Cas9, Cpf1 and C2c1/2/3—What’s next?

Shota Nakade, Takashi Yamamoto, and Tetsushi Sakuma

Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Hiroshima, Japan

ABSTRACT
Since the rapid emergence of clustered regulatory interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) system, developed as a genome engineering tool in 2012–2013, most researchers in the life science field have had a fixated interest in this fascinating technology. CRISPR-Cas9 is an RNA-guided DNA endonuclease system, which consists of Cas9 nuclease defining a few targeting base via protospacer adjacent motif complexed with easily customizable single guide RNA targeting around 20-bp genomic sequence. Although Streptococcus pyogenes Cas9 (SpCas9), one of the Cas9 proteins that applications in genome engineering were first demonstrated, still has wide usage because of its high nuclease activity and broad targeting range, there are several limitations such as large molecular weight and potential off-target effect. In this commentary, we describe various improvements and alternatives of CRISPR-Cas systems, including engineered Cas9 variants, Cas9 homologs, and novel Cas proteins other than Cas9. These variations enable flexible genome engineering with high efficiency and specificity, orthogonal genetic control at multiple gene loci, gene knockdown, or fluorescence imaging of transcripts mediated by RNA targeting, and beyond.

KEYWORDS
C2c2; Cpf1; CRISPR-Cas9; genome engineering; Off-target effect; protospacer adjacent motif

Introduction
Natural CRISPR-Cas systems act as adaptive immunity in archaea and bacteria, which disrupt invading exogenous DNA such as plasmids and phages. CRISPR-Cas mechanisms are currently classified into 2 classes (classes 1 and 2) and 6 types (types I to VI) according to their context. The inactivation of exogenous DNA by CRISPR-Cas systems is conducted by 3 steps; adaptation, expression, and interference. At the adaptation stage, short fragments of exogenous DNA are incorporated into the CRISPR array in the bacterial genome, and act as new spacer sequences. During the expression stage, CRISPR array is transcribed into pre-CRISPR RNA (pre-crRNA), and then processed to produce mature crRNAs. Cas endonuclease(s) expressed from neighboring genomic loci form complexes with mature crRNA, which cleave the secondary invaded exogenous DNA at the interference stage. Especially, type-II CRISPR-Cas system, classified in class 2, i.e. CRISPR-Cas9, requires trans-activating crRNA (tracrRNA) for hybridization partner of crRNA to form complexes with Cas9 nucleases.

CRISPR-Cas9 as a genome engineering tool
After the elucidation of the mechanism of CRISPR-Cas9 system, it was quickly applied for genome engineering. To introduce a site-specific DNA double-strand break (DSB), it is only necessary to express Cas9 nuclease and a chimeric single guide RNA (sgRNA), which often be used instead of crRNA and tracrRNA hybrids. The CRISPR-Cas9 system has various advantages over the past engineered nucleases such as zinc-finger nucleases and transcription activator-like effector nucleases. First, the vector construction becomes quite simple. Unlike the past nucleases, Cas9 can be used as a generic nuclease together with a gene-specific sgRNA. The sgRNA can be expressed from the cassette driven by an RNA polymerase III promoter or introduced as RNA molecules prepared by in vitro transcription or chemical synthesis. In addition, co-introduction of multiple sgRNAs and
Cas9 nuclease can easily induce simultaneous DSBs, resulting in multiple mutagenesis and chromosomal editing such as large deletions, inversions, duplications, and translocations. Despite these advantageous properties of CRISPR-Cas9, there are some limitations such as restriction of target sequence, large protein size and unintended off-target mutagenesis. Regarding the targeted DNA binding of CRISPR-Cas components, not only the base-pairing of crRNA and target strand of dsDNA but also the interaction between Cas nuclease and a few bases of target DNA sequence, defined as a protospacer adjacent motif (PAM), are required. There is no obvious restriction in crRNA target sequence, but some relations between target base components and nuclease activity have been reported, which might limit the range of target DNA sequence. More importantly, particular PAM sequence, whose base specificity differs among the derived species, is absolutely required for the binding of Cas nucleases to the target sites. Streptococcus pyogenes Cas9 (SpCas9), the most commonly used Cas nuclease to perform genome engineering, has 5′-NGG-3′ PAM. This limitation is not so strict, but in some cases, this motif is hard to find around the intended genomic region, especially when the context is highly AT-rich. The large protein size of SpCas9, 1,368 amino acids, is also problematic when delivering with a size-limited viral vector such as the adeno-associated virus (AAV) vector. The third problem with CRISPR-Cas9 is off-target mutations, especially when the context differs among the derived species; VQR, EQR and VRER variants. The VQR variant can recognize 5′-NGAN-3′ PAM sequence. More specifically, it has the highest affinity with 5′-NGAG-3′ sequences, followed by the others (NGAG > NGAT = NGAA > NGAC). The EQR and VRER variants have 5′-NGAG-3′ and 5′-NGCG-3′ PAM sequences, whose target range are relatively limited compared with the VQR variant. The functionality of these 3 variants has already been proved by inducing indels in mammalian cells and zebrafish embryos. In addition, these variants have been broadened their applications as represented by the CORRECT method, which can modify the targeted single nucleotide precisely with sophisticatedly designed 2-step genome engineering.

