.S. (2014) Genome-scale CRISPR-mediated control of gene repression and activation. *Cell*, **159**, 647–661.

Guilinger, J.P., Pattanayak, V., Reyon, D., Tsai, S.Q., Sander, J.D., Joung, J.K. and Liu, D.R. (2014a) Broad specificity profiling of TALENs results in engineered nucleases with improved DNA-cleavage specificity. *Nat. Methods*, **11**, 429–435.

Guilinger, J.P., Thompson, D.B. and Liu, D.R. (2014b) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nat. Biotechnol.* **32**, 577–582.

Gupta, A., Christensen, R.G., Rayla, A.L., Lakshmanan, A., Stormo, G.D. and Wolfe, S.A. (2012) An optimized two-finger archive for ZFN-mediated gene targeting. *Nat. Methods*, **9**, 588–590.

Gupta, A., Hall, V.L., Kok, F.O., Shin, M., McNulty, J.C., Lawson, N.D. and Wolfe, S.A. (2013) Targeted chromosomal deletions and inversions in zebrafish. *Genome Res.* **23**, 1008–1017.

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J.I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa Lyonnet, D., Romana, S., Radford Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L.E., Wissler, M., Prinz, C., Rabbitts, T.H., Le Deist, F., Fischer, A. and Cavazzana Calvo, M. (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*, **302**, 415–419.

Heigwer, F., Kerr, G., Walther, N., Glaeser, K., Pelz, O., Breinig, M. and Boutros, M. (2013) E-TALEN: a web tool to design TALENs for genome engineering. *Nucleic Acids Res.* **41**, e190.

Heigwer, F., Kerr, G. and Boutros, M. (2014) E-CRISP: fast CRISPR target site identification. *Nat. Methods*, **11**, 122–123.

Hockemeyer, D., Wang, H., Kiani, S., Lai, C.S., Gao, Q., Cassady, J.P., Cost, G.J., Zhang, L., Santiago, Y., Miller, J.C., Zeitler, B., Cherone, J.M., Meng, X., Hinkley, S.J., Rebar, E.J., Gregory, P.D., Urnov, F.D. and Jaenisch, R. (2011) Genetic engineering of human pluripotent cells using TALE nucleases. *Nat. Biotechnol.* **29**, 731–734.

Holkers, M., Maggio, I., Liu, J., Janssen, J.M., Miselli, F., Mussolino, C., Recchia, A., Cathomen, T. and Goncalves, M.A. (2013) Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. *Nucleic Acids Res.* **41**, e63.

Hou, Z., Zhang, Y., Propson, N.E., Howden, S.E., Chu, L.F., Sontheimer, E.J. and Thomson, J.A. (2013) Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proc. Natl Acad. Sci. USA*, **110**, 15644–15649.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., Cradick, T.J., Marraffini, L.A., Bao, G. and Zhang, F. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832.

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.R. and Joung, J.K. (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 227–229.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P.S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21**, 635–637.

Jiang, W., Bikard, D., Cox, D., Zhang, F. and Marraffini, L.A. (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.* **31**, 233–239.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, **337**, 816–821.

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E. and Doudna, J. (2013) RNA-programmed genome editing in human cells. *Elife*, **2**, e00471.

Jinek, M., Jiang, F., Taylor, D.W., Sternberg, S.H., Kaya, E., Ma, E., Anders, C., Hauer, M., Zhou, K., Lin, S., Kaplan, M., Iavarone, A.T., Charpentier, E., Nogales, E. and Doudna, J.A. (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science*, **343**, 1247997.

Kim, H. and Kim, J.S. (2014) A guide to genome engineering with programmable nucleases. *Nat. Rev. Genet.* **15**, 321–334.

Kim, Y.G., Cha, J. and Chandrasegaran, S. (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl Acad. Sci. USA*, **93**, 1156–1160.

Kim, H.J., Lee, H.J., Kim, H., Cho, S.W. and Kim, J.S. (2009) Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res.* **19**, 1279–1288.

Kim, J., Lee, H.J. and Carroll, D. (2010) Genome editing with modularly assembled zinc-finger nucleases. *Nat. Methods*, **7**, 91–92.

