

## Review Article

# Editor's cut: DNA cleavage by CRISPR RNA-guided nucleases Cas9 and Cas12a

Thomas Swartjes, Raymond H.J. Staals and  John van der Oost

Laboratory of Microbiology, Wageningen University & Research, Stippeneng 4, 6708 WE Wageningen, Netherlands

**Correspondence:** John van der Oost ([john.vanderoost@wur.nl](mailto:john.vanderoost@wur.nl))



Discovered as an adaptive immune system of prokaryotes, CRISPR–Cas provides many promising applications. DNA-cleaving Cas enzymes like Cas9 and Cas12a, are of great interest for genome editing. The specificity of these DNA nucleases is determined by RNA guides, providing great targeting adaptability. Besides this general method of programmable DNA cleavage, these nucleases have different biochemical characteristics, that can be exploited for different applications. Although Cas nucleases are highly promising, some room for improvement remains. New developments and discoveries like base editing, prime editing, and CRISPR-associated transposons might address some of these challenges.

## Introduction

Horizontal gene transfer plays a crucial role in the evolution of prokaryotes [1–4]. Invading genetic material may give fitness advantages to recipient cells, but also may pose a substantial risk. Therefore, bacteria and archaea have evolved a range of mechanisms to protect themselves from foreign nucleic acids [5]. One such defense mechanism is the CRISPR–Cas system (clustered regularly interspaced short palindromic repeats and CRISPR-associated). CRISPR–Cas has been shown to provide adaptive immunity against mobile genetic elements (e.g. viruses) in three stages: adaptation, expression, and interference (Figure 1) [6,7].

In the adaptation stage (Figure 1A), a complex of Cas1 and Cas2 (and sometimes Cas4) acquires nucleotide stretches called protospacers. Protospacers are selected based on the presence of a protospacer adjacent motif (PAM) and are preferentially derived from foreign DNA or RNA [8–12]. Acquired protospacers are incorporated as spacers in the CRISPR array on the host chromosome, where they are flanked by repeats [13]. Spacer acquisition has been demonstrated to also involve host proteins as well as CRISPR interference itself [14–19].

During the expression stage (Figure 1B), the CRISPR array is transcribed into a long precursor CRISPR RNA (pre-crRNA) that is subsequently processed into small CRISPR RNAs (crRNAs), each containing a single spacer flanked by parts of the repeat. Next, Cas proteins and the mature crRNA (also referred to as the guide RNA) assemble to form a ribonucleoprotein (RNP) complex.

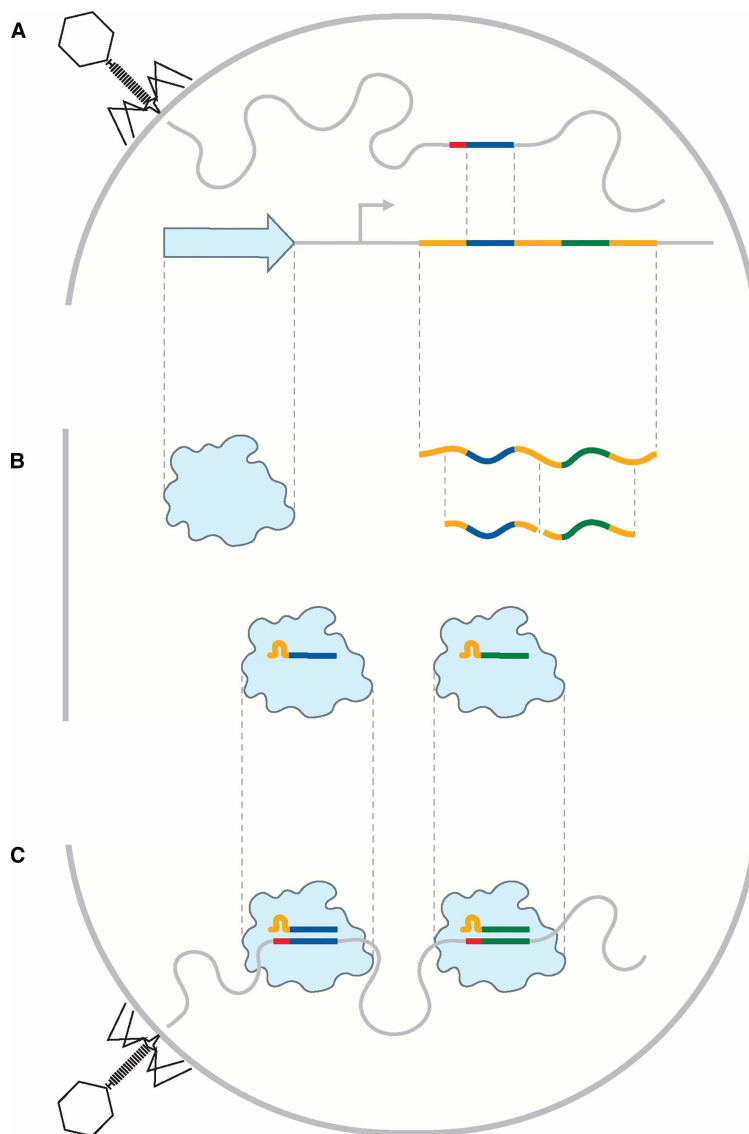
In the interference stage (Figure 1C), RNPs recognize PAMs and interrogate adjacent sequences for complementary to the crRNA. The RNPs then trigger degradation of the foreign DNA or RNA, either by nuclease activity of the RNP itself or through recruitment of another nuclease, for instance, the helicase/nuclease Cas3 [20]. CRISPR–Cas systems are classified in Class 1 (type I, III, IV) and Class 2 (type II, V, VI) [21]. Class 1 systems use multi-subunit Cas complexes (consisting of multiple different Cas proteins) for interference. In contrast, Class 2 systems are characterized by their single, multi-domain protein nucleases.

## Genome editing

Among applications of CRISPR–Cas, genome editing has attracted the most attention by far. Eukaryotic genome editing uses one of two major DNA double-stranded break (DSB) repair pathways: non-homologous end-joining (NHEJ) or homologous recombination (HR) [22]. NHEJ resolves DSBs

Received: 27 September 2019  
 Revised: 5 December 2019  
 Accepted: 6 December 2019

Version of Record published:  
 24 December 2019



**Figure 1. CRISPR–Cas adaptive immunity.**

Schematic overview of the steps involved in adaptive immunity as provided by CRISPR–Cas. **(A)** Adaptation stage, featuring the introduction of viral DNA/RNA (curved gray line) with protospacer 1 (dark blue) and an adjacent PAM (red). Protospacer 1 is incorporated in the bacterial genome as spacer 1 (dark blue) flanked by two repeats (orange) in the CRISPR array alongside spacer 2 (green). **(B)** Expression stage, wherein a Cas protein (light blue) is produced and pre-crRNA is transcribed and processed into mature crRNAs, which subsequently form functional RNP complexes with the Cas protein. **(C)** Interference stage, where a second infection is targeted for degradation by crRNA-guided Cas proteins, binding to matching protospacers adjacent to PAMs.

in the DNA by ligating both ends, often resulting in small insertions or deletions (indels) [23]. In the case of targeted protein-coding genes, indels are likely to cause frameshifts, effectively knocking out these genes. HR, on the other hand, requires a homologous repair template (ssDNA or dsDNA), which is used for more precise DSB repair [22]. For genome editing, one can supply an artificial repair template with the desired sequence, flanked by sequences homologous to the targeted locus. HR will then incorporate the repair template to repair the DSB, thereby introducing the designed edit. Due to these differences in resolving DSBs, NHEJ is useful for knocking out existing genes, and HR can be used for precise insertions or replacement of DNA. Whereas eukaryotic cells possess both HR and NHEJ repair systems, the HR system is highly cell cycle dependent, being

mostly active during S-phase [24]. DNA-cleaving Cas enzymes can be used to create DSBs in desired locations, making them useful in directing genome editing for eukaryotes. In prokaryotes, Cas nucleases are not used to direct DNA repair, but rather to cleave the original DNA sequence. These DSBs kill cells with the original DNA sequence, effectively counter selecting unedited cells and enriching for edited cells.

