A guide to genome engineering with programmable nucleases

Hyongbum Kim[1] *and Jin-Soo Kim*[2,3]

Abstract | Programmable nucleases — including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided engineered nucleases (RGENs) derived from the bacterial clustered regularly interspaced short palindromic repeat (CRISPR)–Cas (CRISPR-associated) system — enable targeted genetic modifications in cultured cells, as well as in whole animals and plants. The value of these enzymes in research, medicine and biotechnology arises from their ability to induce site-specific DNA cleavage in the genome, the repair (through endogenous mechanisms) of which allows high-precision genome editing. However, these nucleases differ in several respects, including their composition, targetable sites, specificities and mutation signatures, among other characteristics. Knowledge of nuclease-specific features, as well as of their pros and cons, is essential for researchers to choose the most appropriate tool for a range of applications.

Homologous recombination
A genetic recombination process in which two similar DNA strands exchange nucleotide sequences.

[1]*Graduate School of Biomedical Science and Engineering, and College of Medicine, Hanyang University, Wangsimni-ro 222, Sungdong-gu, Seoul 133-791, South Korea.*
[2]*Center for Genome Engineering, Institute for Basic Science, Gwanak-ro 1, Gwanak-gu, Seoul 151-747, South Korea.*
[3]*Department of Chemistry, Seoul National University, Gwanak-ro 1, Gwanak-gu, Seoul 151-747, South Korea.*
Correspondence to J.-S.K.
e-mail: jskim01@snu.ac.kr
doi:10.1038/nrg3686
Published online 2 April 2014

Targeted genome engineering (that is, the modification of the genome at a precise, predetermined locus) is broadly applicable to biomedical research, medicine and biotechnology (BOX 1). For example, to determine the functions of specific genes or non-coding elements such as microRNAs (miRNAs), researchers selectively inactivate them and evaluate the phenotypic consequences in cells or whole organisms. Small interfering RNAs (siRNAs) have been widely used to study gene functions; however, gene knockdown by siRNAs is often incomplete[1] and nonspecific[2]. Conventional gene targeting approaches that depend on homologous recombination enable gene inactivation, but the efficiency of such homologous recombination events is extremely low (ranging from 1 in 10^6 to 1 in 10^7) in higher eukaryotic cells, which hampers their routine use.

Programmable nucleases produce site-specific DNA double-strand breaks (DSBs), which enhance the efficiency of homologous recombination by at least two orders of magnitude[3] and/or trigger error-prone non-homologous end-joining (NHEJ), which leads to targeted mutagenesis[4]. The technology of genome editing is evolving rapidly. Until a few years ago, zinc-finger nucleases (ZFNs) were the only practical option available to researchers who are interested in targeted genome engineering. By the end of 2011, when *Nature Methods* chose this technology as "Method of the Year", ZFNs were listed alongside transcription activator-like effector nucleases (TALENs) as tools for genome editing. In January 2013, several groups independently reported a new class of genome editing nucleases — termed RNA-guided engineered nucleases (RGENs) herein to avoid confusion with the original type II clustered regularly interspaced short palindromic repeat (CRISPR)–Cas (CRISPR-associated) adaptive immune system in bacteria — the specificity of which is mostly determined by small guide RNAs rather than by DNA-binding proteins[5–10]. These three types of nucleases share the same mechanism of action: they cleave chromosomal DNA in a site-specific manner, which triggers endogenous DNA repair systems that result in targeted genome modification. However, each nuclease has unique characteristics.

Here, we review the rapidly evolving technology of targeted genome engineering using programmable nucleases. We first describe general features of genome editing that are shared by the nuclease triad. We then compare these nucleases in detail and discuss the pros and cons of each type to provide a guide for choosing the most appropriate tool for a range of genome editing experiments and applications. Finally, we suggest approaches for enhancing the efficiency of programmable nucleases. Other powerful tools of genome engineering — including meganucleases, transposons, recombinases and chemical DNA cutters — are not covered in this Review.

© 2014 Macmillan Publishers Limited. All rights reserved

Non-homologous end-joining
(NHEJ). A repair pathway for DNA double-strand breaks (DSBs) through direct ligation of the break ends without using a homologous template. This error-prone process often causes small insertions and deletions at the DSB site.

Zinc-finger nucleases
(ZFNs). Programmable nucleases composed of the *Fok*I catalytic domain and zinc-finger DNA-binding domains.

Shared features of programmable nucleases

Repair of DNA double-strand breaks. The development of genome editing technology was stimulated by two seminal discoveries. First, it was reported that the efficiency of gene targeting through homologous recombination in mouse stem cells can be enhanced by at least 2–3 orders of magnitude through the introduction of DSBs in the target region using a rare-cutting endonuclease[3]. This marked enhancement in the efficiency of homologous recombination was also observed in other systems using ZFNs[11–13]. Second, it was revealed that DSBs are efficiently repaired by error-prone NHEJ in the absence of a gene targeting vector or a homologous donor DNA[4]. NHEJ-mediated repair or homology-directed repair (HDR) of site-specific DSBs induced by programmable nucleases can thus lead to targeted genetic modifications, including gene disruption, insertion, correction and chromosomal rearrangements (FIG. 1).

Box 1 | Applications of genome editing

Originally developed as artificial restriction endonucleases for gene cloning to replace or complement restriction enzymes[41], zinc-finger nucleases (ZFNs) are now seldom used for this purpose[166]; however, engineered nucleases have become as versatile and indispensable as restriction enzymes in research and biotechnology. The list of non-model organisms with genomes that have been modified successfully using engineered nucleases now includes mosquitoes[167], crickets, silkworms, pigs[168], cows, rabbits and non-human primates[102] among others (reviewed in REF. 38).

Preclinical studies

Engineered nucleases are now used to disrupt not only protein-coding genes but also non-coding elements, including microRNAs[84,169] and long non-coding RNAs[170]. Genetic variations such as single-nucleotide polymorphisms (SNPs) and structural variations can also be created in cell lines and model organisms using engineered nucleases to study the physiological roles of these variations in an otherwise isogenic background[30,31,35,171,172].

Engineered nucleases can be injected directly into one-cell stage embryos to create gene knockout or knock-in animals[7,26–28,66,98,101,102,126,173–176], which bypasses the use of embryonic stem cells. Hence, this technology greatly facilitates the generation of disease models in various animals and in human pluripotent stem cells[29,35,103,136,177], which can be used, for example, for drug target validation and preclinical drug tests. Furthermore, when applied to human pluripotent stem cells, *in vitro* human disease models with isogenic controls can be generated[29,83], which facilitates the investigation of disease pathophysiology and drug screening.

Biotechnology

Engineered nucleases enable the development of genetically improved crops[178–180] and livestock[32,168] that are resistant to diseases or enriched with certain nutrients. For example, pathogen-resistant rice was produced by disrupting pathogen-responsive DNA elements in the rice genome using transcription activator-like effector nucleases (TALENs)[180]. ZFNs allowed the removal of glycoantigens in pigs that elicit acute immune rejection in humans, which paved the way for pig-to-human xenotransplatation[168]. Moreover, engineered nucleases were used to mutate the beta-lactoglobulin (*BLG*) gene in cow that produces allergens in milk[181]. Furthermore, genome editing enables the engineering of cell lines, such as Chinese hamster ovary cells, that are widely used for the production of therapeutic proteins[182].

Therapeutics

Ex vivo and *in vivo* delivery of ZFNs have been used to edit genomes for the treatment of HIV infection in humans[128,183,184] and haemophilia B in mice[19], respectively. Gene correction and addition in patient-derived pluripotent stem cells or somatic cells using programmable nucleases can also provide novel therapeutic opportunities for patients with diverse genetic and acquired diseases. For example, genetic correction has been achieved in cultured human cells that were derived from patients with genetic diseases such as recessive dystrophic epidermolysis bullosa[185], Duchenne muscular dystrophy[186], cystic fibrosis[187], sickle cell disease[171,188], α1-antitrypsin deficiency[136], chronic granulomatous disease[189] and Down syndrome[190].

Gene disruption. Gene knockout — the simplest form of genome editing — can be achieved through error-prone NHEJ, which gives rise to small insertions and deletions (indels) at the nuclease target site (FIG. 1a). Indels often cause frameshifts in the coding region, which disrupt genetic information and result in gene knockout. No additional gene targeting vector is required in this process. Gene disruption is crucial for determining the function of genes in whole organisms and cells (BOX 1); for example, nuclease-induced gene-knockout cell lines have been used to study glycosylation pathways[14], nuclear factor κB (NF-κB) signalling[15] and protein methylation[16].

Gene insertion. Plasmid DNA or integrating viral vectors are often used to insert a gene of interest into the genome. As the insertion site cannot be controlled when these methods are used, the transgene and its regulatory sequence can be incorporated at undesired sites, which may inactivate essential genes or activate proto-oncogenes[17]. Engineered nucleases enhance the efficiency of homologous recombination in a site-specific manner, which allows the targeted insertion of therapeutic genes into genomic 'safe harbours' (REF. 18) or pre-determined sites in the genome[19–21]. To achieve this, the nuclease is co-transfected with a targeting vector, in which the genetic segment to be incorporated is flanked by homology arms with sequences that are identical to those near the target region (FIG. 1a). Alternatively, if defined overhangs are generated by nucleases, then specific sequences can be inserted into the target region by NHEJ-mediated ligation[22,23]. This method is also used for linking DNA sequences that encode reporter genes or peptide tags to endogenous genes to monitor their expression[21] or to facilitate purification of the resulting protein complexes, respectively.

Gene correction and point mutagenesis. Point mutations can be corrected or single-nucleotide variations introduced in the genome through co-delivery of programmable nucleases and targeting vectors[11–13,24] or single-strand oligodeoxynucleotides (ssODNs)[25] (FIG. 1a). Unlike targeting vectors, the preparation of which is cumbersome and time-consuming, ssODNs can be designed and synthesized within a few days. This precise and efficient method streamlines the generation of disease models in animals[26–28] and human cells[29]. Whether ssODNs are incorporated into the genome or whether they are used as templates during DSB repair remains unknown. Understanding the mechanisms behind this process may further improve this convenient method.