Kim, E., Kim, S., Kim, D.H., Choi, B.S., Choi, I.Y. and Kim, J.S. (2012) Precision genome engineering with programmable DNA-nicking enzymes. *Genome Res.* **22**, 1327–1333.

Kim, Y., Kweon, J., Kim, A., Chon, J.K., Yoo, J.Y., Kim, H.J., Kim, S., Lee, C., Jeong, E., Chung, E., Kim, D., Lee, M.S., Go, E.M., Song, H.J., Kim, H., Cho, N., Bang, D., Kim, S. and Kim, J.S. (2013a) A library of TAL effector nucleases spanning the human genome. *Nat. Biotechnol.* **31**, 251–258.

Kim, Y.K., Wee, G., Park, J., Kim, J., Baek, D., Kim, J.S. and Kim, V.N. (2013b) TALEN-based knockout library for human microRNAs. *Nat. Struct. Mol. Biol.* **20**, 1458–1464.

Kim, S., Kim, D., Cho, S.W., Kim, J. and Kim, J.S. (2014) Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* **24**, 1012–1019.

Kim, D., Bae, S., Park, J., Kim, E., Kim, S., Yu, H.R., Hwang, J., Kim, J.I. and Kim, J.S. (2015) Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat. Methods*, **12**, 237–243.

Koike-Yusa, H., Li, Y., Tan, E.P., Velasco-Herrera Mdel, C. and Yusa, K. (2014) Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat. Biotechnol.* **32**, 267–273.

Konermann, S., Brigham, M.D., Trevino, A.E., Hsu, P.D., Heidenreich, M., Cong, L., Platt, R.J., Scott, D.A., Church, G.M. and Zhang, F. (2013) Optical control of mammalian endogenous transcription and epigenetic states. *Nature*, **500**, 472–476.

Konermann, S., Brigham, M.D., Trevino, A.E., Joung, J., Abudayyeh, O.O., Barcena, C., Hsu, P.D., Habib, N., Gootenberg, J.S., Nishimasu, H., Nureki, O. and Zhang, F. (2015) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*, **517**, 583–588.

Koo, T., Lee, J. and Kim, J.S. (2015) Measuring and reducing off-target activities of programmable nucleases including CRISPR-Cas9. *Mol. Cells*, **38**, 475–481.

Krueger, U., Bergauer, T., Kaufmann, B., Wolter, I., Pilk, S., Heider Fabian, M., Kirch, S., Artz Oppitz, C., Isselhorst, M. and Konrad, J. (2007) Insights into effective RNAi gained from large-scale siRNA validation screening. *Oligonucleotides*, **17**, 237–250.

Lagutina, I.V., Valentine, V., Picchione, F., Harwood, F., Valentine, M.B., Villarejo Balcells, B., Carvajal, J. and Grosveld, G.C. (2015) Modeling of the human alveolar rhabdomyosarcoma Pax3-Foxo1 chromosome translocation in mouse myoblasts using CRISPR-Cas9 nuclease. *PLoS Genet.* **11**, e1004951.

Lee, H.J., Kim, E. and Kim, J. (2010) Targeted chromosomal deletions in human cells using zinc finger nucleases. *Genome Res.* **20**, 81–89.

Lee, H.J., Kweon, J., Kim, E., Kim, S. and Kim, J. (2012) Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. *Genome Res.* **22**, 539–548.

Li, H., Haurigot, V., Doyon, Y., Li, T., Wong, S.Y., Bhagwat, A.S., Malani, N., Anguela, X.M., Sharma, R., Ivanciu, L., Murphy, S.L., Finn, J.D., Khazi, F.R., Zhou, S., Paschon, D.E., Rebar, E.J., Bushman, F.D., Gregory, P.D., Holmes, M.C. and High, K.A. (2011) In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature*, **475**, 217–221.

Li, J.F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M. and Sheen, J. (2013a) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat. Biotechnol.* **31**, 688–691.

Li, W., Teng, F., Li, T. and Zhou, Q. (2013b) Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat. Biotechnol.* **31**, 684–686.

Lieber, M.R. (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu. Rev. Biochem.* **79**, 181–211.

