

Bioengineered

ISSN: 2165-5979 (Print) 2165-5987 (Online) Journal homepage: http://www.tandfonline.com/loi/kbie20

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**To cite this article:** Shota Nakade, Takashi Yamamoto & Tetsushi Sakuma (2017) Cas9, Cpf1 and C2c1/2/3–What's next?, Bioengineered, 8:3, 265-273, DOI: 10.1080/21655979.2017.1282018

**To link to this article:** http://dx.doi.org/10.1080/21655979.2017.1282018

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Accepted author version posted online: 31 Jan 2017.
Published online: 31 Jan 2017.

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BIOENGINEERED
2017, VOL. 8, NO. 3, 265–273
https://doi.org/10.1080/21655979.2017.1282018

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COMMENTARY
CRISPR Special Focus

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# Cas9, Cpf1 and C2c1/2/3—What's next?

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**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.

**ARTICLE HISTORY**
Received 3 October 2016
Revised 4 January 2017
Accepted 4 January 2017

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

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## 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.[1] The inactivation of exogenous DNA by CRISPR-Cas systems is conducted by 3 steps; adaptation, expression, and interference.[1] 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.[2]

### *CRISPR-Cas9 as a genome engineering tool*

After the elucidation of the mechanism of CRISPR-Cas9 system, it was quickly applied for genome engineering.,[3,4] 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.[5] The CRISPR-Cas9 system has various advantages over the past engineered nucleases such as zinc-finger nucleases and transcription activator-like effector nucleases.[6] 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[7]. In addition, co-introduction of multiple sgRNAs and

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Cas9 nuclease can easily induce simultaneous DSBs, resulting in multiple mutagenesis and chromosomal editing such as large deletions,[8,9] inversions,[9,10] duplications,[10] and translocations.[11,12] Despite these advantageous properties of CRISPR-Cas9, there still 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.[1] There is no obvious restriction in crRNA target sequence, but some relations between target base components and nuclease activity have been reported,[13] 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.[14] *Streptococcus pyogenes* Cas9 (SpCas9), the most commonly used Cas nuclease to perform genome engineering, has 5′-NGG-3′ PAM.[2] 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.[15] The third problem with CRISPR-Cas9 is off-target mutations,[16] especially for therapeutic applications. Several genome-wide detection methods of off-target DSB introduction such as IDLV integration,[17] GUIDE-seq,[18] HTGTS,[19] BLESS,[20] and Digenome-seq[21] have revealed that the population of cells transfected with the wild-type SpCas9 nuclease often contains various off-target sites, which cannot be easily predicted by *in silico* homology analysis. Therefore, CRISPR-Cas9 system still has room for improvement in terms of flexibility of target design, utility for viral delivery, and specificity of DSB introduction.

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).

## 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[22] and SpCas9-HF.[23] 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.[24]

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.[25] 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.[26] 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.[27]

Functional conversion of SpCas9 has also been demonstrated. The SpCas9 nuclease can be converted to nickase (Cas9n)[5] or nuclease-deficient mutant (dCas9).[28] The Cas9n variant has been used in double-nicking strategy.[29,30] The dCas9 variant has broad applications including FokI-dCas9[31,32] and specific purposes other than conventional genome editing.[33] Of these, single-base editing systems without inducing DSBs, mediated by cytidine deaminase combined with Cas9n or dCas9, as known as “Base Editors,” are

**Table 1.** Summary of various CRISPR-Cas nucleases.

| Nuclease type | Enzyme name | WT/mutants | PAM | Protein size | Typical protospacer length | DNA end | Pros/Cons |
|---|---|---|---|---|---|---|---|
| Cas9 | SpCas9 | WT[5] | 5′-NGG-3′ | 1,368 a.a. | 20 nt | blunt end | Most commonly used/Large protein size |
| | | VQR[25] | 5′-NGAN-3′ | | | | Different PAM specificities/Large protein size |
| | | EQR[25] | 5′-NGAG-3′ | | | | |
| | | VRER[25] | 5′-NGCG-3′ | | | | |
| | SaCas9 | WT[15] | 5′-NNGRRT-3′ | 1,053 a.a. | | | Small protein size/Relatively strict PAM |
| | | KKH[41] | 5′-NNNRRT-3′ | | | | |
| | FnCas9 | WT[43] | 5′-NGG-3′ | 1,629 a.a. | | | Less restrictive PAM/Large protein size, less application examples |
| | | RHA[43] | 5′-YG-3′ | | | | |
| | NmCas9 | WT[69] | 5′-NNNNGATT-3′ | 1,082 a.a. | 24 nt | | Small protein size/Strict PAM |
| | St1Cas9 | WT[70] | 5′-NNAGAAW-3′ | 1,121 a.a. | 20 nt | | Small protein size/Strict PAM |
| | BlatCas9 | WT[42] | 5′-NNNNCNDD-3′ | 1,092 a.a. | 21 nt | | Less restrictive PAM, small protein size/Less application examples |
| Cpf1 | AsCpf1 | WT[44] | 5′-TTTN-3′ | 1,307 a.a. | 23 nt | sticky end | Various unique characteristics (see main text)/Strict PAM |
| | LbCpf1 | WT[44] | 5′-TTTN-3′ | 1,228 a.a. | | | |

recent distinguished achievement.[34,35] Furthermore, unique analyses using Base Editors such as *in situ* direct evolution of endogenous genes have already been reported,[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.[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.[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).[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.[41] In addition, SaCas9 has a size advantage that enables packaging into the AAV vector, because SaCas9 is 315 amino acids smaller than SpCas9[15]. Virginijus 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).[42] His team demonstrated BlatCas9-induced indels in maize. Since BlatCas9s 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.[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.[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.[44] CRISPR-Cpf1 system can be programmed for genome engineering similar with CRISPR-Cas9,

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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[45]) 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.[46] 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.[47,48] 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.[47] 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.[47] 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[47]. 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.[48]

Knockout mice can also be generated using CRISPR-Cpf1, as Young Hoon Sung's group at Asan Medical Center and Jin-Soo Kim's group recently demonstrated.[49,50] Kim's group conducted electroporation of AsCpf1 ribonucleoproteins (RNPs) consisting of recombinant AsCpf1 protein and synthesized crRNA.[50] 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.[49] 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.[51] 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

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Cpf1. They discovered 53 class-2 CRISPR-Cas candidates and categorized them into 3 groups by the context characteristics; C2c1, C2c2 and C2c3[52]. 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.[53] 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.[54] 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.[55] 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.[56,57] Another newly developed system is the structure-guided nuclease (SGN), consisting of FEN1 protein fused to the nuclease domain of FokI.[58] 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.[59] Another report by Peter M. Glazer and colleagues demonstrated genome engineering in cultured cells and mice using $\gamma$-substituted tail-clamp PNA ($\gamma$tcPNA) delivered via nanoparticles.[60] In addition, 5′-tailed duplex DNA, developed by Hiroyuki Kamiya's laboratory, could promote sequence conversion in human cells.[61] 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,[62,63] transcriptional repression or transcriptional activation,[64] analysis of chromatin dynamics using dCas9 fused with fluorescent proteins,[65] epigenome editing with dCas9-epigenetic modifier,[66] and the tracking of cell lineage with DNA barcoding techniques.[67,68] 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.

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