Chromosomal rearrangements. The repair of two concurrent DSBs induced by programmable nucleases can give rise to chromosomal rearrangements or to structural variations in a targeted manner (FIG. 1b,c). Deletions, duplications and inversions of up to a few megabasepairs of chromosomal segments have been achieved using ZFNs[30,31], TALENs[15,32–34] or RGENs[6,33] (that is, the CRISPR–Cas system) in cells and whole

© 2014 Macmillan Publishers Limited. All rights reserved

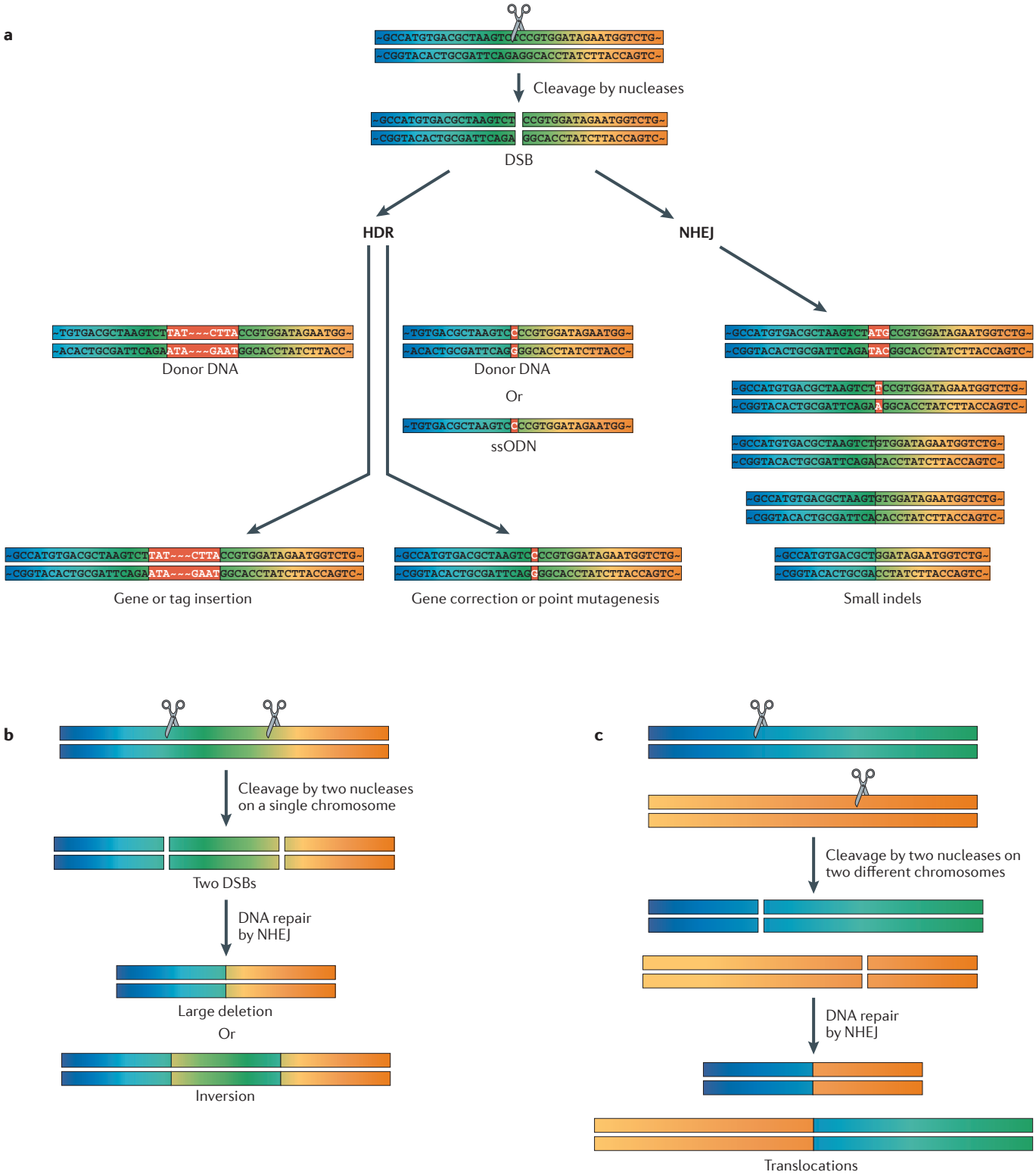


Figure 1 | **Outcome of genome editing using programmable nucleases.** **a** | Nuclease-induced double-strand breaks (DSBs) can lead to sequence insertion, nucleotide correction or change (red box) through homology-directed repair (HDR) in the presence of a donor DNA or a single-strand oligodeoxynucleotide (ssODN), both of which contain homology arms. DSBs can also be repaired through error-prone non-homologous end-joining (NHEJ), which does not require donor DNA or ssODN and consequently often leads to small insertions and deletions (indels). Typical indel sequences and the number of inserted (+3 and +1) or deleted (–2, –4 and –10) bases are shown. **b** | When two DSBs are generated in *cis* on a single chromosome by programmable nucleases, the flanking region can be deleted or inverted. **c** | When two DSBs are generated on two different chromosomes, chromosomal translocations can be induced.

© 2014 Macmillan Publishers Limited. All rights reserved

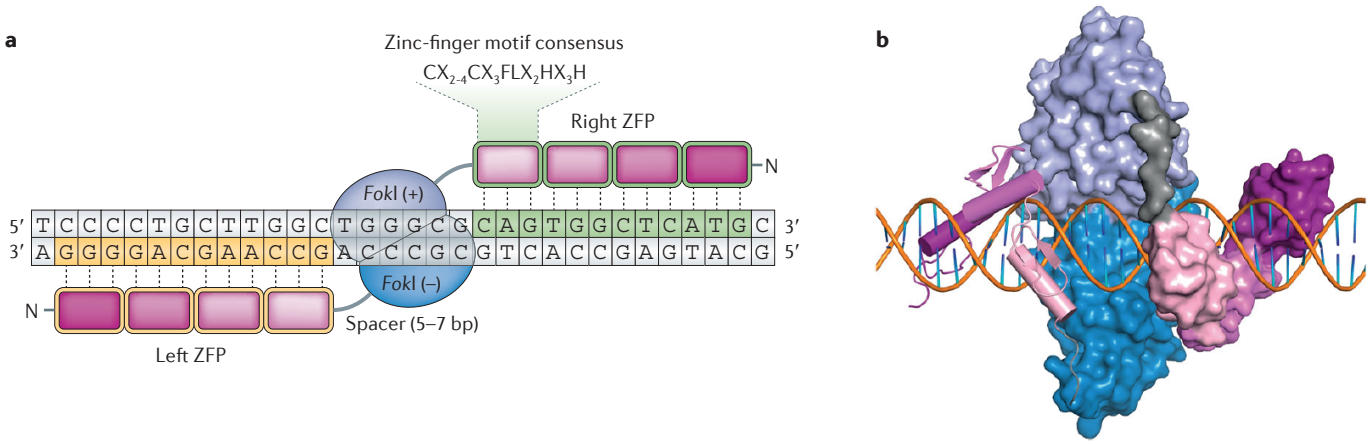


Figure 2 | **Structure of ZFNs.** **a** | A schematic representation of a zinc-finger nuclease (ZFN) pair is shown. Each ZFN is composed of a zinc-finger protein (ZFP) at the amino terminus and the *Fok*I nuclease domain at the carboxyl terminus. In the zinc-finger motif consensus, X represents any amino acid. Target sequences of ZFN pairs are typically 18–36 bp in length, excluding spacers. **b** | A computer model structure of a ZFN pair bound to DNA is shown. Each zinc-finger is shown in shades of pink in ribbon (left) and space-filling (right) representations. The grey region represents the linker between the DNA-binding and catalytic domains. The *Fok*I catalytic domains are shown in blue and purple at the centre using space-filling representations. Part **b** is modified, with permission, from REF. 191 © (2011) Genetics Society of America.

Transcription activator-like effector nucleases (TALENs). Programmable nucleases composed of the *Fok*I catalytic domain and TALE proteins.

RNA-guided engineered nucleases (RGENs). Programmable nucleases composed of the Cas9 protein and a guide RNA.

Clustered regularly interspaced short palindromic repeat (CRISPR). A genomic locus in bacteria or archaea where protospacers and direct repeat sequences are arrayed in tandem. It is associated with adaptive immunity against invading phages and plasmids.

Homology-directed repair (HDR). Template-dependent repair of DNA strand breaks using homologous DNA sequences such as double-strand donor DNA or single-strand oligonucleotides. HDR of programmable nuclease-induced strand breaks leads to precise genome editing, including targeted gene insertion, correction and point mutagenesis.

Insertions and deletions (Indels). Small insertions or deletions of DNA sequences relative to a reference sequence.

*Fok*I
A type IIS restriction enzyme found in *Flavobacterium okeanokoites* that is composed of a separable DNA-binding domain and a nuclease domain that is used to construct ZFNs and TALENs.

organisms, which raises the possibility of correcting genetic defects caused by large chromosomal rearrangements in somatic cells or in stem cells[31]. If DSBs are induced on two different chromosomes, chromosomal translocations can be generated[35,36], which reproduces the aetiology of many cancer-associated mutations. The possibility of unwanted chromosomal rearrangements that arise from off-target DNA cleavage should be considered when programmable nucleases are used for gene or cell therapy.

Zinc-finger nucleases

ZFNs have been used to modify endogenous genes in various organisms, including viruses, bacteria, nematodes, frogs, plants, insects, fish and mammals such as mice, rats and pigs, as well as in cultured mammalian and avian cells (reviewed in REFS 37–40).

Composition. A ZFN has a modular structure that is composed of two domains: a DNA-binding zinc-finger protein (ZFP) domain and the nuclease domain derived from the *Fok*I restriction enzyme[41] (FIG. 2). The structurally separated DNA-binding domain of *Fok*I can be replaced with ZFPs to create ZFNs, and the *Fok*I nuclease domain must dimerize to cleave DNA[42]. Thus, two ZFN monomers are required to form an active nuclease; each monomer must bind to adjacent half-sites that are separated by spacers of 5–7 bp (FIG. 2a). This requirement for dimerization doubles the length of recognition sites, which substantially increases the specificity of ZFNs. However, the wild-type *Fok*I domain can still form homodimers to cleave DNA when one monomer binds to DNA, which often leads to unwanted off-target effects[43,44]. The *Fok*I dimeric interface was artificially modified to generate obligate heterodimeric forms[43,44], which substantially reduced off-target effects and ZFN cytoxicity. *Fok*I domains with enhanced activity were also created by directed evolution[45,46].

The sequence specificity of ZFNs is determined by ZFPs, which consist of tandem arrays of C2H2 zinc-fingers — the most common DNA-binding motif in higher eukaryotes[47]. Each zinc-finger recognizes a 3-bp DNA sequence[48], and 3–6 zinc-fingers are used to generate a single ZFN subunit that binds to DNA sequences of 9–18 bp (FIG. 2a). Importantly, the DNA-binding specificities of zinc-fingers can be altered by mutagenesis[49,50], which is a key feature of constructing a programmable nuclease.

Availability. The co-crystal structure of a ZFP bound to DNA showed that zinc-finger–DNA interactions are modular in nature; each zinc-finger interacts almost independently with a 3-bp DNA sequence[51]. Indeed, new ZFPs with desired specificities can be constructed by modular assembly of pre-characterized zinc-fingers[4,12,52–54]. However, ZFNs created using this fast and convenient method often either lack DNA targeting activity[55] or are cytotoxic owing to off-target effects[56]. Cell-based selection methods and modular assembly methods that account for context dependence between neighbouring zinc-fingers have been developed to yield functional ZFNs[57–60]. Nonetheless, it remains challenging to construct ZFNs with high activity and low cytotoxicity using publicly available resources. Both do-it-yourself kits and custom-made high-quality ZFNs using a proprietary archive of zinc-fingers are available to researchers (TABLE 1).