Lieber, M.R. and Karanjawala, Z.E. (2004) Ageing, repetitive genomes and DNA damage. *Nat. Rev. Mol. Cell Biol.* **5**, 69–75.

Lieber, M.R., Ma, Y., Pannicke, U. and Schwarz, K. (2003) Mechanism and regulation of human non-homologous DNA end-joining. *Nat. Rev. Mol. Cell Biol.* **4**, 712–720.

Maddalo, D., Manchado, E., Concepcion, C.P., Bonetti, C., Vidigal, J.A., Han, Y., Ogrodowski, P., Crippa, A., Rekhtman, N., de Stanchina, E., Lowe, S.W. and Ventura, A. (2014) In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature*, **516**, 423–427.

Maeder, M.L., Thibodeau-Beganny, S., Osiak, A., Wright, D.A., Anthony, R.M., Eichtinger, M., Jiang, T., Foley, J.E., Winfrey, R.J., Townsend, J.A., Unger-Wallace, E., Sander, J.D., Muller-Lerch, F., Fu, F., Pearlberg, J., Gobel, C., Dassie, J.P., Pruett-Miller, S.M., Porteus, M.H., Sgroi, D.C., Iafrate, A.J., Dobbs, D., McCray, P.B. Jr, Cathomen, T., Voytas, D.F. and Joung, J.K. (2008) Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol. Cell*, **31**, 294–301.

Maeder, M.L., Thibodeau-Beganny, S., Sander, J.D., Voytas, D.F. and Joung, J.K. (2009) Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. *Nat. Protoc.* **4**, 1471–1501.

Mak, A.N., Bradley, P., Cernadas, R.A., Bogdanove, A.J. and Stoddard, B.L. (2012) The crystal structure of TAL effector PthXo1 bound to its DNA target. *Science*, **335**, 716–719.

Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I. and Koonin, E.V. (2006) A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. *Biol. Direct*, **1**, 7.

Mali, P., Aach, J., Stranges, P.B., Esvelt, K.M., Moosburner, M., Kosuri, S., Yang, L. and Church, G.M. (2013a) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.* **31**, 833–838.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E. and Church, G.M. (2013b) RNA-guided human genome engineering via Cas9. *Science*, **339**, 823–826.

Maruyama, T., Dougan, S.K., Truttmann, M.C., Bilate, A.M., Ingram, J.R. and Ploegh, H.L. (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat. Biotechnol.* **33**, 538–542.

McConnell Smith, A., Takeuchi, R., Pellenz, S., Davis, L., Maizels, N., Monnat, R.J. Jr and Stoddard, B.L. (2009) Generation of a nicking enzyme that stimulates site-specific gene conversion from the I-AniI LAGLIDADG homing endonuclease. *Proc. Natl Acad. Sci. USA*, **106**, 5099–5104.

McVey, M. and Lee, S.E. (2008) MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet.* **24**, 529–538.

Miller, J.C., Holmes, M.C., Wang, J., Guschin, D.Y., Lee, Y., Rupniewski, I., Beausejour, C.M., Waite, A.J., Wang, N.S., Kim, K.A., Gregory, P.D., Pabo, C.O. and Rebar, E.J. (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat. Biotechnol.* **25**, 778–785.

Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J., Dulay, G.P., Hua, K.L., Ankoudinova, I., Cost, G.J., Urnov, F.D., Zhang, H.S., Holmes, M.C., Zhang, L., Gregory, P.D. and Rebar, E.J. (2011) A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* **29**, 143–148.

Mojica, F.J., Diez-Villasenor, C., Garcia-Martinez, J. and Almendros, C. (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*, **155**, 733–740.

Mussolino, C., Morbitzer, R., Lutge, F., Dannemann, N., Lahaye, T. and Cathomen, T. (2011) A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res.* **39**, 9283–9293.

Niu, Y., Shen, B., Cui, Y., Chen, Y., Wang, J., Wang, L., Kang, Y., Zhao, X., Si, W., Li, W., Xiang, A.P., Zhou, J., Guo, X., Bi, Y., Si, C., Hu, B., Dong, G., Wang, H., Zhou, Z., Li, T., Tan, T., Pu, X., Wang, F., Ji, S., Zhou, Q., Huang, X., Ji, W. and Sha, J. (2014) Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell*, **156**, 836–843.