Functional conversion of SpCas9 has also been demonstrated. The SpCas9 nuclease can be converted to nickase (Cas9n) or nuclease-deficient mutant (dCas9). The Cas9n variant has been used in double-nicking strategy. The dCas9 variant has broad applications including FokI-dCas9 and specific purposes other than conventional genome editing. Of these, single-base editing systems without inducing DSBs, mediated by cytidine deaminase combined with Cas9n or dCas9, as known as “Base Editors,” are

**Improvement of Cas9 nucleases**

**Cas9 variants based on SpCas9**

Amino acid substitution of functionally critical domain is one of the most typical methods to alter the protein function. Feng Zhang at Broad Institute of MIT and Harvard and J. Keith Joung at Massachusetts General Hospital independently developed the highly specific SpCas9 variants, named eSpCas9 and SpCas9-HF. These variants contain several mutations to reduce the non-specific interactions between Cas9 protein and target DNA. In other words, these variants have the minimum binding energy required for the introduction of DSBs into the genome by decreasing the excess energy from wild-type SpCas9. In fact, this strategy was previously demonstrated with TALENs, which enhanced the base recognition specificity by replacing the basic amino acids with glutamines in the C-terminal domain of TALE protein. PAM-altered SpCas9 variants have also been created by amino acid substitutions. J. Keith Joung and colleagues developed SpCas9 variants harboring alternative PAM specificities; VQR, EQR and VRER variants. The VQR variant can recognize 5′-NGAN-3′ PAM sequence. More specifically, it has the highest affinity with 5′-NGAG-3′ sequences, followed by the others (NGAG > NGAT = NGAA > NGAC). The EQR and VRER variants have 5′-NGAG-3′ and 5′-NGCG-3′ PAM sequences, whose target range are relatively limited compared with the VQR variant. The functionality of these 3 variants has already been proved by inducing indels in mammalian cells and zebrafish embryos. In addition, these variants have been broadened their applications as represented by the CORRECT method, which can modify the targeted single nucleotide precisely with sophisticatedly designed 2-step genome engineering.

Considering these limitations, improvement or alternative options of CRISPR-Cas systems have been eagerly desired. In this commentary, we summarize the recent advances in improving Cas9 nucleases and in discovering several new CRISPR-Cas nuclease systems, which can possibly overcome the restrictions and limitations described above (Table 1).
recent distinguished achievement. \cite{34,35} Furthermore, unique analyses using Base Editors such as in situ direct evolution of endogenous genes have already been reported, \cite{36,37} promising the future expanded applications.

Another important functional conversion is RNA-targeting Cas9 (RCas9) system, reported by Jennifer Doudna’s group at University of California. \cite{38} RCas9 requires PAM-presenting oligonucleotide (PAMmer), which can hybridize with a target single-strand RNA molecule and act as the PAM motif, resulting in targeted RNA cleavage, isolation of target RNA molecule, and live cell tracking of target RNA. \cite{39}

**Cas9 homologs derived from other bacteria**

Several Cas9 homologs derived from other bacterial species have been identified and used. Each homolog has different PAM specificities and tracrRNA structures. Some of these homologs form complexes orthogonally with their own sgRNAs, which enable to tether variable functions with respective Cas9s targeting different genomic loci. Moreover, Cas9 homologs often have particular features different from SpCas9.