Targetable sites. Compared with other programmable nucleases, ZFNs are limited by poor targeting density. Although each zinc-finger recognizes a 3-bp DNA sequence, there is no open-source collection of 64 zinc-fingers that covers all possible combinations of triplet sites[53,61]. Furthermore, not all newly assembled ZFNs, especially those with three zinc-fingers, can cleave chromosomal DNA efficiently; successful target sites

© 2014 Macmillan Publishers Limited. All rights reserved

Table 1 | **Resources for programmable nucleases**

	Online resources for nuclease design	Suppliers	
		Non-profit organizations	Companies
ZFNs	• Genome-wide tag scanner for nuclease off-sites • The Segal Laboratory software site • ZFN target site algorithm for identifying sites compatible with the Lawson–Wolfe modular assembly system • Zinc-finger tools • ZiFiT Targeter software	• Addgene	• Sigma-Aldrich • ToolGen
TALENs	• E-TALEN • Genome engineering resources • Scoring algorithm for predicting TALE(N) activity • ToolGen TALEN Designer • ZiFiT Targeter software	• Addgene • TALEN library resource	• Cellectis Bioresearch • Life Technologies • ToolGen • Transposagen Biopharmaceuticals
RGENs	• E-CRISP • Genome engineering resources • RGEN tools • ZiFiT Targeter software	• Addgene	• Life Technologies • Sigma-Aldrich • System Biosciences • ToolGen • Transposagen Biopharmaceuticals

RGEN, RNA-guided engineered nuclease; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease.

are often rich in guanines and consist 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[62]. Various computer programs are available that search for possible ZFN target sites in a given DNA sequence (TABLE 1).

Transcription activator-like effector nucleases

TALENs have been used to modify endogenous genes in various species, including viruses[63], yeast[64,65], plants, nematodes, insects, frogs, fish and mammals such as mice[27,66], rats[67] and pigs, as well as in cultured mammalian cells (reviewed in REFS 37,38,68,69).

Composition. The general structural organization of TALENs is similar to that of ZFNs[70] (FIG. 3). Like ZFNs, TALENs contain the *Fok*I nuclease domain at their carboxyl termini. However, they use a different class of DNA-binding domains known as transcription activator-like effectors (TALEs), which are derived from the plant pathogenic *Xanthomonas* spp. bacterium. TALE-like proteins from *Ralstonia* spp. (which is another phytopathogenic bacteria) can also be engineered to bind to predetermined DNA sequences[71,72]. TALEs are composed of tandem arrays of 33–35 amino acid repeats, each of which recognizes a single base-pair in the major groove[73,74] (FIG. 3b). The nucleotide specificity of each repeat domain is determined by the two amino acids at positions 12 and 13 (REFS 75,76), which are called repeat variable diresidues (RVDs) (FIG. 3). Four different RVD modules — namely Asn-Asn, Asn-Ile, His-Asp and Asn-Gly — are most widely used to recognize guanine, adenine, cytosine and thymine, respectively.

Availability. The one-to-one correspondence between the four RVD modules and the four bases makes it easy to design new TALENs with desired sequence specificities. Web-based computer programs such as E-TALEN[77] are available to help the design of TALENs (TABLE 1). However, the construction of DNA segments that encode TALE arrays can be challenging and time-consuming both because TALENs often consist of up to 20 RVDs and because these highly homologous sequences can recombine with each other in cells[78]. Several methods have been developed for the assembly of custom-designed TALE arrays[79–82]. 'Golden Gate cloning systems' use a type IIS restriction enzyme that cuts outside its recognition sequence, which generates different four-base overhangs at individual DNA segments that encode RVD modules. DNA segments with compatible overhangs are ligated to assemble multiple RVD modules in an ordered array[15,83]. We have developed an improved, one-step Golden Gate cloning method to construct libraries of TALENs that target 18,740 protein-coding genes[15] and 274 miRNA-coding sequences[84] in the human genome. Solid-phase assembly[80,82,85] and ligation-independent cloning[81] methods have also been developed to generate TALENs in a high-throughput manner. Custom-designed TALENs or genome-modified cell lines that are created using TALENs are available from several companies and non-profit academic facilities (TABLE 1).

Targetable sites. TALENs can be designed to target almost any given DNA sequence, which is a crucial advantage of TALENs over other types of nucleases. For example, small DNA sequences (such as enhancers or miRNA-coding sequences) may lack targetable sites for ZFNs or RGENs but can be mutated preferentially using TALENs. The only limitation in the design of TALENs seems to be the requirement for a thymine at the 5′ end of the target sequence, which is recognized by two amino-terminal cryptic repeat folds[73]. Although there have been conflicting reports that emphasize[86] or refute[82,87] the importance of this 5′ thymine, choosing a target sequence with a thymine at the 5′ end is usually recommended. Recently developed TALE variants

Transcription activator-like effectors
(TALEs). DNA-binding proteins with a modular structure derived from *Xanthomonas* spp. (a plant pathogen). Each module is composed of ~34 amino acids and recognizes a single nucleotide. The base specificity is determined by the amino acids at positions 12 and 13 (known as repeat variable diresidues) in each module.

© 2014 Macmillan Publishers Limited. All rights reserved

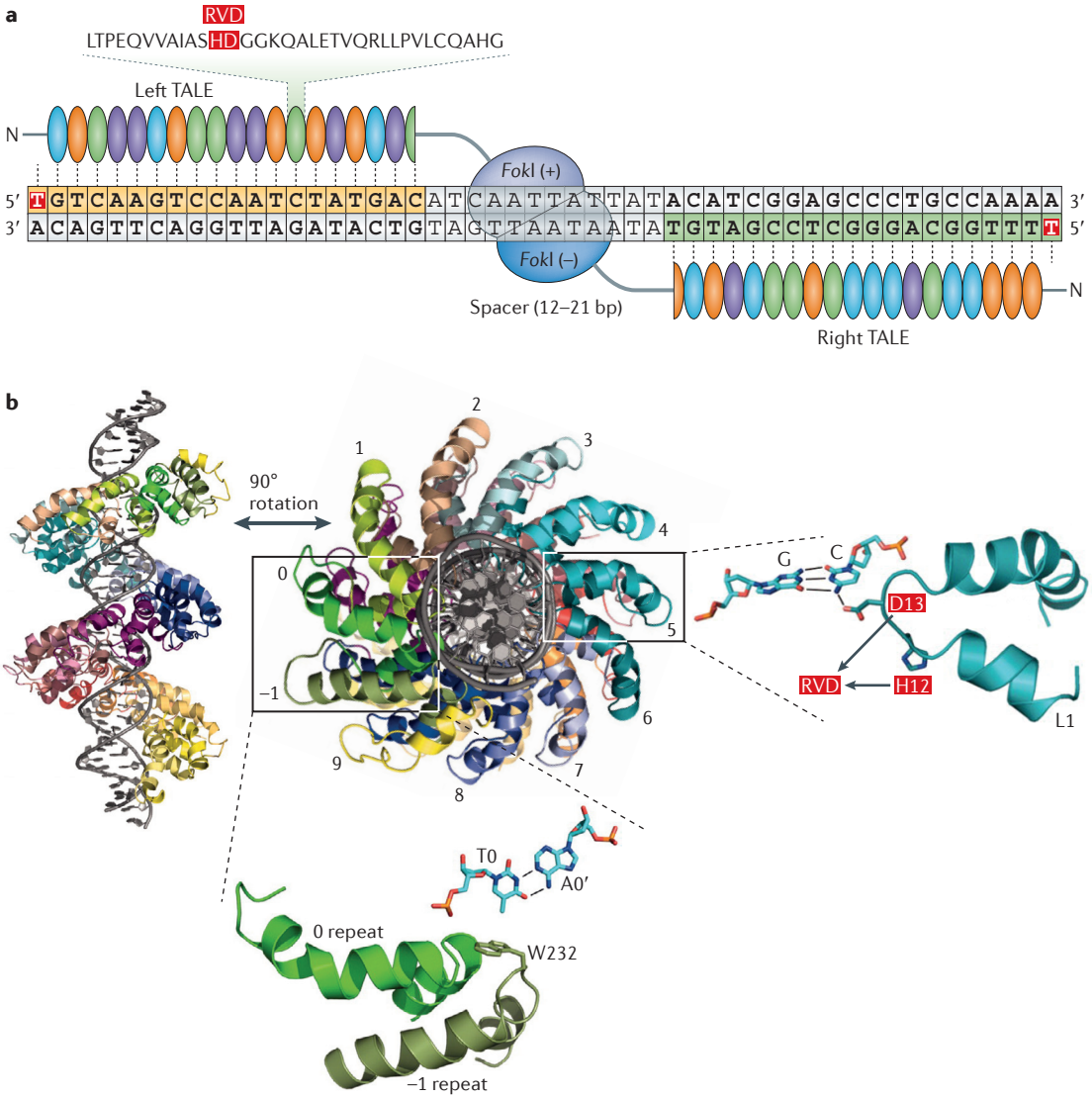


Figure 3 | **Structure of TALENs. a** | A schematic representation of a transcription activator-like effector nuclease (TALEN) pair is shown. Each TALEN is composed of transcription activator-like effectors (TALEs) at the amino terminus and the *FokI* nuclease domain at the carboxyl terminus. Each TALE repeat is comprised of 33–35 amino acids and recognizes a single base pair through the amino acids at positions 12 and 13, which is called the repeat variable diresidue (RVD; shown in red). Target sequences of TALEN pairs are typically 30–40 bp in length, excluding spacers. **b** | In the TALE–DNA co-crystal structure, the RVDs in TALE interact with DNA in the major groove. The amino-terminal repeats (designated as 0 and –1 in the box) contact 5′ thymine. Part **b** is modified, with permission, from REF. 73 © (2012) American Association for the Advancement of Science.

that recognize other bases at the 5′ end would further broaden the range of targetable sites[72,86]. Conventional TALENs cannot cleave target DNA that contains methylated cytosines[15,88]. However, a methylated cytosine is indistinguishable from a thymine in the major groove; hence, the His-Asp RVD repeat (which recognizes cytosines) can be replaced with an Asn-Gly RVD repeat (which recognizes thymines) to generate TALENs that can cleave methylated DNA[89,90].

RNA-guided engineered nucleases

Initially, these RNA-guided systems were used to induce targeted mutagenesis in bacteria, zebrafish embryos and mammalian cells[5–10]. Since then, successful genome editing using RGENs has been expanded to various systems, including plants[91–93], nematodes[94–96], fruitflies[97], mice[28,98–100], rats[98,101], non-human primates[102] and human pluripotent stem cells[9,103].

Composition. In bacteria and archaea, RNA-guided DNA cleavage systems provide adaptive immunity against invading phages or plasmids[104,105]. These organisms often capture small DNA fragments (~20 bp) from the foreign DNA of invading phages or plasmids and insert these sequences (termed protospacers) into their own genome to form a CRISPR. In type II

Protospacers
DNA sequences of 26–72 bp that are initially derived from invading phages and plasmids and that are embedded in the CRISPR loci in bacteria or archaea.