Offringa, R., de Groot, M.J., Haagsman, H.J., Does, M.P., van den Elzen, P.J. and Hooykaas, P.J. (1990) Extrachromosomal homologous recombination and gene targeting in plant cells after Agrobacterium mediated transformation. *EMBO J.* **9**, 3077–3084.

Park, C., Kweon, J., Son, J.S., Lee, J.S., Yoo, J., Cho, S., Kim, J. and Kim, D. (2014) Targeted inversion and reversion of the blood coagulation factor 8 gene in human iPS cells using TALENs. *Proc. Natl Acad. Sci. USA*, **111**, 9253–9258.

Paszkowski, J., Baur, M., Bogucki, A. and Potrykus, I. (1988) Gene targeting in plants. *EMBO J.* **7**, 4021–4026.

Pattanayak, V., Ramirez, C.L., Joung, J.K. and Liu, D.R. (2011) Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nat. Methods*, **8**, 765–770.

Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A. and Liu, D.R. (2013) High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* **31**, 839–843.

Petolino, J.F., Worden, A., Curlee, K., Connell, J., Strange Moynahan, T.L., Larsen, C. and Russell, S. (2010) Zinc finger nuclease-mediated transgene deletion. *Plant Mol. Biol.* **73**, 617–628.

Porteus, M.H. and Baltimore, D. (2003) Chimeric nucleases stimulate gene targeting in human cells. *Science*, **300**, 763.

Puchta, H. (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J. Exp. Bot.* **56**, 1–14.

Puchta, H., Dujon, B. and Hohn, B. (1993) Homologous recombination in plant cells is enhanced by in vivo induction of double strand breaks into DNA by a site-specific endonuclease. *Nucleic Acids Res.* **21**, 5034–5040.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P. and Lim, W.A. (2013a) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*, **152**, 1173–1183.

Qi, Y., Li, X., Zhang, Y., Starker, C.G., Baltes, N.J., Sander, J.D., Reyon, D., Joung, J.K. and Voytas, D.F. (2013b) Targeted deletion and inversion of tandemly arrayed genes in Arabidopsis thaliana using zinc finger nucleases. *G3*, **3**, 1707–1715.

Ramakrishna, S., Kwaku Dad, A.B., Beloor, J., Gopalappa, R., Lee, S.K. and Kim, H. (2014) Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Res.* **24**, 1020–1027.

Ramirez, C.L., Foley, J.E., Wright, D.A., Muller-Lerch, F., Rahman, S.H., Cornu, T.I., Winfrey, R.J., Sander, J.D., Fu, F., Townsend, J.A., Cathomen, T., Voytas, D.F. and Joung, J.K. (2008) Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat. Methods*, **5**, 374–375.

Ramirez, C.L., Certo, M.T., Mussolino, C., Goodwin, M.J., Cradick, T.J., McCaffrey, A.P., Cathomen, T., Scharenberg, A.M. and Joung, J.K. (2012) Engineered zinc finger nickases induce homology-directed repair with reduced mutagenic effects. *Nucleic Acids Res.* **40**, 5560–5568.

Ran, F.A., Hsu, P.D., Lin, C., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y. and Zhang, F. (2013a) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell*, **154**, 1380–1389.

Ran, F.A., Cong, L., Yan, W.X., Scott, D.A., Gootenberg, J.S., Kriz, A.J., Zetsche, B., Shalem, O., Wu, X., Makarova, K.S., Koonin, E.V., Sharp, P.A. and Zhang, F. (2015) In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature*, **520**, 186–191.

Rebar, E.J. and Pabo, C.O. (1994) Zinc finger phage: affinity selection of fingers with new DNA-binding specificities. *Science*, **263**, 671–673.

Reyon, D., Tsai, S.Q., Khayter, C., Foden, J.A., Sander, J.D. and Joung, J.K. (2012) FLASH assembly of TALENs for high-throughput genome editing. *Nat. Biotechnol.* **30**, 460–465.

Rouet, P., Smih, F. and Jasin, M. (1994) Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol. Cell. Biol.* **14**, 8096–8106.