Followings are useful examples of Cas9 homologs. SaCas9, *Staphylococcus aureus* Cas9, has 5’-NNGRRT-3’ PAM (R = G or A). \cite{15,40} Although the target restriction with the PAM sequence of the wild-type SaCas9 is relatively strict, there is already a variant with the relaxed PAM sequence, named KKH SaCas9, developed by the Joung’s group, which recognizes 5’-NNNRRT-3’ PAM. \cite{41} In addition, SaCas9 has a size advantage that enables packaging into the AAV vector, because SaCas9 is 315 amino acids smaller than SpCas9.\cite{15} Virginius Siksnys and colleagues at Vilnius University found another utility Cas9, *Brevibacillus laterosporus* Cas9 (BlatCas9), which recognizes 5’-NNNNCNDD-3’ PAM (D = A, G, or T). \cite{42} His team demonstrated BlatCas9-induced indels in maize. Since BlatCas9’s PAM has one of the broadest targeting ranges among those of various Cas9s reported, further application examples in other organisms are desired. *Francisella novicida* Cas9, FnCas9, consists of 1,629 amino acids, which is the largest Cas9 protein reported ever. \cite{31} Although the PAM sequence of wild-type FnCas9 is the same as that of SpCas9 (5’-NGG-3’), Osamu Nureki and colleagues developed the RHA FnCas9 variant, which has 5’-YG-3’ PAM (Y = T or C). RHA FnCas9 can reportedly introduce indels in mouse zygotes, but it has not shown the cleavage activity in human cells. \cite{43}

**New tools based on other CRISPR-Cas systems**

**CRISPR-Cpf1**

As described earlier, Cas9 is originated from type-II group in class-2 CRISPR systems. The class-2 CRISPR contains a single multifunctional Cas protein, which can bind guidance RNA and target DNA, and cleave the DNA. Among class-2 CRISPR, types II, V and VI systems have been identified. We describe the details about new groups of class-2 CRISPR systems, types V and VI, hereafter.

One kind of endonuclease from the type-V CRISPR-Cas systems, called Cpf1, has been discovered and characterized by Feng Zhang’s team. \cite{44} CRISPR-Cpf1 system can be programmed for genome engineering similar with CRISPR-Cas9.
but it has several unique features. First, Cpf1 cleaves target DNA molecule with a single crRNA alone, not coupled with a tracrRNA. Thus, Cpf1-mediated genome editing with chemically synthesized crRNA can be achieved at lower cost than SpCas9, because the length of crRNA is much shorter than sgRNA for SpCas9 (43 nt vs. ~100 nt). Second, Cpf1 recognizes T-rich PAM sequence at the 5’ side of the protospacer region. Zhang and colleagues have proved that Acidaminococcus sp. BV3L6 Cpf1 (AsCpf1) and Lachnospiraceae bacterium Cpf1 (LbCpf1) can be applied for genome engineering in human cells. These nucleases require 5’-TTTN-3’ (or 5’-TTTV-3’, V = A, C, or G, in some literature) PAM sequences, which are friendly with AT-rich sequences that can hardly be targeted with Cas9s. Third, Cpf1 nucleases produce cohesive ends with 4- or 5-nt overhangs, while SpCas9 produces blunt ends. In this regard, NHEJ-mediated knock-in mediated by the annealing of cohesive ends might be facilitated using Cpf1 proteins. Fourth, Cpf1 protein contains not only the DSB-inducing activity but also an RNase III activity, involving in pre-crRNA processing. This activity can be used for the efficient multiplex genome engineering via tandemly arrayed pre-crRNA-expressing construct, producing multiple mature crRNAs mediated by Cpf1.

Within a half year after the first publication of Cas9 from Zhang’s group, advantageous property of Cpf1 over Cas9 has been reported in terms of targeting specificity. Jin-Soo Kim’s group at Seoul National University and J Keith Joung’s group reported that CRISPR-Cpf1 has worked effectively in human cells and off-target mutagenesis rarely happened. AsCpf1 and LbCpf1 nucleases induced indels with comparable or a little lower efficiencies compared with SpCas9, which is a practically acceptable level of nuclease activity. Regarding the comparison between AsCpf1 and LbCpf1, both Cpf1 nucleases induced indels with the equal efficiency in most cases when the same genomic loci were targeted in U2OS cells. The frequency of off-target mutagenesis with Cpf1 nucleases was also analyzed using the Digenome-seq and GUIDE-seq methods. In the Digenome-seq analysis, following numbers of off-target digestion sites were detected in vitro: 6 ± 3 for LbCpf1 and 12 ± 5 for AsCpf1. These results suggested that Cpf1 nucleases are highly specific in human cells rather than SpCas9, because SpCas9 caused 90 ± 30 digestion events in the previous Digenome-seq analysis. In the GUIDE-seq analysis, Cpf1 nucleases caused undetectable off-target mutagenesis in human cells, which suggests considerably high specificity of Cpf1, consistent with the results of the Digenome-seq analysis.