© 2014 Macmillan Publishers Limited. All rights reserved

CRISPR systems, these CRISPR regions are transcribed as pre-CRISPR RNA (pre-crRNA) and processed to give rise to target-specific crRNA. Invariable target-independent *trans*-activating crRNA (tracrRNA) is also transcribed from the locus and contributes to the processing of pre-crRNA[106]. Both crRNA and tracrRNA are complexed with CRISPR-associated protein 9 (Cas9) to form an active DNA endonuclease, which is often termed dualRNA–Cas9 (FIG. 4). RNA loading induces conformational rearrangements in Cas9 to form a central channel that may accommodate target DNA[107]. The resulting endonuclease cleaves a 23-bp target DNA sequence that is composed of the 20-bp guide sequence in the crRNA (that is, the protospacer) and the 5′-NGG-3′ (or, to a lesser extent, 5′-NAG-3′ (REFS 8,108,109)) sequence known as protospacer adjacent motif (PAM), which is recognized by Cas9 itself[110] (FIG. 4a,b). Interestingly, the dualRNA–Cas9 complex remains tightly bound to cleaved DNA and fails to carry out multiple cleavage[111]. Cas9 proteins derived from species other than *Streptococcus pyogenes* recognize different PAM sequences[6,110,112–114]. crRNA and tracrRNA can be linked to form a single-chain guide RNA (sgRNA)[115], which simplifies the components of RGENs (FIG. 4b).

Availability. A crucial advantage of RGENs over ZFNs and TALENs is their simple design and preparation. New RGEN plasmids are easily prepared by cloning 20-bp guide DNA sequences in a vector that encodes either crRNA or sgRNA[6,103]. Complicated protein engineering is not necessary for making new RGENs because Cas9 remains the same. Furthermore, new RGENs can be made without cloning: crRNA or sgRNA can be prepared by annealing two complementary oligonucleotides followed by *in vitro* transcription[5]. Various RGEN suppliers and resources are listed in TABLE 1.

Targetable sites. RGEN target sites are limited by the requirement for the PAM sequence, which is recognized by Cas9. Thus, the targetable sequences are 5′-X_{20}NGG-3′ (or 5′-X_{20}NAG-3′; where X_{20} corresponds to the 20-bp crRNA sequence). The targetable sites are often further limited by the requirement for a guanine at the 5′ end because guide RNAs are transcribed by RNA polymerase III under the control of the U6 promoter in cells; therefore, RNA transcripts with bases other than guanine at the 5′ end are poorly transcribed. This limitation can be circumvented by making guide RNAs with one or two additional guanine bases at their 5′ ends[5,36]. Considering both Watson and Crick strands, these sequences occur once per 8 bp (or 4 bp if the 5′-NAG-3′ PAM is included) on average. Unlike ZFNs and TALENs, RGENs can cleave methylated DNA[108], but not all sequences that contain the PAM sequence are cleaved efficiently by RGENs in cells[116]. Understanding the molecular basis for this occasional failure is currently an active area of research.

Choosing a programmable nuclease

Each nuclease has its own pros and cons, which are discussed below and summarized in TABLE 2.

Success rate and activity. Not all newly synthesized nucleases are functional and equally efficient. Many ZFNs, especially those created by modular assembly, fail to cleave chromosomal DNA in cultured cells or in whole organisms. Despite continuous improvements in ZFN technology by academic researchers[54,58–60], commercially available ZFNs generally work better than ZFNs created in the laboratory using do-it-yourself kits[54,55]. By contrast, TALENs have a nearly 100% success rate in mammalian cells if one avoids heavily methylated target sites[15]. Nonetheless, mutation frequencies obtained with functional TALENs range from 1% to ~60% in mammalian cells[15,80]. Similarly, RGENs have a broad range of genome editing activities (2.3–79%) in cultured mammalian cells[5,6,9,10,103]. The success rate and activity of the three classes of nucleases depend heavily on the cell type and delivery method. So far, there are no reliable rules to predict nuclease activity before experimental validation. Such rules, if discovered, would greatly facilitate genome editing.

Specificity. As discussed above, the specificity of ZFNs and TALENs can be modulated by changing the number of zinc-fingers and TALE modules, respectively. ZFNs and TALENs that contain more modules are generally believed to recognize longer DNA sequences and thus be more specific than those containing fewer modules. However, it is possible that nucleases with too many modules target many additional sites owing to partial interactions that involve only a portion of the modules. These *Fok*I domain-containing nucleases function in pairs, which supports high specificity. In theory, nucleases that recognize DNA sequences of at least 16 bp would have no off-target effects in the human genome or in other higher eukaryotic genomes 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). In reality, however, all three nucleases can cause off-target mutations[108,117–120]. Furthermore, many laboratory-made ZFNs are cytotoxic, which is probably caused by too many off-target DNA cleavage events in cells[56]. TALENs and RGENs in general are not cytotoxic, and they facilitate isolation of single-cell-derived clones[5,84] or animals with nuclease-induced mutations.

Initial excitement over RGENs has been tempered by several studies that report substantial off-target effects in human cells[108,109,120–122]. 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 there are thousands of potential off-target sites per RGEN in the human genome. Furthermore, off-target DNA cleavage induced by RGENs can cause chromosomal translocations[36]. RGENs tolerate mismatches, especially in the 5′ region upstream of the 10–11-bp seed region that is located next to the PAM sequence. Off-target DNA cleavage may be required by the bacterial and archaeal immune system to recognize and remove hypervariable DNA from foreign invaders. Furthermore, unlike ZFNs and TALENs, RGENs act as monomers — a factor that is not conducive to high specificity.

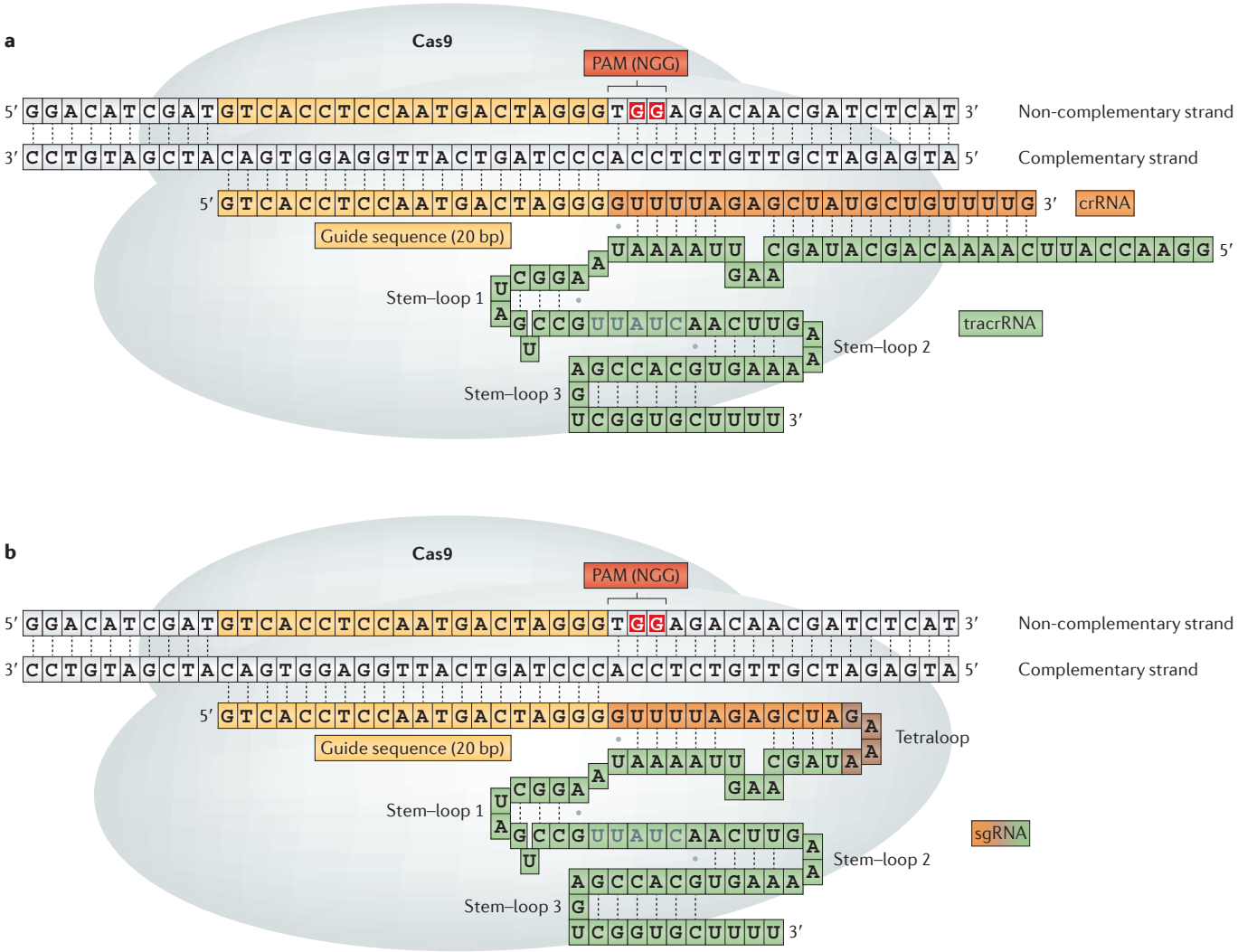
CRISPR RNA
(crRNA). A small RNA transcribed from the CRISPR loci that determines the target-sequence specificity of Cas9 RNA-guided endonulceases.

CRISPR-associated protein 9
(Cas9). A protein derived from bacteria such as *Streptococcus pyogenes*. The Cas9 protein forms an active DNA endonuclease when complexed with guide RNAs.

Protospacer adjacent motif
(PAM). A short (2–5-bp) nucleotide motif adjacent to protospacers that is recognized by Cas9.

Single-chain guide RNA
(sgRNA). A small, single-chain guide RNA that is created by the fusion of CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA).

© 2014 Macmillan Publishers Limited. All rights reserved



© 2014 Macmillan Publishers Limited. All rights reserved

◀ Figure 4 | **Structure of RGENs.** Schematic representations of RNA-guided engineered nucleases (RGENs) are shown. **a** | An RGEN is comprised of CRISPR (clustered regularly interspaced short palindromic repeat)-associated protein 9 (Cas9), a CRISPR RNA (crRNA) and a *trans*-activating crRNA (tracrRNA), which form the dualRNA–Cas9. **b** | Alternatively, an RGEN can contain Cas9 and a single-chain guide RNA (sgRNA). The guide sequence in the crRNA (part **a**) or sgRNA (part **b**) is complementary to a 20-bp target DNA sequence known as protospacer, which is next to the 5′-NGG-3′ (where N represents any nucleotide) sequence known as protospacer adjacent motif (PAM). Grey dots indicate weak bonding. **c** | Target DNA cleaved by an RGEN yielding blunt ends is shown. **d** | A three-dimensional model of Cas9 complexed with DNA is shown. Part **d** courtesy of D. W. Taylor (University of California, Berkeley, USA), J. A. Doudna (University of California, Berkeley, USA) and M. Jinek (University of Zurich, Switzerland).

Multiplex genome editing. Engineered nucleases enable modification of two or more target genes simultaneously in cells or whole organisms. Although ZFNs and TALENs can be used for multiplex gene editing as shown by targeted chromosomal rearrangements using two ZFN pairs or two TALEN pairs, mismatched dimers could form when two or more pairs are introduced into a cell[123], which aggravates off-target effects. RGENs are free of this limitation because they function as monomers. Indeed, two or three genes were disrupted simultaneously using RGENs in mice[28], rats[101] or monkeys[102], which enabled the rapid generation of multigene knockout animals.