Sadelain, M., Papapetrou, E.P. and Bushman, F.D. (2012) Safe harbours for the integration of new DNA in the human genome. *Nat. Rev. Cancer*, **12**, 51–58.

Salomon, S. and Puchta, H. (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. *EMBO J.* **17**, 6086–6095.

Sander, J.D., Dahlborg, E.J., Goodwin, M.J., Cade, L., Zhang, F., Cifuentes, D., Curtin, S.J., Blackburn, J.S., Thibodeau-Beganny, S., Qi, Y., Pierick, C.J., Hoffman, E., Maeder, M.L., Khayter, C., Reyon, D., Dobbs, D., Langenau, D.M., Stupar, R.M., Giraldez, A.J., Voytas, D.F., Peterson, R.T., Yeh, J.R. and Joung, J.K. (2011) Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat. Methods*, **8**, 67–69.

Sapranauskas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P. and Siksnys, V. (2011) The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res.* **39**, 9275–9282.

Sarbajna, S. and West, S.C. (2014) Holliday junction processing enzymes as guardians of genome stability. *Trends Biochem. Sci.* **39**, 409–419.

Schiml, S., Fauser, F. and Puchta, H. (2014) The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. *Plant J.* **80**, 1139–1150.

Schmid-Burgk, J.L., Schmidt, T., Kaiser, V., Honing, K. and Hornung, V. (2013) A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. *Nat. Biotechnol.* **31**, 76–81.

Segal, D.J. and Meckler, J.F. (2013) Genome engineering at the dawn of the golden age. *Annu. Rev. Genomics Hum. Genet.* **14**, 135–158.

Segal, D.J., Dreier, B., Beerli, R.R. and Barbas, C.F. 3rd (1999) Toward controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 5′-GNN-3′ DNA target sequences. *Proc. Natl Acad. Sci. USA*, **96**, 2758–2763.

Segal, D.J., Beerli, R., Blancafort, P., Dreier, B., Effertz, K., Huber, A., Koksch, B., Lund, C.V., Magnenat, L., Valente, D. and Barbas, C.F. (2003) Evaluation of a modular strategy for the construction of novel polydactyl zinc finger DNA-binding proteins. *Biochemistry*, **42**, 2137–2148.

Shah, S.A., Erdmann, S., Mojica, F.J. and Garrett, R.A. (2013) Protospacer recognition motifs: mixed identities and functional diversity. *RNA Biol.* **10**, 891–899.

Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T.S., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G. and Zhang, F. (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*, **343**, 84–87.

Shan, Q., Wang, Y., Li, J., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., Qiu, J.L. and Gao, C. (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 686–688.

Soldner, F., Laganiere, J., Cheng, A.W., Hockemeyer, D., Gao, Q., Alagappan, R., Khurana, V., Golbe, L.I., Myers, R.H., Lindquist, S., Zhang, L., Guschin, D., Fong, L.K., Vu, B.J., Meng, X., Urnov, F.D., Rebar, E.J., Gregory, P.D., Zhang, H.S. and Jaenisch, R. (2011) Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell*, **146**, 318–331.

Sollu, C., Pars, K., Cornu, T.I., Thibodeau-Beganny, S., Maeder, M.L., Joung, J.K., Heilbronn, R. and Cathomen, T. (2010) Autonomous zinc-finger nuclease pairs for targeted chromosomal deletion. *Nucleic Acids Res.* **38**, 8269–8276.

Swiech, L., Heidenreich, M., Banerjee, A., Habib, N., Li, Y., Trombetta, J., Sur, M. and Zhang, F. (2015) In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat. Biotechnol.* **33**, 102–106.

Szczepek, M., Brondani, V., Buchel, J., Serrano, L., Segal, D.J. and Cathomen, T. (2007) Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat. Biotechnol.* **25**, 786–793.

Tsai, S.Q., Wyvekens, N., Khayter, C., Foden, J.A., Thapar, V., Reyon, D., Goodwin, M.J., Aryee, M.J. and Joung, J.K. (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. *Nat. Biotechnol.* **32**, 569–576.