Knockout mice can also be generated using CRISPR-Cpf1, as Young Hoon Sung’s group at Asan Medical Center and Jin-So Kim’s group recently demonstrated. Kim’s group conducted electroporation of AsCpf1 ribonucleoproteins (RNPs) consisting of recombinant AsCpf1 protein and synthesized crRNA. They introduced AsCpf1 or SpCas9 RNPs targeting the Foxn1 locus into mouse one-cell embryos, resulting in 100% mutants for SpCas9 and 64% mutants for AsCpf1. No off-target mutation was detected at the potential sites containing up to 4-bp mismatches against on-target sites. The results indicated that the electroporation of Cpf1 RNPs would become a strong technique to systematically produce knockout mice. In the Sung’s report, knockout mice were generated using AsCpf1 and LbCpf1 via a conventional microinjection technique. They microinjected AsCpf1 or LbCpf1 mRNAs with corresponding crRNA molecules targeting Trp53 and Prkdc loci into mouse embryos. 70–80% of newborns from AsCpf1 or LbCpf1 mRNA-injected embryos harbored mutations in the target genes except for AsCpf1 targeting Prkdc locus (18.2%). Similar to the Kim’s report, off-target mutations were not detected at the 2- to 4-bp-mismatched sites but were detected at only 1-bp mismatched site with the frequency of 18.6% for AsCpf1 and 16.3% for LbCpf1. Taken together, CRISPR-Cpf1 enables genome engineering with high accuracy in human cells and mouse zygotes, although its targeting range is limited because of its stringent PAM sequence.

More recently, homologous recombination-mediated gene knock-in with AsCpf1 and LbCpf1 in mouse N2a cells were reported. Further demonstrations should confirm further advantages and disadvantages of Cpf1 in various genome engineering applications.

**New class-2 CRISPR-Cas systems other than CRISPR-Cpf1**

Although there is no doubt about the usefulness of Cpf1, the pioneer of novel CRISPR-Cas systems, Zhang’s group, has found more options other than
Cpf1. They discovered 53 class-2 CRISPR-Cas candidates and categorized them into 3 groups by the context characteristics; C2c1, C2c2 and C2c3. C2c1 and C2c3 were later grouped in type V, and C2c2 was grouped in the new type VI. Among them, C2c2 nucleases have an especially unique feature that its target molecule is not the double-strand DNA but the single-strand RNA; thus possibly contributing gene knockdown applications. The authors discussed that this RNA-targeting nuclease has several advantages over conventional knockdown methods; i.e., the applicability for a large-scale screening or construction of synthetic regulatory circuits via degradation of transcripts or translational inhibition, transcript tracking using a nuclease-deactivated C2c2 (dC2c2) fused to a fluorescent protein, and delocalization of transcripts by blocking the localization elements.

Furthermore, Doudna’s group discovered that C2c2 contains another RNase activity generating mature crRNAs from CRISPR array transcripts, depending on a distinct functional domain from the domain containing RNA-guided RNase activity. This feature of dual nuclease activity is similar to Cpf1, but interestingly, Cpf1 and C2c2 are evolutionarily unrelated. Therefore, C2c2 can process multiple crRNAs from a single transcript and act as an RNase or a guiding molecule for other RNA-detecting applications without requiring any other molecules such as PAMmer used in RCas9 system.

**Novel nucleases other than CRISPR systems**

Novel candidates for alternative programmable nucleases are not limited to the CRISPR-Cas systems. Chunyu Han at Hebei University of Science and Technology reported that Argonaute protein from Natronobacterium gregoryi (NgAgo) could be used for DNA-guided genome editing. NgAgo-guide DNA system is quite fascinating, because it requires neither PAM sequence nor RNA molecules, which means genome editing can be performed simply by the expression of generic NgAgo protein and introduction of synthetic oligonucleotides on any genomic sequence. However, the reproducibility of NgAgo-mediated genome editing is currently controversial. Another newly developed system is the structure-guided nuclease (SGN), consisting of FEN1 protein fused to the nuclease domain of FokI. The SGN coupled with 2 single-strand DNAs producing the flap ends when they bound to the target DNA sequences can reportedly introduce a DSB, resulting in relatively long deletions (650–2,600 bp). This mutation signature is unique identity for the SGN and might be of benefit particularly for reliable gene knockout. Nevertheless, further characterization is required similar with Argonautes.