Mutation signature. Although ZFNs and TALENs share the same *Fok*I nuclease domain, their mutation patterns are surprisingly different. A comprehensive survey of indels induced through error-prone NHEJ in cells and whole organisms showed that, unlike TALENs that produce deletions much more frequently than insertions (89% versus 1.6% in mammalian cells), ZFNs induce insertions and deletions at comparable frequencies[124]. ZFNs produce defined four- or five-nucleotide 5′ overhangs that are filled in before NHEJ, which frequently yields small insertions. Interestingly, one- or two-nucleotide insertions are often obtained at RGEN target sites[5]. In line with this observation, RGENs occasionally produce one- or two-nucleotide overhangs when they cleave DNA *in vitro*, in addition to the more prevalent blunt ends[115,125] (FIG. 4c). Defined overhangs are often useful for targeted insertions of plasmid DNA at specific sites through NHEJ[22].

Delivery of programmable nucleases. For efficient genome editing, programmable nucleases and/or homologous templates, such as targeting vectors or ssODNs, should be successfully delivered into target cells. Programmable nucleases are delivered into cultured cells, embryos or whole organisms in various forms, including plasmid DNA, *in vitro* transcribed mRNA, viral vectors or purified proteins.

Transient transfection of plasmid DNA through electroporation or liposome transfection is used widely for the delivery of genes that encode programmable nucleases into various cell lines[13,24]. *In vitro* transcribed mRNAs that encode ZFNs, TALENs or Cas9 and guide RNAs are microinjected into one-cell embryos to create knockout or knock-in animals[7,102,126]. Compared with plasmid DNA transfection, mRNA delivery of programmable nucleases can lead to faster expression and avoid unwanted integration of plasmid DNA that encodes the nucleases.

Non-integrating viral vectors — such as integrase-deficient lentivirus vectors (IDLVs), adenoviruses and adeno-associated viruses (AAVs) — can deliver genes encoding programmable nucleases both *in vitro* and *in vivo*. ZFNs are the most compact member of the nuclease triad, and sequences encoding ZFNs can be packaged into small vectors such as AAV[19], which is widely used for gene therapy. IDLVs have successfully delivered ZFNs and homologous donor DNA into cells that are difficult to transfect (for example, human haematopoietic stem cells and embryonic stem cells)[127]. Unfortunately, IDLVs can be incompatible with TALENs because highly homologous TALE repeats often lead to unwanted recombination in cells[78]. Owing to the large cargo size that can be accommodated in adenoviruses, they are an attractive platform for the delivery of programmable nucleases and donor DNA[78,128]. Integrating vectors such as lentivirus have been used for continuous expression of Cas9 and sgRNAs in mammalian cells, and have resulted in almost 100% mutation rates at target sites[129,130]. However, this method is likely to aggravate off-target effects.

Recombinant ZFN proteins have been delivered into mouse and human cells, including primary cells that are difficult to transfect[131,132]. Recombinant Cas9 protein complexed with guide RNA was injected into embryos to induce site-specific mutations in *Caenorhabditis elegans*, zebrafish and mice[96,99]. Unlike DNA-based expression, protein delivery avoids unwanted incorporation of foreign DNA that encodes the nuclease into the host genome and can reduce off-target effects because, unlike plasmid DNA, proteins are rapidly degraded in cells, thus limiting the working time of nucleases[131,133]. Furthermore, the use of nuclease proteins obviates the concern over codon optimization and choice of appropriate promoters when applied to new systems. In this respect, RGENs are more convenient than ZFNs and TALENs because *de novo* purification of Cas9 is not required to construct a new nuclease. Furthermore, the recombinant Cas9 protein can be used in restriction fragment length polymorphism (RFLP) analyses to facilitate genotyping of both nuclease-induced indels and naturally occurring mutations[134].

Improving programmable nucleases

Minimizing off-target effects. Several methods exist to minimize or avoid off-target effects of RGENs and other nucleases. First, it is important to choose unique target sites that lack highly homologous sequences elsewhere in the genome. Whole-exome and whole-genome sequencing analyses showed that ZFNs, TALENs and RGENs that target unique genomic sites do not induce substantial off-target mutations in cells[36,135,136]. A computer algorithm that searches for such sites has been reported for TALENs[15], and web-based programs are available for TALENs[77] and RGENs[108,137,138] (TABLE 1). Second, off-target

© 2014 Macmillan Publishers Limited. All rights reserved

Table 2 | **Comparison of three classes of programmable nucleases***

	ZFNs	TALENs	RGENs
DNA targeting specificity determinant	Zinc-finger proteins	Transcription activator-like effectors	crRNA or sgRNA
Nuclease	*Fok*I	*Fok*I	Cas9
Success rate‡	Low (~24%)	High (>99%)	High (~90%)
Average mutation rate§	Low or variable (~10%)	High (~20%)	High (~20%)
Specificity-determining length of target site	18–36 bp	30–40 bp	22 bp (total length 23 bp)
Restriction in target site	G-rich	Start with T and end with A (owing to the heterodimer structure)	End with an NGG or NAG (lower activity) sequence (that is, PAM)
Design density	One per ~100 bp	At least one per base pair	One per 8 bp (NGG PAM) or 4 bp (NGG and NAG PAM)
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)

Cas9, CRISPR (clustered regularly interspaced short palindromic repeat)-associated protein 9; crRNA, CRISPR RNA; N, any nucleotide; PAM, protospacer adjacent motif; RGEN, RNA-guided engineered nuclease; sgRNA, single-chain guide RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease. *A wide range of success rates and mutation rates (which depend on factors such as the methods used to construct these nucleases, delivery methods and cell lines or organisms) have been reported. The numbers given here are based on our own studies using HEK293 cells[5,15,54,62,124,192]. Mutation frequencies are higher in K562 cells and HeLa cells than in HEK293 cells. ‡The success rate is defined as the proportion of nucleases that induce mutations at frequencies >0.5% in HEK293 cells. §The average mutation rate is based on the frequency of non-homologous end-joining-mediated insertions and deletions obtained at the nuclease target site.

effects of RGENs can be reduced by optimizing nuclease expression levels[108]. Third, the use of either modified guide RNAs with two additional guanines at the 5′ terminus or truncated sgRNAs reduces off-target mutations by orders of magnitude without forgoing mutation efficiencies at target sites[36,139]. Fourth, as mentioned above, the use of recombinant proteins, rather than plasmids that encode them, can further reduce the frequency of off-target mutations because of their rapid degradation in cells[131]. Fifth, nickases (also known as DNA-nicking enzymes) that induce single-strand breaks (SSBs; that is, 'nicks') can minimize off-target mutations (see below).

Programmable nickases. The repair of DSBs that are induced by programmable nucleases through NHEJ inevitably gives rise to uncontrolled and unwanted indels at both target sites and off-target sites even in the presence of homologous donor DNA. To avoid these undesirable mutations, nickases that produce SSBs rather than DSBs have been developed. SSBs can stimulate HDR without activating the error-prone NHEJ pathway and can essentially prevent the formation of unwanted indels[140,141].

The first programmable nickases, which were derived from ZFNs by introducing a mutation at the active site of the *Fok*I domain in one subunit, were tested in cultured human cells[142–144]. This mutant monomer itself is catalytically inactive but can still activate the other wild-type subunit when they form a heterodimer, which generates a SSB at the target site. As expected, these nickases (termed ZFNickases) do not cause unwanted DSBs or mutations: a genome-wide DNA damage test using antibodies specific to the phosphorylated histone H2A.X and tumour protein p53-binding protein 1 — both of which are markers of DNA damage (including DSBs) — showed that DSBs are not induced by the nickases[143]. Deep sequencing revealed that ZFNickases do not generate indels at target sites or off-target sites[142]. These nickases allow precise genome editing in the presence of targeting vectors through HDR, albeit at lower efficiency than the corresponding ZFNs[142–144]. Other programmable nickases have been reported: TALE–MutH has been generated by conjugating a TALE array with MutH (which is a naturally occurring monomeric site-specific nickase) and tested *in vitro*[145]. RNA-guided engineered nickases (RGENickases) that are composed of a mutant (Asp10Ala) form of Cas9 (REF. 146) also led to high-fidelity HDR with negligible NHEJ-driven mutations[6,9]. Paired ZFNickases or RGENickases that produce two SSBs on different DNA strands, which generates a composite DSB, can lead to precise genome modification without forgoing editing efficiencies compared with their corresponding nucleases in human and mouse cells[23,36,109,142].

Enhancement of nuclease activity. The efficiency of nuclease-driven mutagenesis can be enhanced by various methods. First, high levels of nuclease expression in cultured cells increase mutation efficiencies, although this may aggravate off-target effects. For example, a simple and useful way to enhance the activity of ZFNs and TALENs is to culture mammalian cells at 30 °C rather than at 37 °C, which increases nuclease expression levels[147]. Similarly, a proteasomal inhibitor markedly

Nickases
Enzymes that generate DNA single-strand breaks (that is, 'nicks').

© 2014 Macmillan Publishers Limited. All rights reserved

increases the frequency of ZFN-induced mutations by inhibiting ZFN degradation in human cells[148]. The use of an appropriate promoter for ZFN expression also enhances mutation frequency, probably by optimizing ZFN expression in human cell lines[149]. Second, co-expression of DNA end-processing exonucleases with programmable nucleases raises the efficiency of targeted gene disruption in cells and whole animals[150,151].

Evaluating and enriching nuclease activity. Most primary cells and stem cells are poorly transfected, which hampers the use of engineered nucleases in these cell types. Reporter plasmids allow isolation of cells that are co-transfected with nuclease-encoding plasmids; for example, cells containing TALEN-directed genetic modifications have been enriched by the selection of transfected cells either through flow cytometric sorting using vectors that express fluorescent proteins or through antibiotic selection using antibiotic resistance factors[32,83,152]. Furthermore, nuclease target sequences can be incorporated in reporter plasmids to monitor mutagenic events in cells and to enrich for mutant cells. We have developed various methods to select mutant cells using these surrogate reporters by flow cytometry, magnetic separation or hygromycin selection[153–155]. These reporters express GFP only when frameshifting indels are generated through mutagenic NHEJ at the target sequence that is incorporated in the plasmids. For magnetic separation and hygromycin selection, the reporters encode H-2K^k (a surface antigenic marker protein) and the hygromycin resistance gene, respectively, in addition to GFP. An elaborate fluorescent reporter system, aptly termed 'traffic light', supports flow cytometric analyses of the choice of DSB repair pathways (that is, homologous recombination versus NHEJ)[156]. siRNA or small-molecule screens implemented with the traffic light system can identify factors that regulate DSB repair mechanisms. Of note, most of these auxiliary methods for improving programmable nucleases are compatible with all three types of nucleases, although some of them have not yet been tested with TALENs and RGENs.

Conclusions and prospects

Technologies for 'reading', 'writing' and editing (that is, sequencing, synthesizing and modifying, respectively) genomes are being developed in parallel and are in stride with each other. Genome editing has come of age and is increasingly affordable and available. ZFNs — the founding member of genome editing tools — have been developed and improved by researchers in academia and industry for almost two decades. The insights gained in the course of these efforts are often applicable to TALENs and RGENs. Potential users can either obtain these nucleases at affordable prices from several vendors or construct their own nucleases with (or even without) standard recombinant DNA technology. We also note that DNA-binding moieties used for making programmable nucleases can be exploited in applications other than genome editing; for example, sequence-specific DNA-binding proteins or ribonucleoproteins can be fused to various effector domains to regulate expression of specific genes[157], modify epigenomes in a targeted manner[158–160] or visualize chromatin dynamics[161,162].