Where most other DNA nucleases rely on protein–DNA binding for target recognition, Cas nucleases use crRNA–DNA complementarity to determine specificity. New crRNAs can be designed and produced rapidly, providing superior versatility for target specificity of Cas nucleases. In this review, we focus on Class 2 Cas nucleases like Cas9 and Cas12a because these combine targeting versatility with the simplicity of single protein nucleases, making them the preferred tools for genome editing.

## Cas9

Because of its early discovery, the most frequently used Cas protein is the type II nuclease Cas9. The characterization of Cas9 from *Streptococcus pyogenes* (SpCas9) highlighted the potential of CRISPR–Cas for genome editing applications [25,26]. Since then, Cas9 has been used for applications in organisms from all domains of life, ranging from improving microbial cell factories and crop plants to clinical trials aimed at repairing genetic disorders in human patients [27–36].

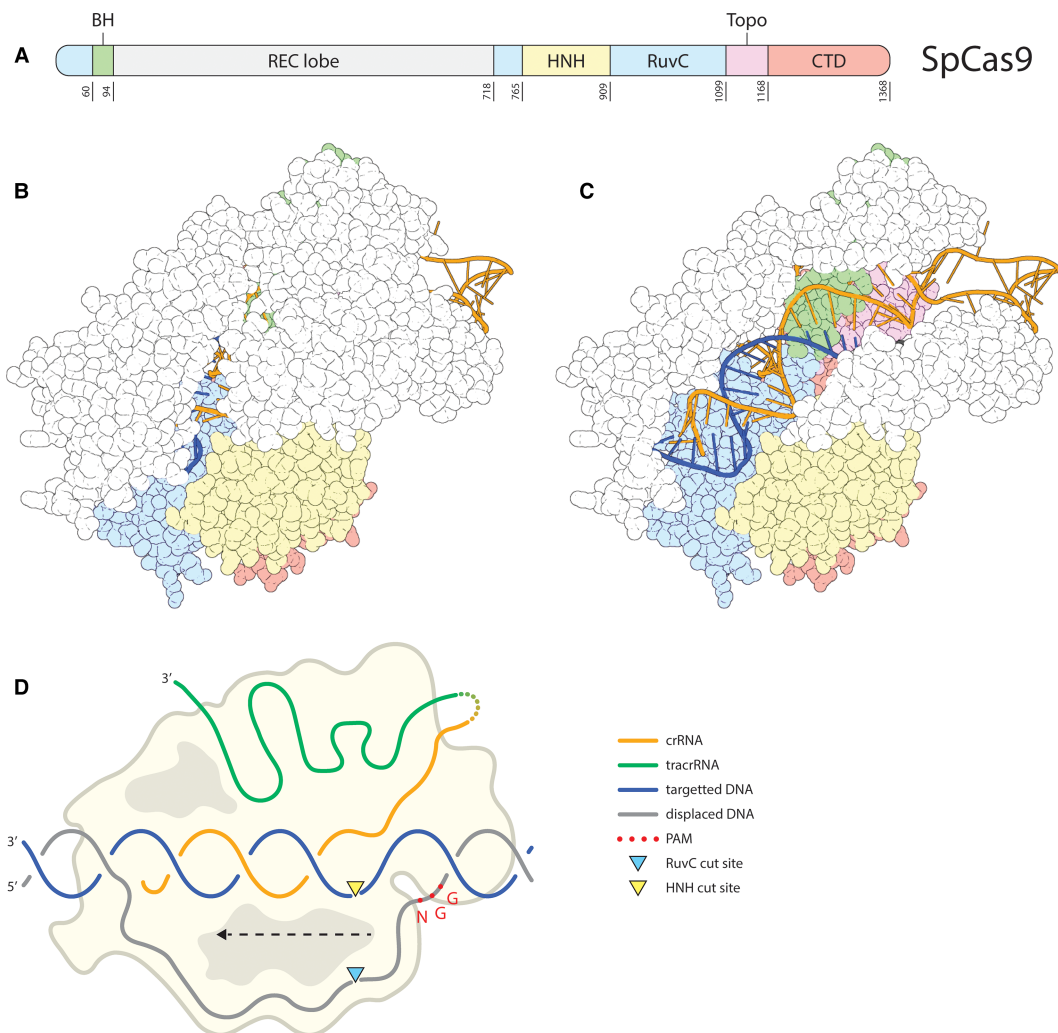
Cas9 is a single, multi-domain DNA nuclease that is guided by RNA [25]. The Cas9 protein can be divided into the recognition (REC) lobe and the nuclease (NUC) lobe [37] (Figure 2A). The NUC lobe consists of a PAM-interacting (PI) domain and two nuclease domains: HNH and RuvC. The archetype SpCas9 protein is a polypeptide of 1368 amino acids, but shorter variants from other organisms have also been described [38–40].

In type II systems, CRISPRs are transcribed as a single pre-crRNA. The maturation of the guides requires a transactivating crRNA (tracrRNA), which partially base pairs to the repeat regions of the pre-crRNA [41]. In the presence of Cas9, these partial dsRNA fragments are processed by the non-Cas ribonuclease RNase III [41]. To circumvent this processing step, the mature tracrRNA and crRNA can be fused by a short synthetic linker, and expressed as a single-guide RNA (sgRNA) that binds Cas9 to form a functional RNP complex [25].

The resulting RNP complex surveys the cells for a complementary target sequence. Single-molecule analysis revealed that the Cas9 RNP complex encounters DNA stretches through three-dimensional diffusion [42]. In the absence of a PAM, these RNP–DNA interactions are very short-lived [42]. If the RNP encounters a PAM, however, binding lasts substantially longer [42]. The PAM can, therefore, be regarded as an initial quality control that Cas9 uses in its surveillance for DNA targets and might allow the nuclease to find target DNA sequences more efficiently. SpCas9 recognizes its PAM (5'-NGG), directly downstream of the target sequence (Figure 2D) [25]. As the 5'-NGG motif occurs quite often, the PAM is generally not limiting Cas9 applications, unless a very precise cleavage position is desired. It should be noted that PAM recognition by Cas9 can be either strict or very relaxed in different Cas9 orthologs [39,43–45].

PAM binding by Cas9 enables local separation of the DNA strands directly upstream of the PAM, allowing interrogation of the target sequence for base pairing with the crRNA [42,46]. Successful base-pairing interactions between the interrogated target DNA strand and the crRNA extend the melting of the DNA strands [42]. The DNA strand displacement proceeds towards the PAM-distal region, eventually resulting in a complete R-loop configuration [42] (Figure 2B–D). Because of the direction (PAM proximal to distal), PAM-proximal matching between crRNA and the target DNA are disproportionately important for RNP binding [42,47]. This important PAM-proximal stretch is referred to as the seed region; in case of mismatches between this seed and the target strand, R-loop formation, and subsequent cleavage are generally aborted [25,42,47]. A certain level of mismatches in the PAM-distal target sequence are tolerated, which is advantageous for its natural role in defense against viruses with high mutation rates. However, in the case of genome editing, this tolerance can result in undesired binding/cleavage of imperfectly matching DNA sequences, referred to as off-targets (see below) [48,49]. More specifically, efficient DNA binding by Cas9 requires at least nine continuous matches between crRNA and target sequence [47]. Sufficient base pairing between crRNA and the DNA target strand stabilizes the R-loop, which is further stabilized by the interaction between the displaced DNA strand and charged residues of the Cas9 protein [37,50]. If the R-loop extends to the most PAM-distal bases, a conformational change is induced that poises the HNH domain in the NUC lobe for cleavage [51].

The HNH domain then cleaves the target strand, which consistently creates a break between the 3rd and 4th nucleotides upstream of the PAM [25,52]. The conformational change in the HNH domain is also required for



**Figure 2. SpCas9.**

(A) The layout of the SpCas9 protein from C- (left) to N-terminus (right). Featuring the REC lobe (gray) and the domains of the NUC lobe: RuvC (light blue), BH (green), HNH (yellow), Topo-homology (Topo, pink), CTD (C-terminal domain, red). The last residue position for each domain is given below the domains. (B) Full structure (PDB: 4UN3 [46]) of SpCas9 in complex with sgRNA (orange), target DNA strand (dark blue), and displaced DNA strand (black, but barely visible here). Protein domains are colored in accordance with (A). Visualized using PyMOL [109]. (C) Same as B with some residues from the REC lobe removed to allow a view of the sgRNA and the target DNA strand. (D) Schematic overview of the R-loop formed by Cas9 RNP. DNA strands are colored in accordance with (B). The crRNA (orange) and tracrRNA (green) are shown connected by a linker (dashed lines between them) to form a sgRNA. The direction of R-loop formation is indicated with a dashed arrow. Approximate cleavage positions are represented by colored triangles.