On the other hand, nucleic acid-based, protein-free genome editing reagents are also the attractive alternatives. Two independent studies suggest potential applicability of peptide nucleic acid (PNA) for targeted DNA modification. Makoto Komiyama and colleagues have developed an artificial restriction DNA cutter (ARCUt), which is a chemically synthesizable DSB-inducing system based on pseudo-complementary PNA (pcPNA) and Ce(IV)/EDTA complex. Another report by Peter M. Glazer and colleagues demonstrated genome engineering in cultured cells and mice using γ-substituted tail-clamp PNA (γtcPNA) delivered via nanoparticles. In addition, 5′-tailed duplex DNA, developed by Hiroyuki Kamiya’s laboratory, could promote sequence conversion in human cells. Although these alternative technologies have not been fully characterized compared with the standard genome editing tools such as CRISPR-Cas9, they might enable synthetic mass production of custom-made reagents with low cost and tissue/cell type-specific targeted delivery for gene therapy applications in the future.

**Conclusions**

Application of genome editing is now broadly expanded using CRISPR-Cas9, such as genome-wide functional screening based on gene knockout, transcriptional repression or transcriptional activation, analysis of chromatin dynamics using dCas9 fused with fluorescent proteins, epigenome editing with dCas9-epigenetic modifier, and the tracking of cell lineage with DNA barcoding techniques. Behind such application development is the fact that the history of technological improvements of basic genome editing tools. The evolving nucleases, expounded in this commentary, must contribute future epoch-making applications.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
Funding
This study was supported in part by the Grant-in-Aid for Young Scientists (B) (16K18478) to T.S. and the Grant-in-Aid for JSPS Research Fellow (16J03164) to S.N. from Japan Society for the Promotion of Science.

ORCID
Shota Nakade http://orcid.org/0000-0003-4721-9408

References
[1] Mohanraju P, Makarova KS, Zetsche B, Zhang F, Koonin EV, van der Oost J. Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. Science 2016; 353(6299):aad5147; PMID:27493190; https://doi.org/10.1126/science.aad5147
[2] Delcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor Rnase III. Nature 2011; 471(7340):602-7; PMID:21455174; https://doi.org/10.1038/nature09886
[3] Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. Multiplex genome engineering using CRISPR/Cas9 systems. Science 2013; 339(6121):819-23; PMID:23287718; https://doi.org/0.1126/science.1231143
[4] Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. Science 2013; 339(6121):823-6; PMID:23287722; https://doi.org/10.1126/science.1232033
[5] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012; 337(6096):816-21; PMID:22745249; https://doi.org/10.1126/science.1232782
[6] Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 2013; 339(6121):823-6; PMID:23287718; https://doi.org/10.1126/science.1258596
[7] Aida T, Chiyoo K, Usami T, Ishikubo H, Imahashi R, Wada Y, Tanaka KF, Sakuma T, Yamamoto T, Tanaka K. Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice. Genome Biol 2015; 16:87; PMID:25924609; https://doi.org/10.1186/s13059-015-0653-x
[8] Sakuma T, Nishikawa A, Kume S, Chayama K, Yamamoto T. Multiplex genome engineering in human cells using all-in-one CRISPR/Cas9 vector system. Sci Rep 2014; 4:5400; PMID:24954249; https://doi.org/10.1038/srep05400
[9] Ota S, Hisano Y, Ikawa Y, Kawahara A. Multiple genome modifications by the CRISPR/Cas9 system in zebrafish. Genes Cells 2014; 19(7):555-64; PMID:24848337; https://doi.org/10.1111/gtc.12154
[10] Li J, Shou J, Guo Y, Tang Y, Wu Y, Jia Z, Zhai Y, Chen Z, Xu Q, Wu Q. Efficient inversions and duplications of mammalian regulatory DNA elements and gene clusters by CRISPR/Cas9. J Mol Cell Biol 2015; 7(4):284-98; PMID:25757625; https://doi.org/10.1093/jmcb/mjv016
[11] Choi PS, Meyerson M. Targeted genomic rearrangements using CRISPR/Cas9 technology. Nat Commun 2014; 5:3728; PMID:24759083; https://doi.org/10.1038/ncomms4728
[12] Maddalo D, Manchado E, Concepcion CP, Bonetti C, Vidigal JA, Han YC, Ogrodowski P, Crippa A, Rekhtman N, de Chastine E, et al. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. Nature 2014; 516(7531):423-7; PMID:25337876; https://doi.org/10.1038/nature13902
[13] Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ, Root DE. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nat Biotechnol 2014; 32(12):1262-7; PMID:25184501; https://doi.org/10.1038/nbt.3026
[14] Gazisnas 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 2012; 109(39):E2579-86; PMID:22949671; https://doi.org/10.1073/pnas.1208507109
[15] Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, et al. In vivo genome editing using Staphylococcus aureus Cas9. Nature 2015; 520(7546):186-91; PMID:25830891; https://doi.org/10.1038/nature14299
[16] Fu Y, Foden JA, Khayter C, Maeder ML, Reynon D, Joung JK, Sander JD. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 2013; 31(9):822-6; PMID:23792628; https://doi.org/10.1038/nbt.2623
[17] Wang X, Wang Y, Wu X, Wang J, Wang Y, Qiu Z, Chang T, Huang H, Lin RJ, Yee JK. Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENs using integrase-defective lentiviral vectors. Nat Biotechnol 2015; 33(2):175-8; PMID:25599175; https://doi.org/10.1038/nbt.3027
[18] Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Vyvekens N, Khayter C, Iafrate AJ, Le LP, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas9 nucleases. Nat Biotechnol 2015; 33(2):187-97; PMID:25513782; https://doi.org/10.1038/nbt.3117
[19] Frock RL, Hu J, Meyers RM, Ho YJ, Kii E, Alt FW. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. Nat Biotechnol 2015; 33(2):179-86; PMID:25503383; https://doi.org/10.1038/nbt.3101
[20] Crosetto N, Mitra A, Silva MJ, Bienko M, Dojer N, Wang Q, Karaca E, Chiarle R, Skrzypczak M, Ginalska K, et al. Nucleotide-resolution DNA double-strand break
mapping by next-generation sequencing. Nat Methods 2013; 10(4):361-5; PMID:23503052; https://doi.org/10.1038/nmeth.2408