Nonetheless, there is plenty of room for improvement in this technology. First, the activity and specificity of programmable nucleases could be further optimized. For example, biophysical and biochemical studies on RGENs[107,111,163] could pave the way for the design of next-generation genome editing tools. Methods for identifying, in an unbiased manner, off-target mutations induced by nucleases[117,119] are essential for the development of high-precision nucleases. Second, DSB repair pathways could be manipulated either genetically or pharmacologically to drive nuclease-induced mutations in a controlled manner. Methods for enhancing the efficiency of HDR over NHEJ — the dominant DSB repair system in higher eukaryotes — would be of particular interest in many applications. Third, a genome-scale library of nucleases[15,116,129,130] would enable both drug target identification and target validation, and replace siRNA screens, which are limited by false-positive and false-negative results[164]. Fourth, more efficient methods of nuclease expression and delivery, such as protein transduction[131] *ex vivo* or *in vivo*, would further broaden the use of this technology.

Programmable nucleases have the potential to change the genetic landscape of life forms around us, including crops, flowers, fish, poultry, livestock, pets and humans. Breeders have been manipulating the genomes of animals and plants, albeit randomly, for thousands of years. In a way, traditional breeders are like blind watchmakers — a term introduced by Richard Dawkins[165]. With engineered nucleases, molecular breeders can now modify animal and plant genomes in a targeted manner to improve or alter essentially any trait at will. Thus, with programmable nucleases, artificial selection can now be driven by desired genotypes a priori rather than by unpredictable phenotypes a posteriori: watchmakers will no longer be blind. In the future, it may even be possible to use engineered nucleases with improved efficiency and precision in germline gene therapy for the treatment of otherwise incurable and fatal diseases, which opens a new era of human genetic modification.

1. Krueger, U. *et al.* Insights into effective RNAi gained from large-scale siRNA validation screening. *Oligonucleotides* **17**, 237–250 (2007).
2. Jackson, A. L. *et al.* Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotech.* **21**, 635–637 (2003).
3. Rouet, P., Smih, F. & Jasin, M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol. Cell. Biol.* **14**, 8096–8106 (1994).
4. Bibikova, M., Golic, M., Golic, K. G. & Carroll, D. Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics* **161**, 1169–1175 (2002).
This paper describes targeted gene knockout in animals using engineered nucleases.
5. Cho, S. W., Kim, S., Kim, J. M. & Kim, J. S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nature Biotech.* **31**, 230–232 (2013).
6. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).
7. Hwang, W. Y. *et al.* Efficient genome editing in zebrafish using a CRISPR–Cas system. *Nature Biotech.* **31**, 227–229 (2013).
8. Jiang, W., Bikard, D., Cox, D., Zhang, F. & Marraffini, L. A. RNA-guided editing of bacterial genomes using CRISPR–Cas systems. *Nature Biotech.* **31**, 233–239 (2013).

© 2014 Macmillan Publishers Limited. All rights reserved

9. Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826 (2013).
10. Jinek, M. *et al.* RNA-programmed genome editing in human cells. *Elife* **2**, e00471 (2013).
References 5, 6, 9 and 10 show that RGENs can cleave chromosomal DNA and induce site-specific mutations efficiently in human cells.
11. Bibikova, M. *et al.* Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol. Cell. Biol.* **21**, 289–297 (2001).
12. Bibikova, M., Beumer, K., Trautman, J. K. & Carroll, D. Enhancing gene targeting with designed zinc finger nucleases. *Science* **300**, 764 (2003).
13. Porteus, M. H. & Baltimore, D. Chimeric nucleases stimulate gene targeting in human cells. *Science* **300**, 763 (2003).
14. Steentoft, C. *et al.* Mining the O-glycoproteome using zinc-finger nuclease-glycoengineered SimpleCell lines. *Nature Methods* **8**, 977–982 (2011).
15. Kim, Y. *et al.* A library of TAL effector nucleases spanning the human genome. *Nature Biotech.* **31**, 251–258 (2013).
This paper presents a genome-scale library of TALENs that target human protein-coding genes.
16. Kernstock, S. *et al.* Lysine methylation of VCP by a member of a novel human protein methyltransferase family. *Nature Commun.* **3**, 1038 (2012).
17. Hacein-Bey-Abina, S. *et al.* LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415–419 (2003).
18. Lombardo, A. *et al.* Site-specific integration and tailoring of cassette design for sustainable gene transfer. *Nature Methods* **8**, 861–869 (2011).
19. Li, H. *et al. In vivo* genome editing restores haemostasis in a mouse model of haemophilia. *Nature* **475**, 217–221 (2011).
This study shows that ZFNs can be delivered *in vivo* and lead to gene correction in mice.
20. Hockemeyer, D. *et al.* Genetic engineering of human pluripotent cells using TALE nucleases. *Nature Biotech.* **29**, 731–734 (2011).
21. Doyon, J. B. *et al.* Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited mammalian cells. *Nature Cell Biol.* **13**, 331–337 (2011).
22. Maresca, M., Lin, V. G., Guo, N. & Yang, Y. Obligate ligation-gated recombination (ObLiGaRe): custom-designed nuclease-mediated targeted integration through nonhomologous end joining. *Genome Res.* **23**, 539–546 (2013).
23. Ran, F. A. *et al.* Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380–1389 (2013).
24. Urnov, F. D. *et al.* Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**, 646–651 (2005).
This milestone paper in the field of genome editing presents efficient gene correction using ZFNs in human cell lines.
25. Chen, F. *et al.* High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nature Methods* **8**, 753–755 (2011).
This paper describes a convenient method to introduce user-defined variations in a gene of interest using ssODNs.
26. Cui, X. *et al.* Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nature Biotech.* **29**, 64–67 (2011).
27. Wefers, B. *et al.* Direct production of mouse disease models by embryo microinjection of TALENs and oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **110**, 3782–3787 (2013).
28. Wang, H. *et al.* One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910–918 (2013).
29. Soldner, F. *et al.* Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell* **146**, 318–331 (2011).
30. Lee, H. J., Kim, E. & Kim, J. S. Targeted chromosomal deletions in human cells using zinc finger nucleases. *Genome Res.* **20**, 81–89 (2010).
31. Lee, H. J., Kweon, J., Kim, E., Kim, S. & Kim, J. S. Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. *Genome Res.* **22**, 539–548 (2012).
32. Carlson, D. F. *et al.* Efficient TALEN-mediated gene knockout in livestock. *Proc. Natl. Acad. Sci. USA* **109**, 17382–17387 (2012).
33. Xiao, A. *et al.* Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Res.* **41**, e141 (2013).
34. Gupta, A. *et al.* Targeted chromosomal deletions and inversions in zebrafish. *Genome Res.* **23**, 1008–1017 (2013).
35. Brunet, E. *et al.* Chromosomal translocations induced at specified loci in human stem cells. *Proc. Natl. Acad. Sci. USA* **106**, 10620–10625 (2009).
References 30, 31 and 35 show that programmable nucleases enable targeted chromosomal rearrangements in human cell lines to induce deletions, inversions, duplications and translocations.
36. Cho, S. W. *et al.* Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res.* **24**, 132–141 (2014).
37. Gaj, T., Gersbach, C. A. & Barbas, C. F. 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* **31**, 397–405 (2013).
38. Segal, D. J. & Meckler, J. F. Genome engineering at the dawn of the golden age. *Annu. Rev. Genom. Hum. Genet.* **14**, 135–158 (2013).
39. Perez-Pinera, P., Ousterout, D. G. & Gersbach, C. A. Advances in targeted genome editing. *Curr. Opin. Chem. Biol.* **16**, 268–277 (2012).
40. Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. *Nature Rev. Genet.* **11**, 636–646 (2010).
41. Kim, Y. G., Cha, J. & Chandrasegaran, S. Hybrid restriction enzymes: zinc finger fusions to *Fok*I cleavage domain. *Proc. Natl. Acad. Sci. USA* **93**, 1156–1160 (1996).
42. Bitinaite, J., Wah, D. A., Aggarwal, A. K. & Schildkraut, I. *Fok*I dimerization is required for DNA cleavage. *Proc. Natl. Acad. Sci. USA* **95**, 10570–10575 (1998).
43. Miller, J. C. *et al.* An improved zinc-finger nuclease architecture for highly specific genome editing. *Nature Biotech.* **25**, 778–785 (2007).
44. Szczepek, M. *et al.* Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nature Biotech.* **25**, 786–793 (2007).
References 43 and 44 introduce obligatory heterodimeric *Fok*I variants to enhance the specificity of ZFNs and TALENs.
45. Guo, J., Gaj, T. & Barbas, C. F. 3rd. Directed evolution of an enhanced and highly efficient *Fok*I cleavage domain for zinc finger nucleases. *J. Mol. Biol.* **400**, 96–107 (2010).
46. Doyon, Y. *et al.* Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nature Methods* **8**, 74–79 (2011).
47. Tupler, R., Perini, G. & Green, M. R. Expressing the human genome. *Nature* **409**, 832–833 (2001).
48. Wolfe, S. A., Nekludova, L. & Pabo, C. O. DNA recognition by Cys_2His_2 zinc finger proteins. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 183–212 (2000).
49. Desjarlais, J. R. & Berg, J. M. Redesigning the DNA-binding specificity of a zinc finger protein: a data base-guided approach. *Proteins* **12**, 101–104 (1992).
50. Rebar, E. J. & Pabo, C. O. Zinc finger phage: affinity selection of fingers with new DNA-binding specificities. *Science* **263**, 671–673 (1994).
This paper describes a powerful method to generate sequence-specific ZFPs using phage display and paved the way for the construction of ZFNs.
51. Pavletich, N. P. & Pabo, C. O. Zinc finger–DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science* **252**, 809–817 (1991).
52. Segal, D. J. *et al.* Evaluation of a modular strategy for the construction of novel polydactyl zinc finger DNA-binding proteins. *Biochemistry* **42**, 2137–2148 (2003).
53. Bae, K. H. *et al.* Human zinc fingers as building blocks in the construction of artificial transcription factors. *Nature Biotech.* **21**, 275–280 (2003).
54. Kim, J. S., Lee, H. J. & Carroll, D. Genome editing with modularly assembled zinc-finger nucleases. *Nature Methods* **7**, 91 (2010).
55. Ramirez, C. L. *et al.* Unexpected failure rates for modular assembly of engineered zinc fingers. *Nature Methods* **5**, 374–375 (2008).
56. Cornu, T. I. *et al.* DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol. Ther.* **16**, 352–358 (2008).
57. Maeder, M. L. *et al.* Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol. Cell* **31**, 294–301 (2008).
58. Sander, J. D. *et al.* Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nature Methods* **8**, 67–69 (2011).
59. Gupta, A. *et al.* An optimized two-finger archive for ZFN-mediated gene targeting. *Nature Methods* **9**, 588–590 (2012).
60. Bhakta, M. S. *et al.* Highly active zinc-finger nucleases by extended modular assembly. *Genome Res.* **23**, 530–538 (2013).
61. Segal, D. J., Dreier, B., Beerli, R. R. & Barbas, C. F. 3rd. 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 (1999).
62. Kim, H. J., Lee, H. J., Kim, H., Cho, S. W. & Kim, J. S. Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res.* **19**, 1279–1288 (2009).
63. Bloom, K., Ely, A., Mussolino, C., Cathomen, T. & Arbuthnot, P. Inactivation of hepatitis B virus replication in cultured cells and *in vivo* with engineered transcription activator-like effector nucleases. *Mol. Ther.* **21**, 1889–1897 (2013).
64. Li, T. *et al.* TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and *Fok*I DNA-cleavage domain. *Nucleic Acids Res.* **39**, 359–372 (2011).
65. Christian, M. *et al.* Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* **186**, 757–761 (2010).
66. Sung, Y. H. *et al.* Knockout mice created by TALEN-mediated gene targeting. *Nature Biotech.* **31**, 23–24 (2013).
67. Tesson, L. *et al.* Knockout rats generated by embryo microinjection of TALENs. *Nature Biotech.* **29**, 695–696 (2011).
68. Sun, N. & Zhao, H. Transcription activator-like effector nucleases (TALENs): a highly efficient and versatile tool for genome editing. *Biotechnol. Bioeng.* **110**, 1811–1821 (2013).
69. Joung, J. K. & Sander, J. D. TALENs: a widely applicable technology for targeted genome editing. *Nature Rev. Mol. Cell Biol.* **14**, 49–55 (2013).
70. Miller, J. C. *et al.* A TALE nuclease architecture for efficient genome editing. *Nature Biotech.* **29**, 143–148 (2011).
This paper shows the first successful gene knockout in human cells using an improved TALEN architecture.
71. Li, L. *et al.* Characterization and DNA-binding specificities of *Ralstonia* TAL-like effectors. *Mol. Plant* **6**, 1318–1330 (2013).
72. Doyle, E. L. *et al.* TAL effector specificity for base 0 of the DNA target is altered in a complex, effector- and assay-dependent manner by substitutions for the tryptophan in cryptic repeat −1. *PLoS ONE* **8**, e82120 (2013).
73. Mak, A. N., Bradley, P., Cernadas, R. A., Bogdanove, A. J. & Stoddard, B. L. The crystal structure of TAL effector PthXo1 bound to its DNA target. *Science* **335**, 716–719 (2012).
74. Deng, D. *et al.* Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* **335**, 720–723 (2012).
75. Boch, J. *et al.* Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **326**, 1509–1512 (2009).
76. Moscou, M. J. & Bogdanove, A. J. A simple cipher governs DNA recognition by TAL effectors. *Science* **326**, 1501 (2009).
References 75 and 76 present the code of TALE–DNA interactions and paved the way for the construction of TALENs.
77. Heigwer, F. *et al.* E-TALEN: a web tool to design TALENs for genome engineering. *Nucleic Acids Res.* **41**, e190 (2013).
78. Holkers, M. *et al.* Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. *Nucleic Acids Res.* **41**, e63 (2013).
79. Cermak, T. *et al.* Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* **39**, e82 (2011).
80. Reyon, D. *et al.* FLASH assembly of TALENs for high-throughput genome editing. *Nature Biotech.* **30**, 460–465 (2012).