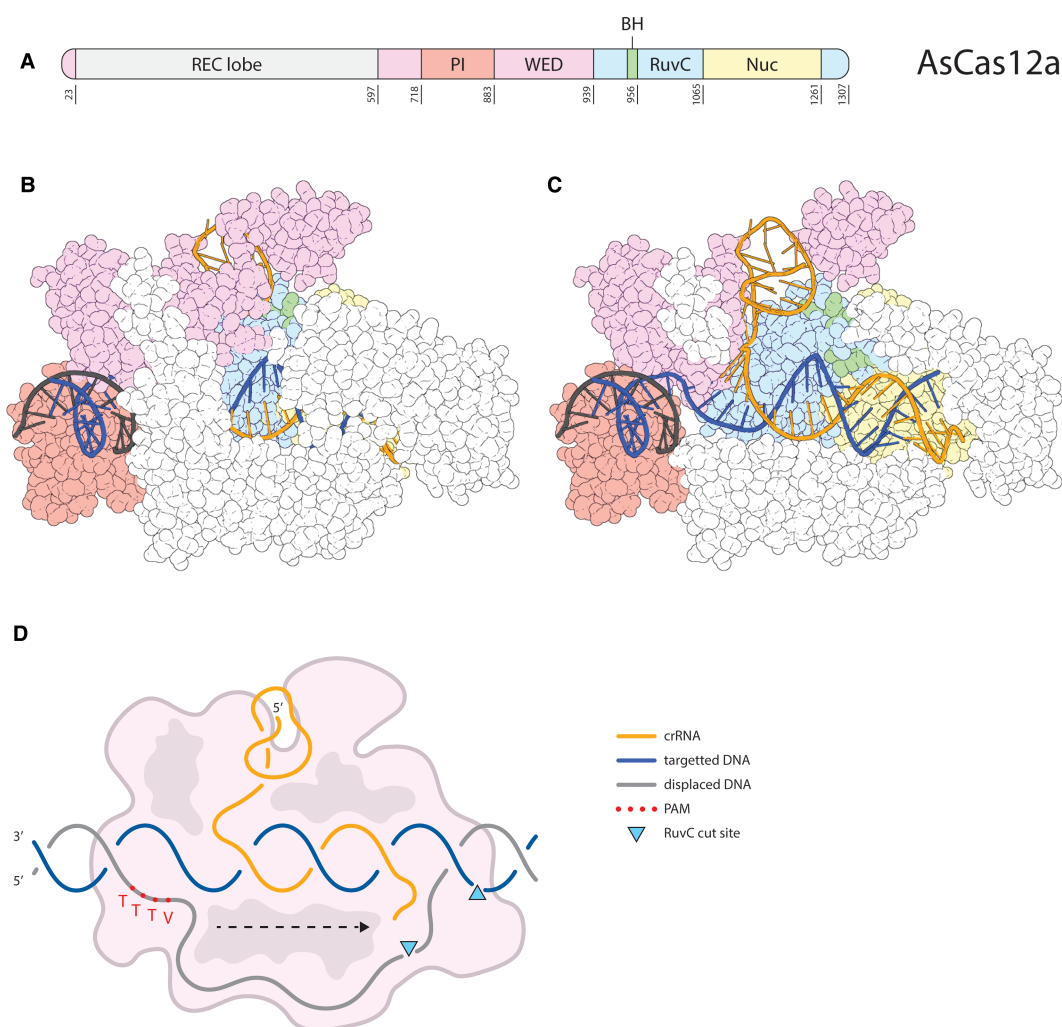
nuclease activity of the RuvC domain which cleaves the displaced strand, generally between either the 3rd and 4th or between the 4th and 5th bases upstream of the PAM [25,53,52]. Collectively, this results in a DSB with blunt ends or a short overhang. Notably, cleavage by Cas9 falls within the seed region of the target sequence [25]. If the dsDNA break is repaired through NHEJ, the resulting insertions or deletions will lead to disruption of the seed region, and hence the altered target sequence is no longer cleaved.

After cleavage, Cas9 remains stably bound to both ends of the protospacer. While the targeted strand is in heteroduplex with the crRNA, the cleaved ends of the displaced strand are less structured and might engage the RuvC active site repeatedly. This would explain the observed time-dependent exonuclease activity on the displaced strand after initial cleavage [52].

## Cas12a

Although Cas9 has proven to be a powerful tool for genome editing, other Cas nucleases may be interesting alternatives. The type V nuclease Cas12a (previously called Cpf1) is a good example of this. Despite having been characterized several years later than Cas9, Cas12a offers some advantages that may favor the latter enzyme for specific applications. One of the most used variants of Cas12a is the enzyme from *Acidaminococcus* sp. BV3L6 (AsCas12a). Like Cas9, Cas12a has by now been applied in a wide range of species including bacteria, yeast, plants, and in human cells [54–58].

As a Class 2 nuclease, Cas12a is a single multi-domain protein that induces RNA-guided dsDNA cleavage [59]. Like Cas9, the Cas12a structure can be subdivided in a REC and a NUC lobe [60,61] (Figure 3A). The NUC lobe of Cas12a lacks an HNH domain, but does contain a RuvC domain, for DNA cleavage [60]. In



**Figure 3. AsCas12a.**

(A) The layout of the AsCas12a protein from C- (left) to N-terminus (right). Featuring the REC lobe (gray) and the domains of the NUC lobe: WED (pink), PI (red), RuvC (light blue), BH (green), Nuc (yellow). The last residue position for each domain is given below the domains. (B) Full structure (PDB: 5B43 [60]) of AsCas12a in complex with crRNA (orange), target DNA strand (dark blue), and displaced DNA strand (black). Protein domains are colored in accordance with (A). Visualized using PyMOL [109]. (C) Same as B with some residues from the REC lobe and WED domain removed to allow a view of the sgRNA and DNA. (D) Schematic overview of the R-loop formed by Cas12a RNP. DNA and RNA strands are colored in accordance with (B). The direction of R-loop formation is indicated with a dashed arrow. Approximate RuvC cleavage positions in the DNA are represented by (light blue) triangles, but both strands are cleaved by a single catalytic site [62].



addition, the NUC lobe features a Wedge (WED) domain and a 'Nuclease' (Nuc) domain, which turned out not to cleave DNA [62]. AsCas12a is 1307 amino acids long, making it almost as large as SpCas9.

Unlike Cas9, Cas12a does not require a tracrRNA (nor RNase III) for pre-crRNA processing [59]. However, other type V nucleases (e.g. Cas12b) do need tracrRNAs [63]. Cas12a possesses a ribonuclease site in the WED domain, that allows for autonomous processing of pre-crRNA to mature crRNAs [64]. Cas12a crRNAs are substantially shorter than the Cas9 guide RNA (sgRNA or crRNA + tracrRNA). Collectively, this allows compact Cas12a CRISPR arrays which can be used to target multiple sites simultaneously [65]. Once bound to the Cas12a protein, the repeat-derived part of the crRNA adopts a 5' pseudoknot structure [66]. Within the spacer-derived part of the crRNA, the PAM-proximal (seed) region is pre-ordered by interactions with the protein, exposing these nucleotides for base-pairing interactions with the DNA target strand [62].

In contrast with Cas9, the Cas12a RNP seems to follow the DNA through intermittent contact during its survey for protospacers [67], although it would be best to compare DNA surveillance by both nucleases in a single study. Cas12a enzymes recognize target sequence-upstream T-rich PAMs (5'-TTTV for AsCas12a) on the displaced strand [59] (Figure 3D). The T-rich PAM of Cas12a expands the targeting space that Cas9 offers.