[21] Kim D, Bae S, Park J, Kim E, Kim S, Yu HR, Hwang J, Kim JI, Kim JS. Digene-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. Nat Methods 2015; 12(3):237-43; PMID:25664545; https://doi.org/10.1038/nmeth.3284

[22] Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. Science 2016; 351(6268):84-8; PMID:26628643; https://doi.org/10.1126/science.aad5227

[23] Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 2016; 529(7587):490-5; PMID:26735016; https://doi.org/10.1038/nature16526

[24] Guilinger JP, Pattanayak V, Reyon D, Tsai SQ, Sander JD, Joung JK, Liu DR. Broad specificity profiling of TALENs results in engineered nucleases with improved DNA-cleavage specificity. Nat Methods 2014; 11(4):429-35; PMID:24531420; https://doi.org/10.1038/nmeth.2845

[25] Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JR, et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 2015; 523(7561):481-5; PMID:26098369; https://doi.org/10.1038/nature14592

[26] Feng Y, Chen C, Han Y, Chen Z, Lu X, Liang F, Li S, Qin W, Lin S. Expanding CRISPR/Cas9 Genome Editing Capacity in Zebrafish Using SaCas9. G3 (Bethesda) 2016; 6(8):2517-21; PMID:27317783; https://doi.org/10.1534/g3.116.031914

[27] Paquet D, Kwart D, Chen A, Sproul A, Jacob S, Olsen KM, Gregg A, Noggle S, Tessier-Lavigne M. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. Nature 2016; 533(7601):125-9; PMID:27120160; https://doi.org/10.1038/nature17664

[28] Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 2013; 154(2):442-51; PMID:23849981; https://doi.org/10.1016/j.cell.2013.06.044

[29] Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 2013; 31(9):833-8; PMID:23907171; https://doi.org/10.1038/nbt.2675

[30] Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 2013; 154(6):1380-9; PMID:23992846; https://doi.org/10.1016/j.cell.2013.08.021

[31] Tsai SQ, Wyvekens N, Khayer C, Foden JA, Thapar V, Reyon D, Goodwin MJ, Aryee MJ, Joung JK. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol 2014; 32(6):569-76; PMID:24770325; https://doi.org/10.1038/nbt.2908

[32] Guilinger JP, Thompson DB, Liu DR. Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. Nat Biotechnol 2014; 32(6):577-82; PMID:24770324; https://doi.org/10.1038/nbt.2909

[33] Barrangou R, Doudna JA. Applications of CRISPR technologies in research and beyond. Nat Biotechnol 2016; 34(9):933-941; PMID:27606440; https://doi.org/10.1038/nbt.3659

[34] Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 2016; 533(7603):420-4; PMID:27096365; https://doi.org/10.1038/nature17946

[35] Nishida K, Araoz T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M, Haru KY, et al. Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science 2016; 353(6305):420-4; PMID:27492474; https://doi.org/10.1126/science.aad58729

[36] Ma Y, Zhang J, Yin W, Zhang Z, Song Y, Chang X. Targeted AID-mediated mutagenesis (TAM) enables efficient genomic diversification in mammalian cells. Nat Methods 2016; 13(12):1029-1035; PMID:27723754; https://doi.org/10.1038/nmeth.4027