© 2014 Macmillan Publishers Limited. All rights reserved

81. Schmid-Burgk, J. L., Schmidt, T., Kaiser, V., Honing, K. & Hornung, V. A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. *Nature Biotech.* **31**, 76–81 (2013).
82. Briggs, A. W. *et al.* Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. *Nucleic Acids Res.* **40**, e117 (2012).
83. Ding, Q. *et al.* A TALEN genome-editing system for generating human stem cell-based disease models. *Cell Stem Cell* **12**, 238–251 (2013).
84. Kim, Y. K. *et al.* TALEN-based knockout library for human microRNAs. *Nature Struct. Mol. Biol.* **20**, 1458–1464 (2013).
This paper presents a genome-scale library of TALENs that target human miRNA sequences.
85. Wang, Z. *et al.* An integrated chip for the high-throughput synthesis of transcription activator-like effectors. *Angew. Chem. Int. Ed. Engl.* **51**, 8505–8508 (2012).
86. Lamb, B. M., Mercer, A. C. & Barbas, C. F. 3rd. Directed evolution of the TALE N-terminal domain for recognition of all 5′ bases. *Nucleic Acids Res.* **41**, 9779–9785 (2013).
87. Sun, N., Liang, J., Abil, Z. & Zhao, H. Optimized TAL effector nucleases (TALENs) for use in treatment of sickle cell disease. *Mol. Biosyst.* **8**, 1255–1263 (2012).
88. Bultmann, S. *et al.* Targeted transcriptional activation of silent *oct4* pluripotency gene by combining designer TALEs and inhibition of epigenetic modifiers. *Nucleic Acids Res.* **40**, 5368–5377 (2012).
89. Valton, J. *et al.* Overcoming transcription activator-like effector (TALE) DNA binding domain sensitivity to cytosine methylation. *J. Biol. Chem.* **287**, 38427–38432 (2012).
90. Deng, D. *et al.* Recognition of methylated DNA by TAL effectors. *Cell Res.* **22**, 1502–1504 (2012).
91. Li, J. F. *et al.* Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nature Biotech.* **31**, 688–691 (2013).
92. Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D. & Kamoun, S. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nature Biotech.* **31**, 691–693 (2013).
93. Shan, Q. *et al.* Targeted genome modification of crop plants using a CRISPR–Cas system. *Nature Biotech.* **31**, 686–688 (2013).
94. Friedland, A. E. *et al.* Heritable genome editing in *C. elegans* via a CRISPR–Cas9 system. *Nature Methods* **10**, 741–743 (2013).
95. Dickinson, D. J., Ward, J. D., Reiner, D. J. & Goldstein, B. Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nature Methods* **10**, 1028–1034 (2013).
96. Cho, S. W., Lee, J., Carroll, D., Kim, J. S. & Lee, J. Heritable gene knockout in *Caenorhabditis elegans* by direct injection of Cas9–sgRNA ribonucleoproteins. *Genetics* **195**, 1177–1180 (2013).
97. Gratz, S. J. *et al.* Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* **194**, 1029–1035 (2013).
98. Li, D. *et al.* Heritable gene targeting in the mouse and rat using a CRISPR–Cas system. *Nature Biotech.* **31**, 681–683 (2013).
99. Sung, Y. H. *et al.* Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. *Genome Res.* **24**, 125–131 (2014).
100. Shen, B. *et al.* Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res.* **23**, 720–723 (2013).
101. Li, W., Teng, F., Li, T. & Zhou, Q. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR–Cas systems. *Nature Biotech.* **31**, 684–686 (2013).
102. Niu, Y. *et al.* Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* **156**, 836–843 (2014).
103. Ding, Q. *et al.* Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. *Cell Stem Cell* **12**, 393–394 (2013).
104. Makarova, K. S., Grishin, N. V., Shabalina, S. A., Wolf, Y. I. & Koonin, E. V. 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 (2006).
105. Barrangou, R. *et al.* CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709–1712 (2007).
106. Deltcheva, E. *et al.* CRISPR RNA maturation by *trans*-encoded small RNA and host factor RNase III. *Nature* **471**, 602–607 (2011).
107. Jinek, M. *et al.* Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* http://dx.doi.org/10.1126/science.1247997 (2014).
108. Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotech.* **31**, 827–832 (2013).
109. Mali, P. *et al.* CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nature Biotech.* **31**, 833–838 (2013).
References 23, 36 and 109 present paired Cas9 nickases that produce two offset SSBs on opposite DNA strands to enhance the specificity of RNA-guided genome editing, which is a strategy originally implemented using paired ZFNickases (reference 142).
110. Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J. & Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* **155**, 733–740 (2009).
111. Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. & Doudna, J. A. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62–67 (2014).
112. Shah, S. A., Erdmann, S., Mojica, F. J. & Garrett, R. A. Protospacer recognition motifs: mixed identities and functional diversity. *RNA Biol.* **10**, 891–899 (2013).
113. Hou, Z. *et al.* Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. *Proc. Natl. Acad. Sci. USA* **110**, 15644–15649 (2013).
114. Fonfara, I. *et al.* Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR–Cas systems. *Nucleic Acids Res.* **42**, 2577–2590 (2014).
115. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
This paper shows that the Cas9 protein is guided by small RNAs to cleave DNA in a targeted manner *in vitro*, which paved the way for RNA-guided genome editing both in cells and *in vivo*.
116. Koike-Yusa, H., Li, Y., Tan, E.-P., Velasco-Herrera, M. D. C. & Yusa, K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nature Biotech.* **32**, 267–273 (2013).
117. Pattanayak, V., Ramirez, C. L., Joung, J. K. & Liu, D. R. Revealing off-target cleavage specificities of zinc-finger nucleases by *in vitro* selection. *Nature Methods* **8**, 765–770 (2011).
118. Mussolino, C. *et al.* A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res.* **39**, 9283–9293 (2011).
119. Gabriel, R. *et al.* An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nature Biotech.* **29**, 816–823 (2011).
References 117 and 119 demonstrate two different methods to identify off-target sites of programmable nucleases in the human genome.
120. Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR–Cas nucleases in human cells. *Nature Biotech.* **31**, 822–826 (2013).
121. Cradick, T. J., Fine, E. J., Antico, C. J. & Bao, G. CRISPR/Cas9 systems targeting β-globin and *CCR5* genes have substantial off-target activity. *Nucleic Acids Res.* **41**, 9584–9592 (2013).
122. Pattanayak, V. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature Biotech.* **31**, 839–843 (2013).
References 36, 109 and 120–122 show that RGENs induce off-target mutations in human cells.
123. Sollu, C. *et al.* Autonomous zinc-finger nuclease pairs for targeted chromosomal deletion. *Nucleic Acids Res.* **38**, 8269–8276 (2010).
124. Kim, Y., Kweon, J. & Kim, J. S. TALENs and ZFNs are associated with different mutation signatures. *Nature Methods* **10**, 185 (2013).
125. Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. USA* **109**, E2579–E2586 (2012).
126. Meng, X., Noyes, M. B., Zhu, L. J., Lawson, N. D. & Wolfe, S. A. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nature Biotech.* **26**, 695–701 (2008).
127. Lombardo, A. *et al.* Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nature Biotech.* **25**, 1298–1306 (2007).
128. Perez, E. E. *et al.* Establishment of HIV-1 resistance in $CD4^+$ T cells by genome editing using zinc-finger nucleases. *Nature Biotech.* **26**, 808–816 (2008).
This study develops ZFNs that disrupt the chemokine (C-C motif) receptor 5 (*CCR5*) gene in T cells to prevent HIV infection.
129. Shalem, O. *et al.* Genome-scale CRISPR–Cas9 knockout screening in human cells. *Science* **343**, 84–87 (2014).
130. Wang, T., Wei, J. J., Sabatini, D. M. & Lander, E. S. Genetic screens in human cells using the CRISPR–Cas9 system. *Science* **343**, 80–84 (2014).
References 116, 129 and 130 describe genome-scale gene knockout screening in human and murine cell lines.
131. Gaj, T., Guo, J., Kato, Y., Sirk, S. J. & Barbas, C. F. 3rd. Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. *Nature Methods* **9**, 805–807 (2012).
132. Chen, Z. *et al.* Receptor-mediated delivery of engineered nucleases for genome modification. *Nucleic Acids Res.* **41**, e182 (2013).
133. Pruett-Miller, S. M., Reading, D. W., Porter, S. N. & Porteus, M. H. Attenuation of zinc finger nuclease toxicity by small-molecule regulation of protein levels. *PLoS Genet.* **5**, e1000376 (2009).
134. Kim, J. M., Kim, D., Kim, S. & Kim, J. S. Genotyping with CRISPR–Cas-derived RNA-guided endonucleases. *Nature Commun.* **5**, 3157 (2014).
135. Li, T. *et al.* Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. *Nucleic Acids Res.* **39**, 6315–6325 (2011).
136. Yusa, K. *et al.* Targeted gene correction of α1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* **478**, 391–394 (2011).
137. Bae, S., Park, J. & Kim, J. S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* http://dx.doi.org/10.1093/bioinformatics/btu048 (2014).
138. Heigwer, F., Kerr, G. & Boutros, M. E-CRISP: fast CRISPR target site identification. *Nature Methods* **11**, 122–123 (2014).
139. Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M. & Joung, J. K. Improving CRISPR–Cas nuclease specificity using truncated guide RNAs. *Nature Biotech.* **32**, 279–284 (2014).
140. McConnell Smith, A. *et al.* 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 (2009).
141. Davis, L. & Maizels, N. DNA nicks promote efficient and safe targeted gene correction. *PLoS ONE* **6**, e23981 (2011).
142. Kim, E. *et al.* Precision genome engineering with programmable DNA-nicking enzymes. *Genome Res.* **22**, 1327–1333 (2012).
143. Wang, J. *et al.* Targeted gene addition to a predetermined site in the human genome using a ZFN-based nicking enzyme. *Genome Res.* **22**, 1316–1326 (2012).
144. Ramirez, C. L. *et al.* Engineered zinc finger nickases induce homology-directed repair with reduced mutagenic effects. *Nucleic Acids Res.* **40**, 5560–5568 (2012).
145. Gabsalilow, L., Schierling, B., Friedhoff, P., Pingoud, A. & Wende, W. Site- and strand-specific nicking of DNA by fusion proteins derived from MutH and I-SceI or TALE repeats. *Nucleic Acids Res.* **41**, e83 (2013).
146. Sapranauskas, R. *et al.* The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res.* **39**, 9275–9282 (2011).
147. Doyon, Y. *et al.* Transient cold shock enhances zinc-finger nuclease-mediated gene disruption. *Nature Methods* **7**, 459–460 (2010).
148. Ramakrishna, S., Kim, Y. H. & Kim, H. Stability of zinc finger nuclease protein is enhanced by the proteasome inhibitor MG132. *PLoS ONE* **8**, e54282 (2013).
149. Herrmann, F. *et al.* p53 gene repair with zinc finger nucleases optimised by yeast 1-hybrid and validated by Solexa sequencing. *PLoS ONE* **6**, e20913 (2011).