Tsai, S.Q., Zheng, Z., Nguyen, N.T., Liebers, M., Topkar, V.V., Thapar, V., Wyvekens, N., Khayter, C., Iafrate, A.J., Le, L.P., Aryee, M.J. and Joung, J.K. (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat. Biotechnol.* **33**, 187–197.

Veres, A., Gosis, B.S., Ding, Q., Collins, R., Ragavendran, A., Brand, H., Erdin, S., Cowan, C.A., Talkowski, M.E. and Musunuru, K. (2014) Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human

stem cell clones detected by whole-genome sequencing. *Cell Stem Cell*, **15**, 27–30.

Wah, D.A., Bitinaite, J., Schildkraut, I. and Aggarwal, A.K. (1998) Structure of FokI has implications for DNA cleavage. *Proc. Natl Acad. Sci. USA*, **95**, 10564–10569.

Wang, J., Friedman, G., Doyon, Y., Wang, N.S., Li, C.J., Miller, J.C., Hua, K.L., Yan, J.J., Babiarz, J.E., Gregory, P.D. and Holmes, M.C. (2012) Targeted gene addition to a predetermined site in the human genome using a ZFN-based nicking enzyme. *Genome Res.* **22**, 1316–1326.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M., Cheng, A.W., Zhang, F. and Jaenisch, R. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*, **153**, 910–918.

Wang, X., Wang, Y., Wu, X., Wang, J., Wang, Y., Qiu, Z., Chang, T., Huang, H., Lin, R.J. and Yee, J.K. (2015) Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENs using integrase-defective lentiviral vectors. *Nat. Biotechnol.* **33**, 175–178.

Wefers, B., Meyer, M., Ortiz, O., Hrabe de Angelis, M., Hansen, J., Wurst, W. and Kuhn, R. (2013) Direct production of mouse disease models by embryo microinjection of TALENs and oligodeoxynucleotides. *Proc. Natl Acad. Sci. USA*, **110**, 3782–3787.

Wolfe, S.A., Nekludova, L. and Pabo, C.O. (2000) DNA recognition by Cys2His2 zinc finger proteins. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 183–212.

Xiao, A., Wang, Z., Hu, Y., Wu, Y., Luo, Z., Yang, Z., Zu, Y., Li, W., Huang, P., Tong, X., Zhu, Z., Lin, S. and Zhang, B. (2013) Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Res.* **41**, e141.

Xie, K., Zhang, J. and Yang, Y. (2014) Genome-wide prediction of highly specific guide RNA spacers for CRISPR-Cas9-mediated genome editing in model plants and major crops. *Mol. Plant*, **7**, 923–926.

Xie, K., Minkenberg, B. and Yang, Y. (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc. Natl Acad. Sci. USA*, **112**, 3570–3575.

Yang, L., Guell, M., Byrne, S., Yang, J.L., De Los Angeles, A., Mali, P., Aach, J., Kim-Kiselak, C., Briggs, A.W., Rios, X., Huang, P.Y., Daley, G. and Church, G. (2013) Optimization of scarless human stem cell genome editing. *Nucleic Acids Res.* **41**, 9049–9061.

Yin, H., Xue, W., Chen, S., Bogorad, R.L., Benedetti, E., Grompe, M., Koteliansky, V., Sharp, P.A., Jacks, T. and Anderson, D.G. (2014) Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat. Biotechnol.* **32**, 551–553.

Yu, C., Liu, Y., Ma, T., Liu, K., Xu, S., Zhang, Y., Liu, H., La Russa, M., Xie, M., Ding, S. and Qi, L.S. (2015) Small molecules enhance CRISPR genome editing in pluripotent stem cells. *Cell Stem Cell*, **16**, 142–147.

Zalatan, J.G., Lee, M.E., Almeida, R., Gilbert, L.A., Whitehead, E.H., La Russa, M., Tsai, J.C., Weissman, J.S., Dueber, J.E., Qi, L.S. and Lim, W.A. (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell*, **160**, 339–350.

Zuris, J.A., Thompson, D.B., Shu, Y., Guilinger, J.P., Bessen, J.L., Hu, J.H., Maeder, M.L., Joung, J.K., Chen, Z.Y. and Liu, D.R. (2015) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat. Biotechnol.* **33**, 73–80.