[37] Hess GT, Frêraud L, Han K, Lee CH, Li A, Cinprich KA, Montgomery SB, Bassik MC. Directed evolution using dCas9-targeted somatic hypermutation in mammalian cells. Nat Methods 2016; 13(12):1036-1042; PMID:27798611; https://doi.org/10.1038/nmeth.4038

[38] O’Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, Doudna JA. Programmable RNA recognition and cleavage by CRISPR/Cas9. Nature 2014; 516(7530):263-6; PMID:25274302; https://doi.org/10.1038/nature13769

[39] Nelles DA, Fang MY, O’Connell MR, Xu JL, Markmiller SJ, Doudna JA, Yeo GW. Programmable RNA Tracking in Live Cells with CRISPR/Cas9. Cell 2016; 165(2):488-96; PMID:26997482; https://doi.org/10.1016/j.cell.2016.02.054

[40] Nishimasu H, Cong L, Yan WX, Ran FA, Zetsche B, Li Y, Kurabayashi A, Ishitani R, Zhang F, Nureki O. Crystal Structure of Staphylococcus aureus Cas9. Cell 2015; 162(5):1113-26; PMID:26317473; https://doi.org/10.1016/j.cell.2015.08.007

[41] Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JR, et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 2015; 523(7561):442-51; PMID:26098369; https://doi.org/10.1038/nature14592
[42] Karvelis T, Gasiunas G, Young J, Bigelyte G, Silanskas A, Cigan M, Siksny S. Rapid characterization of CRISPR-Cas9 protospacer adjacent motif sequence elements. Genome Biol 2015; 16:253; PMID:26585795; https://doi.org/10.1186/s13059-015-0188-7

[43] Hirano H, Gootenberg JS, Horii T, Abudayyeh OO, Shmakov S, Makarova KS, Wolf YI, Kleinstiver BP, Tsai SQ, Prew MS, Nguyen NT, Kim D, Kim J, Hur JK, Been KW, Yoon SH, Kim JS. Targeted mutagenesis in mice by electroporation of Cpf1 ribonucleoproteins. Nat Biotechnol 2016; 34(8):807-8; PMID:27272385; https://doi.org/10.1038/nbt.3614

[44] Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Esletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 2015; 163(3):759-71; PMID:26422227; https://doi.org/10.1016/j.cell.2015.09.038

[45] Kim HK, Song M, Lee J, Menon AV, Jung S, Kang YM, Choi JW, Woo E, Koh HC, Nam JW, Kim H. In vivo high-throughput profiling of CRISPR-Cpf1 activity. Nat Methods 2017; 14(2):153–159; PMID:27992409; https://doi.org/10.1038/nmeth.4104

[46] Fonfarà I, Richter H, Bratović M, Le Rhun A, Charpentier E. The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. Nature 2016; 532(7600):517-21; PMID:27096362; https://doi.org/10.1038/17945

[47] Kim D, Kim J, Hur JK, Been KW, Yoon SH, Kim JS. Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. Nat Biotechnol 2016; 34 (8):863-8; PMID:2772384; https://doi.org/10.1038/nbt.3609

[48] Kleinstiver BP, Tsai SQ, Prew MS, Nguyen NT, Welch MM, Lopez JM, McCaw ZR, Aryee MJ, Joung JK. Genome-wide specificities of CRISPR-Cas Cpf1 endonucleases in human cells. Nat Biotechnol 2016; 34 (8):869-74; PMID:27347757; https://doi.org/10.1038/nbt.3620

[49] Kim Y, Cheong SA, Lee JG, Lee SW, Lee MS, Baek IJ, Sung YH. Generation of knockout mice by Cpf1-mediated gene targeting. Nat Biotechnol 2016; 34(8):808-10; PMID:27272387; https://doi.org/10.1038/nbt.3614

[50] Hur JK, Kim K, Been KW, Baek G, Ye S, Hur JW, Ryu SM, Lee YS, Kim JS. Targeted mutagenesis in mice by electroporation of Cpf1 ribonucleoproteins. Nat Biotechnol 2016; 34 (8):807-8; PMID:27272385; https://doi.org/10.1038/nbt.3596

[51] Tóth E, Weinhardt N, Bencsura P, Huszár K, Kulcsár PI, Tálas A, Fodor E, Welker E. Cpf1 nucleases demonstrate robust activity to induce DNA modification by exploiting homology directed repair pathways in mammalian cells. Biol Direct 2016; 11:46; PMID:27630115; https://doi.org/10.1186/s13062-016-0147-0