© 2014 Macmillan Publishers Limited. All rights reserved

150. Certo, M. T. *et al.* Coupling endonucleases with DNA end-processing enzymes to drive gene disruption. *Nature Methods* **9**, 973–975 (2012).
151. Mashimo, T. *et al.* Efficient gene targeting by TAL effector nucleases coinjected with exonucleases in zygotes. *Sci. Rep.* **3**, 1253 (2013).
152. Frank, S., Skryabin, B. V. & Greber, B. A modified TALEN-based system for robust generation of knock-out human pluripotent stem cell lines and disease models. *BMC Genomics* **14**, 773 (2013).
153. Kim, H., Um, E., Cho, S. R., Jung, C. & Kim, J. S. Surrogate reporters for enrichment of cells with nuclease-induced mutations. *Nature Methods* **8**, 941–943 (2011).
154. Kim, H. *et al.* Magnetic separation and antibiotics selection enable enrichment of cells with ZFN/TALEN-induced mutations. *PLoS ONE* **8**, e56476 (2013).
155. Ramakrishna, S. *et al.* Surrogate reporter-based enrichment of cells containing RNA-guided Cas9 nuclease-induced mutations. *Nature Commun.* **5**, 3414 (2014).
156. Certo, M. T. *et al.* Tracking genome engineering outcome at individual DNA breakpoints. *Nature Methods* **8**, 671–676 (2011).
157. Beerli, R. R., Dreier, B. & Barbas, C. F. 3rd. Positive and negative regulation of endogenous genes by designed transcription factors. *Proc. Natl. Acad. Sci. USA* **97**, 1495–1500 (2000).
158. Mendenhall, E. M. *et al.* Locus-specific editing of histone modifications at endogenous enhancers. *Nature Biotech.* **31**, 1133–1136 (2013).
159. Konermann, S. *et al.* Optical control of mammalian endogenous transcription and epigenetic states. *Nature* **500**, 472–476 (2013).
160. Maeder, M. L. *et al.* Targeted DNA demethylation and activation of endogenous genes using programmable TALE–TET1 fusion proteins. *Nature Biotech.* **31**, 1137–1142 (2013).
161. Miyanari, Y., Ziegler-Birling, C. & Torres-Padilla, M. E. Live visualization of chromatin dynamics with fluorescent TALEs. *Nature Struct. Mol. Biol.* **20**, 1321–1324 (2013).
162. Chen, B. *et al.* Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* **155**, 1479–1491 (2013).
163. Nishimasu, H. *et al.* Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949 (2014).
164. Sigoillot, F. D. & King, R. W. Vigilance and validation: keys to success in RNAi screening. *ACS Chem. Biol.* **6**, 47–60 (2011).
165. Dawkins, R. *The Blind Watchmaker: Why the Evidence of Evolution Reveals a Universe without Design* (Norton & Company, 1986).
166. Zeevi, V., Tovkach, A. & Tzfira, T. Increasing cloning possibilities using artificial zinc finger nucleases. *Proc. Natl Acad. Sci. USA* **105**, 12785–12790 (2008).
167. Smidler, A. L., Terenzi, O., Soichot, J., Levashina, E. A. & Marois, E. Targeted mutagenesis in the malaria mosquito using TALE nucleases. *PLoS ONE* **8**, e74511 (2013).
168. Hauschild, J. *et al.* Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proc. Natl. Acad. Sci. USA* **108**, 12013–12017 (2011).
169. Hu, R., Wallace, J., Dahlem, T. J., Grunwald, D. J. & O'Connell, R. M. Targeting human microRNA genes using engineered Tal-effector nucleases (TALENs). *PLoS ONE* **8**, e63074 (2013).
170. Gutschner, T., Baas, M. & Diederichs, S. Noncoding RNA gene silencing through genomic integration of RNA destabilizing elements using zinc finger nucleases. *Genome Res.* **21**, 1944–1954 (2011).
171. Zou, J., Mali, P., Huang, X., Dowey, S. N. & Cheng, L. Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. *Blood* **118**, 4599–4608 (2011).
172. Simsek, D. *et al.* DNA ligase III promotes alternative nonhomologous end-joining during chromosomal translocation formation. *PLoS Genet.* **7**, e1002080 (2011).
173. Carbery, I. D. *et al.* Targeted genome modification in mice using zinc-finger nucleases. *Genetics* **186**, 451–459 (2010).
174. Yang, H. *et al.* One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* **154**, 1370–1379 (2013).
175. Geurts, A. M. *et al.* Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* **325**, 433 (2009).
176. Doyon, Y. *et al.* Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nature Biotech.* **26**, 702–708 (2008).
177. Zou, J. *et al.* Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell* **5**, 97–110 (2009).
178. Shukla, V. K. *et al.* Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature* **459**, 437–441 (2009).
179. Townsend, J. A. *et al.* High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature* **459**, 442–445 (2009).
180. Li, T., Liu, B., Spalding, M. H., Weeks, D. P. & Yang, B. High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nature Biotech.* **30**, 390–392 (2012).
181. Yu, S. *et al.* Highly efficient modification of beta-lactoglobulin (*BLG*) gene via zinc-finger nucleases in cattle. *Cell Res.* **21**, 1638–1640 (2011).
182. Santiago, Y. *et al.* Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. *Proc. Natl. Acad. Sci. USA* **105**, 5809–5814 (2008).
183. Holt, N. *et al.* Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to *CCR5* control HIV-1 *in vivo*. *Nature Biotech.* **28**, 839–847 (2010).
184. Maier, D. A. *et al.* Efficient clinical scale gene modification via zinc finger nuclease-targeted disruption of the HIV co-receptor CCR5. *Hum. Gene Ther.* **24**, 245–258 (2013).
185. Osborn, M. J. *et al.* TALEN-based gene correction for epidermolysis bullosa. *Mol. Ther.* **21**, 1151–1159 (2013).
186. Ousterout, D. G. *et al.* Reading frame correction by targeted genome editing restores dystrophin expression in cells from Duchenne muscular dystrophy patients. *Mol. Ther.* **21**, 1718–1726 (2013).
187. Schwank, G. *et al.* Functional repair of *CFTR* by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* **13**, 653–658 (2013).
188. Sebastiano, V. *et al. In situ* genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. *Stem Cells* **29**, 1717–1726 (2011).
189. Zou, J. *et al.* Oxidase-deficient neutrophils from X-linked chronic granulomatous disease iPS cells: functional correction by zinc finger nuclease-mediated safe harbor targeting. *Blood* **117**, 5561–5572 (2011).
190. Jiang, J. *et al.* Translating dosage compensation to trisomy 21. *Nature* **500**, 296–300 (2013).
191. Carroll, D. Genome engineering with zinc-finger nucleases. *Genetics* **188**, 773–782 (2011).
192. Kim, S., Lee, M. J., Kim, H., Kang, M. & Kim, J. S. Preassembled zinc-finger arrays for rapid construction of ZFNs. *Nature Methods* **8**, 7 (2011).

Acknowledgements

This work was supported by the National Research Foundation of Korea (2013-000718 to J.S.K. and 2011-0013568 to H.K.), Health Technology Research and Development Project by the Korean Ministry of Health and Welfare (H10C1740 to H.K.), and Converging Research Center Program funded by the Korean Ministry of Science, ICT and Future Planning (2013K000275 to H.K.). The authors thank three anonymous referees for comments and all the researchers, including our collaborators and competitors, who have contributed to the development of programmable nucleases in the past 20 years and regret the need to omit many relevant papers owing to the page limitation.

Competing interests statement

The authors declare competing interests: see Web version for details.

FURTHER INFORMATION

Addgene: http://www.addgene.org
E-CRISP: http://www.e-crisp.org/E-CRISP/
E-TALEN: http://www.e-talen.org/E-TALEN/
Genome engineering laboratory: http://gel.snu.ac.kr
Genome engineering resources: http://www.genome-engineering.org/
Genome-wide tag scanner for nuclease off-sites: http://ccg.vital-it.ch/tagger/targetsearch.html
RGEN tools: http://www.rgenome.net/
Scoring algorithm for predicting TALE(N) activity: http://baolab.bme.gatech.edu/Research/BioinformaticTools/TAL_targeter.html
TALEN library resource: http://www.talenlibrary.net/
The Segal Laboratory software site: http://zinc.genomecenter.ucdavis.edu:8080/Plone
ToolGenTALEN Designer: http://www.toolgen.co.kr/talen_designer/
ZFN target site algorithm: http://pgfe.umassmed.edu/ZFPmodularsearch.html
ZiFit Targeter Software: http://zifit.partners.org/
Zinc-finger tools: http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

© 2014 Macmillan Publishers Limited. All rights reserved