PAM binding by Cas12a causes local melting of the target sequence DNA directly downstream of the PAM [60–62]. This allows the interrogation of the downstream sequence for complementarity to the crRNA. The crRNA base pairs to the DNA target strand, further melting the DNA, extending the heteroduplex towards the PAM-distal end, eventually forming a full R-loop [67] (Figure 3B–D). This directionality results in a PAM-proximal seed region where mismatches are more deleterious to binding and cleavage [68]. However, compared with Cas9, Cas12a requires more matching bases between crRNA and target DNA for stable binding [47,68,69]. Especially, PAM-distal mismatches are much less tolerated by Cas12a than by Cas9 [69]. Cas12a thus has a less pronounced seed region and provides more specificity, as observed *in vivo* [70,69]. Only when target DNA and crRNA are sufficiently complementary, the Cas12a RNP forms a stable R-loop structure, enabling DNA cleavage [67,68].

Unlike Cas9, Cas12a likely uses a single RuvC nuclease domain to cleave both strands of DNA [62]. The Cas12a RuvC domain is thought to first cleave the displaced strand, generally between the 16th and 17th nucleotides downstream from the PAM, then proceeding to cleave the target strand between the 23rd and 24th nucleotides [67,69,71]. However, the initial cleavage sites have been found to vary slightly and subsequent trimming (see below) is thought to take place as well [69]. The Nuc domain seems to be involved in orienting the target strand DNA close to the active site that resides in the RuvC domain [62]. This is supported by the observation that a mutation in the Nuc domain (R1226A for AsCas12a) prevents cleavage of the target strand, effectively converting Cas12a into a nickase [60].

Cas12a thus induces PAM-distal cleavage with a 5' overhang of variable length. It is worth noting that Cas12a cleaves outside the seed region, while Cas9 cleaves within its seed region. Because of this, Zetsche et al. [59] suggested that DNA cleavage by Cas12a could tolerate small indels created by NHEJ, resulting in multiple rounds of DSB formation, as such enhancing the chance of HR to occur. However, given the high observed *in vivo* specificity of Cas12a, this now seems unlikely [70].

After cleavage, the Cas12a RNP remains bound to the PAM-proximal cleavage product [68]. This allows the displaced strand to be trimmed likely due to subsequent interactions with the RuvC catalytic site [69]. In contrast with the relatively stable interaction of Cas9 with its cleavage products, the Cas12a PAM-distal cleavage product is readily released after cleavage [42,47,68]. This release of the PAM-distal DNA might allow ssDNA molecules to interact with the still active RuvC domain, possibly explaining the observed non-specific cleavage activity on ssDNA by Cas12a after cleavage of an initial specific dsDNA target [72,73]. This conditional, collateral ssDNA activity is unlikely to be problematic as DNA hardly occurs in a single-stranded state *in vivo*. Indeed, Cas12a has been found to be less toxic than Cas9 in some contexts [54,74].

## Challenges for genome editing

Although both Cas9 and Cas12a are highly promising tools, some room for improvement remains for their optimal performance in genome editing. One main constraint of Cas nucleases is that the targeting space is limited by the requirement for the presence of a properly positioned PAM. To overcome this limitation, rational engineering and/or random mutagenesis have been used to produce Cas9 and Cas12a enzymes with altered, or less stringent PAM recognition [46,75–78]. It is tempting to propose efforts towards a hypothetical PAM-less Cas nuclease. However, the PAM recognition is generally coupled to the initial opening of the dsDNA target. In addition, more permissive Cas nucleases would spend more time on non-target sequences [42]. Because of this, there might be a strict trade-off between PAM range and on-target activity.

In contrast with Cas nucleases, prokaryotic Argonaute proteins are a class of guided nucleases that do not require a PAM for their activity. Because currently characterized prokaryotic Argonaute proteins do not trigger DNA unwinding, their dsDNA cleavage activity is far lower than for Cas9 and Cas12a [79,80]. Hence, it will be a major challenge to develop an efficient PAM-independent Cas nuclease.

An alternative to PAM-less nucleases is to work towards a comprehensive 'toolbox' of Cas nucleases that each recognize a different PAM. A Cas nuclease with an appropriate PAM could then be chosen based on the desired target site. The current targeting range is most limiting for applications where the exact location of cleavage is critical, including single nucleotide engineering by base editing (see below) [75].

The presence of a PAM does not always guarantee efficient protospacer cleavage. In addition to the PAM, the nature of the crRNA/protospacer sequence has been found to strongly affect the cleavage efficiency of Cas nucleases [48,49]. Apart from target accessibility related to chromatin structure, the variable parts of the (pre-) crRNA sequences may result in the formation of inappropriate secondary structures. Mis-folding of these guides will affect the formation of functional RNP complexes, resulting in decreased editing efficiency.

However, high cleavage efficacy does not always result in efficient eukaryotic genome editing. HR efficiency in eukaryotic cells varies substantially depending on the cell type and on the cell cycle phase. In addition, the NHEJ system may outcompete the HR system in repairing DSBs, which might lead to low levels of the desired HR. In conclusion, for precise genome editing, HR efficiency is a major bottleneck, rather than DSB formation by Cas nucleases. Hence, apart from strategies to improve HR, HR-independent strategies (see below) may be promising alternatives [81].

Possibly the most important challenge for applications of Cas nucleases is to solve the problem of off-target cleavage. Indeed sequences partially complementary to the crRNA have been shown to be cleaved at relevant rates [48,82]. For human therapeutics, it is critical that these off-targets are reduced to the bare minimum to enable safe genome editing. Compared with Cas9, Cas12a is substantially more specific due to its decreased tolerance for mismatches during DNA/RNA heteroduplex formation, at least *in vivo* [70]. Characterization of more natural Cas nucleases might reveal even more specific enzymes. In addition, rational engineering combined with laboratory evolution has been used to successfully generate several Cas9 variants with substantially increased specificity [50,83–88].

## Beyond DNA cleavage by Cas9 and Cas12a

Several exciting developments (Table 1) have recently been established in the field of Class II CRISPR nucleases. These developments further highlight the potential of the programmable DNA-specificity of Cas nucleases. Besides Cas9 and Cas12a, other Cas nucleases may prove useful genome editing tools, including Cas12b and Cas12e nucleases for which genome editing has already been demonstrated [63,89]. In addition, a designed variant of a Class 1 complex (Cascade fused to a FokI nuclease domain) has recently been described as an alternative genome editing system [90].

Active nucleases like Cas9 and Cas12a can be converted into nickases or be rendered catalytically inactive by mutating conserved residues in the catalytic sites [25,91,59,60]. Binding of such catalytically inactive Cas nucleases can be used to inhibit gene transcription (CRISPR inhibition, CRISPRi), e.g. by blocking a promoter to prevent binding of RNA polymerase [91–93]. On the other hand, fusions of a Cas nuclease to the RNA polymerase omega subunit can be used to enhance the recruitment of RNA polymerase, promoting expression (CRISPR activation, CRISPRa) of downstream genes [92–94]. This type of targeted gene regulation can be used to study gene functions and is a versatile tool in the field of synthetic biology.

Nickase and catalytically inactive versions of Cas nucleases also offer a tantalizing alternative to the DSB-induced genome editing. The programmable specificity of Cas nucleases can be used to direct fused cytidine or adenine deaminase enzymes to specific DNA stretches. Such base editing has so far been reported for conversions of C\*G pairs to T\*A (using cytidine deaminase fused Cas9 or Cas12a), or A\*T to G\*C (using an evolved and engineered DNA adenine deaminase fused Cas9) [95–98].