[52] Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Minakhin L, Joung J, Konermann S, Severinov K, et al. Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. Mol Cell 2015; 60(3):385-97; PMID:26593719; https://doi.org/10.1016/j.molcel.2015.10.008

[53] Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DB, Shmakov S, Makarova KS, Semenova E, Minakhin L, et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science 2016; 353(6299):aaf5573; PMID:27256883; https://doi.org/10.1126/science.aaf5573

[54] East-Seletsky A, O’Connell MR, Knight SC, Burstein D, Cate JH, Tjian R, Doudna JA. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. Nature 2016; 538(7624):270-273; PMID:27669025; https://doi.org/10.1038/nature19802

[55] Gao F, Shen XZ, Jiang F, Wu Y, Han C. DNA-guided genome editing using the Natronobacterium gregoryi Argonauta. Nat Biotechnol 2016; 34(7):678-73; PMID:27136078; https://doi.org/10.1038/nbt.3547

[56] Cyranoski D. Replications, ridicule and a recluse: the controversy over NgAgo gene-editing intensifies. Nature 2016; 536(7615):136-7; PMID:27510204; https://doi.org/10.1038/536136a

[57] Lee SH, Turchiano G, Ata H, Nowsheen S, Romito M, Lou Z, Ryu SM, Ekker SC, Cathomen T, Kim JS. Failure to detect DNA-guided genome editing using Natronobacterium gregoryi Argonauta. Nat Biotechnol 2016; 35:17-18; PMID:27893702; https://doi.org/10.1038/nbt.3753

[58] Varshney GK, Burgess SM. DNA-guided genome editing using structure-guided endonucleases. Genome Biol 2016; 17(1):187; PMID:27640875; https://doi.org/10.1186/s13059-016-1055-4

[59] Komiyama M. Cut-and-Paste of DNA Using an Artificial Restriction DNA Cutter. Int J Mol Sci 2013; 14(2):3343-57; PMID:23385238; https://doi.org/10.3390/ijms14023343

[60] Bahal R, Ali McNeer N, Quijano E, Liu Y, Sulkowski P, Turchick A, Lu YC, Bhunia DC, Manna A, Greiner DL, et al. In vivo correction of anaemia in β-thalassemic mice by γDNA-mediated gene editing with nanoparticle delivery. Nat Commun 2016; 7:13304; PMID:27782131; https://doi.org/10.1038/ncomms13304

[61] Suzuki T, Imada T, Nishigaki N, Kobayashi M, Matsuoka I, Kamiya H. Cleavage of Target DNA Promotes Sequence Conversion with a Tailed Duplex. Biol Pharm Bull 2016; 39(8):1392-5; PMID:27476948; https://doi.org/10.1248/bpb.b16-00325

[62] East-Seletsky A, O’Connell MR, Knight SC, Burstein D, Cate JH, Tjian R, Doudna JA. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. Nature 2016; 538(7624):270-273; PMID:27669025; https://doi.org/10.1038/nature19802

[63] Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells. Science 2014; 343(6166):84-7; PMID:24336571; https://doi.org/10.1126/science.1247005

[64] Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH, Guimaraes C, Panning B, Ploegh HL, Bassik MC, et al. Genome-Scale CRISPR-Mediated Control of
Gene Repression and Activation. Cell 2014; 159(3):647-61; PMID:25307932; https://doi.org/10.1016/j.cell.2014.09.029

Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW, Park J, Blackburn EH, Weissman JS, Qi LS, Huang B. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell 2013; 155(7):1479-91; PMID:24360272; https://doi.org/10.1016/j.cell.2013.12.001

Hilton IB, D’Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nat Biotechnol 2015; 33(5):510-7; PMID:25849900; https://doi.org/10.1038/nbt.3199

McKenna A, Findlay GM, Gagnon JA, Horwitz MS, Schier AF, Shendure J. Whole-organism lineage tracing by combinatorial and cumulative genome editing. Science 2016; 353(6298):aaf7907; PMID:27229144; https://doi.org/10.1126/science.aaf7907

Perli SD, Cui CH, Lu TK. Continuous genetic recording with self-targeting CRISPR-Cas in human cells. Science 2016; 353(6304):aag0511; PMID:27540006; https://doi.org/10.1126/science.aag0511

Hou Z, Zhang Y, Propson NE, Howden SE, Chu LF, Sontheimer EJ, Thomson JA. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. Proc Natl Acad Sci USA 2013; 110(39):15644-9; PMID:23940360; https://doi.org/10.1073/pnas.1313587110

Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. Nat Methods 2013; 10(11):1116-21; PMID:24076762; https://doi.org/10.1038/nmeth.2681