Compared with DSB-repair-based genome editing, base editing offers major advantages. Most importantly, base conversions do not rely on HR, NHEJ or other mechanisms of DSB repair. Therefore, base editing could theoretically reach higher efficiencies as it is less dependent on cell type and cell cycle phase. In addition, because no DSB is introduced with base editing, it is unlikely to result in major chromosome rearrangements [99]. Recently, cytosine base editing (but not adenine base editing) was found to induce substantial off-target base substitutions on DNA, possibly due to protospacer independent base conversions [100,101]. In addition, both types of base editors were found to convert off-target bases in cellular RNA, although such RNA activity

**Table 1 Genome editing strategies**

Strategy	Edit type	Features
NHEJ	Small indels [23]	<ul style="list-style-type: none"> <li>• Triggered by DSBs</li> <li>• No control over indels</li> <li>• Useful for knock-outs</li> </ul>
HR	Replacements [22]	<ul style="list-style-type: none"> <li>• Triggered by DSBs</li> <li>• Cell-cycle dependent</li> <li>• Requires repair template</li> </ul>
Counter selection	Selection only	<ul style="list-style-type: none"> <li>• Relies on HR</li> <li>• Enriches edited cells</li> <li>• Useful for prokaryotes</li> </ul>
Base editing [95–97]	Base substitutions	<ul style="list-style-type: none"> <li>• Independent of DNA repair</li> <li>• Single base substitutions</li> <li>• Sensitive to off-targets [100,101]</li> </ul>
Prime editing [104]	Replacements	<ul style="list-style-type: none"> <li>• Independent of DNA repair</li> <li>• Versatile for small edits</li> <li>• Control over exact edit</li> </ul>
Transposition [107,108]	Insertions	<ul style="list-style-type: none"> <li>• Requires transposon proteins</li> <li>• Large insertions possible</li> <li>• So far only in prokaryotes</li> </ul>

Overview of the six main strategies for genome editing based on CRISPR–Cas, listing the type of edit they introduce and three main features per strategy.

could be reduced through protein engineering [102,103]. These studies highlight the importance of genome/transcriptome wide off-target analysis on single cell-derived DNA. Another challenge for base editing is the requirement for precise targeting, which is often limited by PAM availability (see above). Lastly, it is currently difficult to target a single base position for conversion, instead, current base editors may convert any appropriate base within a certain window (although these windows can be very narrow). Whereas this is no problem for gene disruption, it may be a major hurdle for precision gene therapy. Future optimization of base editors is required to address these challenges.

The recent development of prime editing potentially offers a much more versatile method for DSB-independent genome editing [104]. Prime editing uses an extended version of the guide RNA in conjunction with a nickase version of Cas9 fused to reverse transcriptase. The RNA guides the Cas9 fusion to the complementary target on the genome, where Cas9 nicks the displaced strand. The reverse transcriptase then uses the extra part of the guide RNA as a primer, incorporating the guide-encoded edit in the DNA of the displaced strand. Prime editing enables small insertions, deletions, and replacements to be made independent from NHEJ or HR.

Another exciting development is the application of recently discovered CRISPR-associated transposons for both Class I and Class II CRISPR systems [105–108]. These transposons rely on RNA-guided Cas enzymes to direct transposition to positions downstream from protospacers. The involved Cas proteins do not introduce DSBs, but rather guide the transposon proteins [107]. This targeted transposase activity has already been used to integrate large stretches of DNA in the genome of *Escherichia coli* at high efficiency [107,108]. Although it requires transposon proteins in addition to the Cas nuclease protein(s), this method provides a promising alternative to HR-based genomic insertions. The next challenge will be to successfully use these systems for programmable integrations in mammalian cells.

## Perspectives

- The CRISPR–Cas defense system of prokaryotes holds great promise for genome editing in a broad spectrum of organisms, across the entire tree of life. In fact, Cas nuclease-based cleavage and binding of DNA have already proven extremely useful in fundamental research and in microbial and plant biotechnology.



- Apart from the initial biochemical and genetic analyses, a range of high-resolution structures and single-molecule studies have shed light on the molecular mechanism of DNA cleavage by Class 2 Cas nucleases. It has also become apparent that there is room for improving targeting space, specificity, and efficiency for genome editing.
- Engineering by rational design and/or laboratory evolution may result in improved variants of the current set of Cas nucleases, potentially addressing recent challenges. Moving forward, we expect more developments in engineering Cas nucleases for applications like base editing and prime editing. Finally, it is anticipated that future research will reveal CRISPR systems with new Cas proteins, potentially offering new functions as exemplified by the newly discovered role in RNA-guided transposition.

## Abbreviations

AsCas12a, *Acidaminococcus* sp. BV3L6 Cas12a; BH domain, bridge-helix domain; Cas, CRISPR-associated; CRISPR, clustered regularly interspaced short palindromic repeats; CRISPRa, CRISPR activation; CRISPRi, CRISPR inhibition; crRNA, CRISPR RNA; CTD, C-terminal domain; DSB, double-stranded break; HR, homologous recombination; Indels, insertions and deletions; NHEJ, non-homologous end-joining; Nuc domain, 'Nuclease' domain; NUC lobe, nuclease lobe; PAM, protospacer adjacent motif; PI domain, PAM-interacting domain; pre-crRNA, precursor CRISPR RNA; REC lobe, recognition lobe; RNP, ribonucleoprotein; sgRNA, single-guide RNA; SpCas9, *Streptococcus pyogenes* Cas9; Topo domain, Topo-homology domain; tracrRNA, transactivating crRNA; WED domain, Wedge domain.

## Author contributions

T.S. wrote the first draft of the manuscript. All authors have reviewed and revised the manuscript and approved the final version.

## Funding

R.H.J.S. supported by the Dutch Research Council (NWO) Veni grant (016.Veni.171.047 to R.H.J.S.). J.v.d.O. supported by the NWO Spinoza prize (SPI 93-537 to J.v.d.O) and the European Research Council (ERC) (834279-ARGO-ERC-2018-ADG to J.v.d.O).

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

## References

- 1 de la Cruz, F. and Davies, J. (2000) Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends Microbiol.* **8**, 128–133 [https://doi.org/10.1016/S0966-842X\(00\)01703-0](https://doi.org/10.1016/S0966-842X(00)01703-0)
- 2 Gogarten, J.P., Doolittle, W.F. and Lawrence, J.G. (2002) Prokaryotic evolution in light of gene transfer. *Mol. Biol. Evol.* **19**, 2226–2238 <https://doi.org/10.1093/oxfordjournals.molbev.a004046>
- 3 Chibani-Chennoufi, S., Bruttin, A., Dillmann, M.-L. and Brüssow, H. (2004) Phage-host interaction: an ecological perspective. *J. Bacteriol.* **186**, 3677–3686 <https://doi.org/10.1128/JB.186.12.3677-3686.2004>
- 4 Koonin, E.V. and Wolf, Y.I. (2008) Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. *Nucleic Acids Res.* **36**, 6688–6719 <https://doi.org/10.1093/nar/gkn668>
- 5 Koonin, E.V., Makarova, K.S. and Wolf, Y.I. (2017) Evolutionary genomics of defense systems in archaea and bacteria. *Annu. Rev. Microbiol.* **71**, 233–261 <https://doi.org/10.1146/annurev-micro-090816-093830>
- 6 Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S. et al. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709–1712 <https://doi.org/10.1126/science.1138140>
- 7 van der Oost, J., Jore, M.M., Westra, E.R., Lundgren, M. and Brouns, S.J.J. (2009) CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends Biochem. Sci.* **34**, 401–407 <https://doi.org/10.1016/j.tibs.2009.05.002>
- 8 Nuñez, J.K., Kranzusch, P.J., Noeske, J., Wright, A.V., Davies, C.W. and Doudna, J.A. (2014) Cas1–Cas2 complex formation mediates spacer acquisition during CRISPR–Cas adaptive immunity. *Nat. Struct. Mol. Biol.* **21**, 528–534 <https://doi.org/10.1038/nsmb.2820>
- 9 Bolotin, A., Quinquis, B., Sorokin, A. and Ehrlich, S.D. (2005) Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **151**, 2551–2561 <https://doi.org/10.1099/mic.0.28048-0>

- 10 Horvath, P., Romero, D.A., Coûté-Monvoisin, A.-C., Richards, M., Deveau, H., Moineau, S. et al. (2008) Diversity, activity, and evolution of CRISPR Loci in *Streptococcus thermophilus*. *J. Bacteriol.* **190**, 1401–1412 <https://doi.org/10.1128/JB.01415-07>
- 11 Mojica, F.J.M., Díez-Villaseñor, C., García-Martínez, J. and Almendros, C. (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* **155**, 733–740 <https://doi.org/10.1099/mic.0.023960-0>
- 12 Marraffini, L.A. and Sontheimer, E.J. (2010) Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* **463**, 568–571 <https://doi.org/10.1038/nature08703>
- 13 Yosef, I., Goren, M.G. and Qimron, U. (2012) Proteins and DNA elements essential for the CRISPR adaptation process in *Escherichia coli*. *Nucleic Acids Res.* **40**, 5569–5576 <https://doi.org/10.1093/nar/gks216>
- 14 Levy, A., Goren, M.G., Yosef, I., Auster, O., Manor, M., Amitai, G. et al. (2015) CRISPR adaptation biases explain preference for acquisition of foreign DNA. *Nature* **520**, 505–510 <https://doi.org/10.1038/nature14302>
- 15 Radović, M., Killelea, T., Savitskaya, E., Wettstein, L., Bolt, E.L. and Ivančić-Baće, I. (2018) CRISPR–cas adaptation in *Escherichia coli* requires RecBCD helicase but not nuclease activity, is independent of homologous recombination, and is antagonized by 5' ssDNA exonucleases. *Nucleic Acids Res.* **46**, 10173–10183 <https://doi.org/10.1093/nar/gky799>
- 16 Swarts, D.C., Mosterd, C., van Passel, M.W.J. and Brouns, S.J.J. (2012) CRISPR interference directs strand specific spacer acquisition. *PLoS ONE* **7**, e35888 <https://doi.org/10.1371/journal.pone.0035888>
- 17 Datsenko, K.A., Pougach, K., Tikhonov, A., Wanner, B.L., Severinov, K. and Semenova, E. (2012) Molecular memory of prior infections activates the CRISPR/Cas adaptive bacterial immunity system. *Nat. Commun.* **3**, 1–7 <https://doi.org/10.1038/ncomms1937>
- 18 Staats, R.H.J., Jackson, S.A., Biswas, A., Brouns, S.J.J., Brown, C.M. and Fineran, P.C. (2016) Interference-driven spacer acquisition is dominant over naive and primed adaptation in a native CRISPR–Cas system. *Nat. Commun.* **7**, 12853 <https://doi.org/10.1038/ncomms12853>
- 19 Künne, T., Kieper, S.N., Bannenberg, J.W., Vogel, A.I.M., Miellet, W.R., Klein, M. et al. (2016) Cas3-derived target DNA degradation fragments fuel primed CRISPR adaptation. *Mol. Cell.* **63**, 852–864 <https://doi.org/10.1016/j.molcel.2016.07.011>
- 20 Marraffini, L.A. (2015) CRISPR–Cas immunity in prokaryotes. *Nature* **526**, 55–61 <https://doi.org/10.1038/nature15386>
- 21 Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders, S.J. et al. (2015) An updated evolutionary classification of CRISPR–Cas systems. *Nat. Rev. Microbiol.* **13**, 722–736 <https://doi.org/10.1038/nrmicro3569>
- 22 Scully, R., Panday, A., Elango, R. and Willis, N.A. (2019) DNA double-strand break repair-pathway choice in somatic mammalian cells. *Nat. Rev. Mol. Cell Biol.* **20**, 698–714 <https://doi.org/10.1038/s41580-019-0152-0>
- 23 Ceccaldi, R., Rondinelli, B. and D'Andrea, A.D. (2016) Repair pathway choices and consequences at the double-strand break. *Trends Cell Biol.* **26**, 52–64 <https://doi.org/10.1016/j.tcb.2015.07.009>
- 24 Karanam, K., Kafri, R., Loewer, A. and Lahav, G. (2012) Quantitative live cell imaging reveals a gradual shift between DNA repair mechanisms and a maximal use of HR in mid S phase. *Mol. Cell.* **47**, 320–329 <https://doi.org/10.1016/j.molcel.2012.05.052>
- 25 Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 <https://doi.org/10.1126/science.1225829>
- 26 Gasiunas, G., Barrangou, R., Horvath, P. and Siksnys, V. (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl Acad. Sci. U.S.A.* **109**, E2579–E2586 <https://doi.org/10.1073/pnas.1208507109>
- 27 Xie, K. and Yang, Y. (2013) RNA-guided genome editing in plants using a CRISPR–Cas system. *Mol. Plant.* **6**, 1975–1983 <https://doi.org/10.1093/mp/ssl119>
- 28 Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F. et al. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910–918 <https://doi.org/10.1016/j.cell.2013.04.025>
- 29 Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z. et al. (2013) Targeted genome modification of crop plants using a CRISPR–Cas system. *Nat. Biotechnol.* **31**, 686–688 <https://doi.org/10.1038/nbt.2650>
- 30 Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J.D.G. and Kamoun, S. (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* **31**, 691–693 <https://doi.org/10.1038/nbt.2655>
- 31 Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E. et al. (2013) RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826 <https://doi.org/10.1126/science.1232033>
- 32 Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J. et al. (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat. Biotechnol.* **31**, 688–691 <https://doi.org/10.1038/nbt.2654>
- 33 Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D. et al. (2013) Efficient genome editing in zebrafish using a CRISPR–Cas system. *Nat. Biotechnol.* **31**, 227–229 <https://doi.org/10.1038/nbt.2501>
- 34 Friedland, A.E., Tzur, Y.B., Esvelt, K.M., Colaiácovo, M.P., Church, G.M. and Calarco, J.A. (2013) Heritable genome editing in *C. elegans* via a CRISPR–Cas9 system. *Nat. Methods* **10**, 741–743 <https://doi.org/10.1038/nmeth.2532>
- 35 Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N. et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 <https://doi.org/10.1126/science.1231143>
- 36 Bassett, A.R., Tibbit, C., Ponting, C.P. and Liu, J.-L. (2013) Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep.* **4**, 220–228 <https://doi.org/10.1016/j.celrep.2013.06.020>
- 37 Nishimasu, H., Ran, F.A., Hsu, P.D., Konermann, S., Shehata, S.I., Dohmae, N. et al. (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949 <https://doi.org/10.1016/j.cell.2014.02.001>
- 38 Hou, Z., Zhang, Y., Propson, N.E., Howden, S.E., Chu, L.-F., Sontheimer, E.J. et al. (2013) Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. *Proc. Natl Acad. Sci. U.S.A.* **110**, 15644–15649 <https://doi.org/10.1073/pnas.1313587110>
- 39 Ran, F.A., Cong, L., Yan, W.X., Scott, D.A., Gootenberg, J.S., Kriz, A.J. et al. (2015) *In vivo* genome editing using *Staphylococcus aureus* Cas9. *Nature* **520**, 186–191 <https://doi.org/10.1038/nature14299>
- 40 Kim, E., Koo, T., Park, S.W., Kim, D., Kim, K., Cho, H.-Y. et al. (2017) *In vivo* genome editing with a small Cas9 orthologue derived from *Campylobacter jejuni*. *Nat. Commun.* **8**, 14500 <https://doi.org/10.1038/ncomms14500>
- 41 Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y., Pirzada, Z.A. et al. (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **471**, 602–607 <https://doi.org/10.1038/nature09886>

- 42 Sternberg, S.H., Redding, S., Jinek, M., Greene, E.C. and Doudna, J.A. (2014) DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62–67 <https://doi.org/10.1038/nature13011>
- 43 Hirano, H., Gootenberg, J.S., Horii, T., Abudayyeh, O.O., Kimura, M., Hsu, P.D. et al. (2016) Structure and engineering of *Francisella novicida* Cas9. *Cell* **164**, 950–961 <https://doi.org/10.1016/j.cell.2016.01.039>
- 44 Harrington, L.B., Paez-Espino, D., Staahl, B.T., Chen, J.S., Ma, E., Kyrpides, N.C. et al. (2017) A thermostable Cas9 with increased lifetime in human plasma. *Nat. Commun.* **8**, 1424 <https://doi.org/10.1038/s41467-017-01408-4>
- 45 Mougiakos, I., Mohanraju, P., Bosma, E.F., Vrouwe, V., Finger Bou, M., Naduthodi, M.I.S. et al. (2017) Characterizing a thermostable Cas9 for bacterial genome editing and silencing. *Nat. Commun.* **8**, 1647 <https://doi.org/10.1038/s41467-017-01591-4>
- 46 Anders, C., Niewoehner, O., Duerst, A. and Jinek, M. (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* **513**, 569–573 <https://doi.org/10.1038/nature13579>
- 47 Singh, D., Sternberg, S.H., Fei, J., Doudna, J.A. and Ha, T. (2016) Real-time observation of DNA recognition and rejection by the RNA-guided endonuclease Cas9. *Nat. Commun.* **7**, 12778 <https://doi.org/10.1038/ncomms12778>
- 48 Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V. et al. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832 <https://doi.org/10.1038/nbt.2647>
- 49 Mandal, P.K., Ferreira, L.M.R., Collins, R., Meissner, T.B., Boutwell, C.L., Friesen, M. et al. (2014) Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. *Cell Stem Cell*. **15**, 643–652 <https://doi.org/10.1016/j.stem.2014.10.004>
- 50 Slaymaker, I.M., Gao, L., Zetsche, B., Scott, D.A., Yan, W.X. and Zhang, F. (2016) Rationally engineered Cas9 nucleases with improved specificity. *Science* **351**, 84–88 <https://doi.org/10.1126/science.1252227>
- 51 Yang, M., Peng, S., Sun, R., Lin, J., Wang, N. and Chen, C. (2018) The conformational dynamics of Cas9 governing DNA cleavage are revealed by single-molecule FRET. *Cell Rep.* **22**, 372–382 <https://doi.org/10.1016/j.celrep.2017.12.048>
- 52 Stephenson, A.A., Raper, A.T. and Suo, Z. (2018) Bidirectional degradation of DNA cleavage products catalyzed by CRISPR/Cas9. *J. Am. Chem. Soc.* **140**, 3743–3750 <https://doi.org/10.1021/jacs.7b13050>
- 53 Sternberg, S.H., LaFrance, B., Kaplan, M. and Doudna, J.A. (2015) Conformational control of DNA target cleavage by CRISPR–Cas9. *Nature* **527**, 110–113 <https://doi.org/10.1038/nature15544>
- 54 Ungerer, J. and Pakrasi, H.B. (2016) Cpf1 is a versatile tool for CRISPR genome editing across diverse species of cyanobacteria. *Sci. Rep.* **6**, 39681 <https://doi.org/10.1038/srep39681>
- 55 Yan, M.-Y., Yan, H.-Q., Ren, G.-X., Zhao, J.-P., Guo, X.-P. and Sun, Y.-C. (2017) CRISPR–Cas12a-assisted recombineering in bacteria. *Appl. Environ. Microbiol.* **83**, e00947-17 <https://doi.org/10.1128/AEM.00947-17>
- 56 Świat, M.A., Dashko, S., den Ridder, M., Wijsman, M., van der Oost, J., Daran, J.-M. et al. (2017) Fncpf1: a novel and efficient genome editing tool for *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **45**, 12585–12598 <https://doi.org/10.1093/nar/gkx1007>
- 57 Begemann, M.B., Gray, B.N., January, E., Gordon, G.C., He, Y., Liu, H. et al. (2017) Precise insertion and guided editing of higher plant genomes using Cpf1 CRISPR nucleases. *Sci. Rep.* **7**, 11606 <https://doi.org/10.1038/s41598-017-11760-6>
- 58 Kim, D., Kim, J., Hur, J.K., Been, K.W., Yoon, S. and Kim, J.-S. (2016) Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. *Nat. Biotechnol.* **34**, 863–868 <https://doi.org/10.1038/nbt.3609>
- 59 Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P. et al. (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR–Cas system. *Cell* **163**, 759–771 <https://doi.org/10.1016/j.cell.2015.09.038>
- 60 Yamano, T., Nishimasu, H., Zetsche, B., Hirano, H., Slaymaker, I.M., Li, Y. et al. (2016) Crystal structure of Cpf1 in complex with guide RNA and target DNA. *Cell* **165**, 949–962 <https://doi.org/10.1016/j.cell.2016.04.003>
- 61 Gao, P., Yang, H., Rajashankar, K.R., Huang, Z. and Patel, D.J. (2016) Type V CRISPR–Cas Cpf1 endonuclease employs a unique mechanism for crRNA-mediated target DNA recognition. *Cell Res.* **26**, 901–913 <https://doi.org/10.1038/cr.2016.88>
- 62 Swarts, D.C., van der Oost, J. and Jinek, M. (2017) Structural basis for guide RNA processing and seed-dependent DNA targeting by CRISPR–Cas12a. *Mol. Cell*. **66**, 221–233.e4 <https://doi.org/10.1016/j.molcel.2017.03.016>
- 63 Strecker, J., Jones, S., Koopal, B., Schmid-Burgk, J., Zetsche, B., Gao, L. et al. (2019) Engineering of CRISPR–Cas12b for human genome editing. *Nat. Commun.* **10**, 212 <https://doi.org/10.1038/s41467-018-08224-4>
- 64 Fonfara, I., Richter, H., Bratovič, M., Le Rhun, A. and Charpentier, E. (2016) The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature* **532**, 517–521 <https://doi.org/10.1038/nature17945>
- 65 Adiego-Pérez, B., Randazzo, P., Daran, J.M., Verwaal, R., Roubos, J.A., Daran-Lapujade, P. et al. (2019) Multiplex genome editing of microorganisms using CRISPR–Cas. *FEMS Microbiol. Lett.* **366**, fnz086. <https://doi.org/10.1093/femsle/fnz086>
- 66 Dong, D., Ren, K., Qiu, X., Zheng, J., Guo, M., Guan, X. et al. (2016) The crystal structure of Cpf1 in complex with CRISPR RNA. *Nature* **532**, 522–526 <https://doi.org/10.1038/nature17944>
- 67 Jeon, Y., Choi, Y.H., Jang, Y., Yu, J., Goo, J., Lee, G. et al. (2018) Direct observation of DNA target searching and cleavage by CRISPR–Cas12a. *Nat. Commun.* **9**, 1–11 <https://doi.org/10.1038/s41467-018-05245-x>
- 68 Singh, D., Mallon, J., Poddar, A., Wang, Y., Tippiana, R., Yang, O. et al. (2018) Real-time observation of DNA target interrogation and product release by the RNA-guided endonuclease CRISPR Cpf1 (Cas12a). *Proc. Natl Acad. Sci. U.S.A.* **115**, 5444–5449 <https://doi.org/10.1073/pnas.1718686115>
- 69 Strohkendl, I., Saifuddin, F.A., Rybarski, J.R., Finkelstein, I.J. and Russell, R. (2018) Kinetic basis for DNA target specificity of CRISPR–Cas12a. *Mol. Cell*. **71**, 816–824.e3 <https://doi.org/10.1016/j.molcel.2018.06.043>
- 70 Kleinstiver, B.P., Tsai, S.Q., Prew, M.S., Nguyen, N.T., Welch, M.M., Lopez, J.M. et al. (2016) Genome-wide specificities of CRISPR–Cas Cpf1 nucleases in human cells. *Nat. Biotechnol.* **34**, 869–874 <https://doi.org/10.1038/nbt.3620>
- 71 Swarts, D.C. and Jinek, M. (2018) Cas9 versus Cas12a/Cpf1: structure–function comparisons and implications for genome editing. *Wiley Interdiscip. Rev. RNA*. **9**, e1481 <https://doi.org/10.1002/wrna.1481>
- 72 Li, S.-Y., Cheng, Q.-X., Liu, J.-K., Nie, X.-Q., Zhao, G.-P. and Wang, J. (2018) CRISPR–cas12a has both cis - and trans -cleavage activities on single-stranded DNA. *Cell Res.* **28**, 491–493 <https://doi.org/10.1038/s41422-018-0022-x>
- 73 Chen, J.S., Ma, E., Harrington, L.B., Costa, M.D., Tian, X., Palefsky, J.M. et al. (2018) CRISPR–cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* **360**, 436–439 <https://doi.org/10.1126/science.aar6245>

- 74 Jiang, Y., Qian, F., Yang, J., Liu, Y., Dong, F., Xu, C. et al. (2017) CRISPR–cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nat. Commun.* **8**, 15179 <https://doi.org/10.1038/ncomms15179>
- 75 Kleinstiver, B.P., Prew, M.S., Tsai, S.Q., Topkar, V.V., Nguyen, N.T., Zheng, Z. et al. (2015) Engineered CRISPR–Cas9 nucleases with altered PAM specificities. *Nature* **523**, 481–485 <https://doi.org/10.1038/nature14592>
- 76 Kleinstiver, B.P., Prew, M.S., Tsai, S.Q., Nguyen, N.T., Topkar, V.V., Zheng, Z. et al. (2015) Broadening the targeting range of *Staphylococcus aureus* CRISPR–Cas9 by modifying PAM recognition. *Nat. Biotechnol.* **33**, 1293–1298 <https://doi.org/10.1038/nbt.3404>
- 77 Gao, L., Cox, D.B.T., Yan, W.X., Manteiga, J.C., Schneider, M.W., Yamano, T. et al. (2017) Engineered Cpf1 variants with altered PAM specificities. *Nat. Biotechnol.* **35**, 789–792 <https://doi.org/10.1038/nbt.3900>
- 78 Hu, J.H., Miller, S.M., Geurts, M.H., Tang, W., Chen, L., Sun, N. et al. (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* **556**, 57–63 <https://doi.org/10.1038/nature26155>
- 79 Hegge, J.W., Swarts, D.C. and Oost van der, J. (2018) Prokaryotic Argonaute proteins: novel genome-editing tools? *Nat. Rev. Microbiol.* **16**, 5–11 <https://doi.org/10.1038/nrmicro.2017.73>
- 80 Hegge, J.W., Swarts, D.C., Chandradoss, S.D., Cui, T.J., Kneppers, J., Jinek, M. et al. (2019) DNA-guided DNA cleavage at moderate temperatures by *Clostridium butyricum* Argonaute. *Nucleic Acids Res.* **47**, 5809–5821 <https://doi.org/10.1093/nar/gkz306>
- 81 Lin, S., Staahl, B.T., Alla, R.K. and Doudna, J.A. (2014) Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife* **3**, e04766 <https://doi.org/10.7554/eLife.04766>
- 82 Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K. et al. (2013) High-frequency off-target mutagenesis induced by CRISPR–Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822–826 <https://doi.org/10.1038/nbt.2623>
- 83 Kleinstiver, B.P., Pattanayak, V., Prew, M.S., Tsai, S.Q., Nguyen, N.T., Zheng, Z. et al. (2016) High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **529**, 490–495 <https://doi.org/10.1038/nature16526>
- 84 Casini, A., Olivieri, M., Petris, G., Montagna, C., Reginato, G., Maule, G. et al. (2018) A highly specific SpCas9 variant is identified by *in vivo* screening in yeast. *Nat. Biotechnol.* **36**, 265–271 <https://doi.org/10.1038/nbt.4066>
- 85 Chen, J.S., Dagdas, Y.S., Kleinstiver, B.P., Welch, M.M., Sousa, A.A., Harrington, L.B. et al. (2017) Enhanced proofreading governs CRISPR–Cas9 targeting accuracy. *Nature* **550**, 407–410 <https://doi.org/10.1038/nature24268>
- 86 Lee, J.K., Jeong, E., Lee, J., Jung, M., Shin, E., Kim, Y. et al. (2018) Directed evolution of CRISPR–Cas9 to increase its specificity. *Nat. Commun.* **9**, 1–10 <https://doi.org/10.1038/s41467-018-05477-x>
- 87 Kim, S., Bae, T., Hwang, J. and Kim, J.-S. (2017) Rescue of high-specificity Cas9 variants using sgRNAs with matched 5' nucleotides. *Genome Biol.* **18**, 218 <https://doi.org/10.1186/s13059-017-1355-3>
- 88 Singh, D., Wang, Y., Mallon, J., Yang, O., Fei, J., Poddar, A. et al. (2018) Mechanisms of improved specificity of engineered Cas9s revealed by single-molecule FRET analysis. *Nat. Struct. Mol. Biol.* **25**, 347–354 <https://doi.org/10.1038/s41594-018-0051-7>
- 89 Liu, J.-J., Orlova, N., Oakes, B.L., Ma, E., Spinner, H.B., Baney, K.L.M. et al. (2019) Casx enzymes comprise a distinct family of RNA-guided genome editors. *Nature* **566**, 218–223 <https://doi.org/10.1038/s41586-019-0908-x>
- 90 Cameron, P., Coons, M.M., Klompe, S.E., Lied, A.M., Smith, S.C., Vidal, B. et al. (2019) Harnessing type I CRISPR–Cas systems for genome engineering in human cells. *Nat. Biotechnol.* **37**, 1471–1477 <https://doi.org/10.1038/s41587-019-0310-0>
- 91 Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P. et al. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173–1183 <https://doi.org/10.1016/j.cell.2013.02.022>
- 92 Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F. and Marraffini, L.A. (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR–Cas system. *Nucleic Acids Res.* **41**, 7429–7437 <https://doi.org/10.1093/nar/gkt520>
- 93 Kampmann, M. (2018) CRISPRi and CRISPRa screens in mammalian cells for precision biology and medicine. *ACS Chem. Biol.* **13**, 406–416 <https://doi.org/10.1021/acschembio.7b00657>
- 94 Dove, S.L. and Hochschild, A. (1998) Conversion of the  $\omega$  subunit of *Escherichia coli* RNA polymerase into a transcriptional activator or an activation target. *Genes Dev.* **12**, 745–754 <https://doi.org/10.1101/gad.12.5.745>
- 95 Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A. and Liu, D.R. (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420 <https://doi.org/10.1038/nature17946>
- 96 Nishida, K., Arazoe, T., Yachie, N., Banno, S., Kakimoto, M., Tabata, M. et al. (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science* **353**, aaf8729 <https://doi.org/10.1126/science.aaf8729>
- 97 Gaudelli, N.M., Komor, A.C., Rees, H.A., Packer, M.S., Badran, A.H., Bryson, D.I. et al. (2017) Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* **551**, 464–471 <https://doi.org/10.1038/nature24644>
- 98 Li, X., Wang, Y., Liu, Y., Yang, B., Wang, X., Wei, J. et al. (2018) Base editing with a Cpf1–cytidine deaminase fusion. *Nat. Biotechnol.* **36**, 324–327 <https://doi.org/10.1038/nbt.4102>
- 99 Komor, A.C., Zhao, K.T., Packer, M.S., Gaudelli, N.M., Waterbury, A.L., Koblan, L.W. et al. (2017) Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T:A base editors with higher efficiency and product purity. *Sci. Adv.* **3**, eaao4774 <https://doi.org/10.1126/sciadv.aao4774>
- 100 Zuo, E., Sun, Y., Wei, W., Yuan, T., Ying, W., Sun, H. et al. (2019) Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. *Science* **364**, 289–292 <https://doi.org/10.1126/science.aav9973>
- 101 Jin, S., Zong, Y., Gao, Q., Zhu, Z., Wang, Y., Qin, P. et al. (2019) Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. *Science* **364**, 292–295 <https://doi.org/10.1126/science.aaw7166>
- 102 Grünwald, J., Zhou, R., Garcia, S.P., Iyer, S., Lareau, C.A., Aryee, M.J. et al. (2019) Transcriptome-wide off-target RNA editing induced by CRISPR-guided DNA base editors. *Nature* **569**, 433–437 <https://doi.org/10.1038/s41586-019-1161-z>
- 103 Rees, H.A., Wilson, C., Doman, J.L. and Liu, D.R. (2019) Analysis and minimization of cellular RNA editing by DNA adenine base editors. *Sci. Adv.* **5**, eaax5717 <https://doi.org/10.1126/sciadv.aax5717>
- 104 Anzalone, A.V., Randolph, P.B., Davis, J.R., Sousa, A.A., Koblan, L.W., Levy, J.M. et al. (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576**, 149–157 <https://doi.org/10.1038/s41586-019-1711-4>
- 105 Peters, J.E., Makarova, K.S., Shmakov, S. and Koonin, E.V. (2017) Recruitment of CRISPR–Cas systems by Tn7-like transposons. *Proc. Natl Acad. Sci. U.S.A.* **114**, E7358–E7366 <https://doi.org/10.1073/pnas.1709035114>

- 106 Faure, G., Shmakov, S.A., Yan, W.X., Cheng, D.R., Scott, D.A., Peters, J.E. et al. (2019) CRISPR–cas in mobile genetic elements: counter-defence and beyond. *Nat. Rev. Microbiol.* **17**, 513–525 <https://doi.org/10.1038/s41579-019-0204-7>
- 107 Strecker, J., Ladha, A., Gardner, Z., Schmid-Burgk, J.L., Makarova, K.S., Koonin, E.V. et al. (2019) RNA-guided DNA insertion with CRISPR-associated transposases. *Science* **365**, 48–53 <https://doi.org/10.1126/science.aax9181>
- 108 Klompe, S.E., Vo, P.L.H., Halpin-Healy, T.S. and Sternberg, S.H. (2019) Transposon-encoded CRISPR–Cas systems direct RNA-guided DNA integration. *Nature* **571**, 219–225 <https://doi.org/10.1038/s41586-019-1323-z>
- 109 Schrödinger, L.L.C. (2019) The PyMOL Molecular Graphics System, Version 2.3. 2019