Expanding Genome Editing Beyond Traditional CRISPR Nucleases

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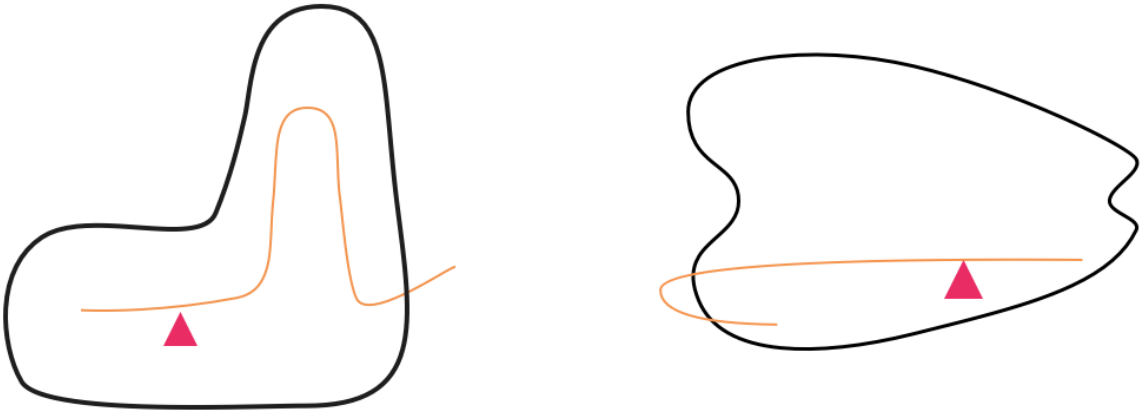


Most CRISPR research to date has focused on *Streptococcus pyogenes* Cas9 (SpCas9), the first CRISPR nuclease used to edit eukaryotic DNA (Jinek, Chylinski et al. 2012; Cong, Ran et al. 2013; Mali, Yang et al. 2013). Recombinant nuclease studies, predictive algorithm development and therapeutic applications are generally catered to this nuclease due to its early success both *in vitro* and *in vivo*. SpCas9 alone has led to ample excitement over the possibilities of CRISPR both in the lab and the clinic.

However, other Cas9 orthologs and Cas family nucleases have since been discovered in other species of bacteria. These nucleases offer some benefits over SpCas9, particularly in the context of eukaryotic genome editing, and can be used orthogonally for more complex genome editing experiments (Esvelt, Mali et al. 2013). The utility of this approach has already been demonstrated in large-scale genetic screens (Najm, Strand, Donovan, Hegde, Sanson et al. 2017).

One of the key benefits of using CRISPR nucleases other than SpCas9 is that many are smaller than SpCas9, which may aid in CRISPR delivery. These nucleases also recognize different PAM sites than SpCas9 (NGG), potentially opening up the targeting range of the nuclease for new genome editing options (**Table 1**). Others feature a tolerance for high temperatures and environments such as human serum which can be hostile to exogenous proteins. In this article, we will explore these nucleases and their unique features compared to standard SpCas9 approaches.

Discovering Cas9 in Other Species

One of the most famous orthologs of SpCas9 was found in *Staphylococcus aureus* early in the history of CRISPR genome editing (Cong, Ran et al. 2013). Its PAM specificities (NNGRRT) and other qualities were characterized in-depth not long after (Friedland et al. 2015). SaCas9, like many other orthologs, is significantly smaller (3.16 kb) than traditional SpCas9 (4.1 kb) (**Table 1**). This helps researchers looking for new and effective ways

to deliver CRISPR, particularly in a therapeutic context.

Ran, Cong, Yan and coauthors demonstrated this principle by delivering SaCas9 and a guide RNA *in vivo* in a single adeno-associated virus (AAV) vector to successfully edit the cholesterol gene *Pcsk9* in mice (Ran, Cong, Yan et al. 2015). This approach is far more difficult with SpCas9 because AAV has a limited cargo size, and SpCas9 is 1 kb larger than SaCas9. Using just one (instead of multiple) vectors maintains efficiency of expression and editing. The smaller size of SaCas9 therefore opens doors for further *in vivo* work with AAV, a common vector of choice. Another team reiterated this point by packaging four guides with SaCas9 into a single AAV vector to target and remove HIV-1 provirus in mice (Yin, Zhang, Qu et al. 2017).

Cas9 Orthologs

SPECIES OF ORIGIN	NAME	SIZE	PRIMARY PAM RECOGNITION
Streptococcus pyogenes	SpCas9	4.1 kb	NGG
Staphylococcus aureus	SaCas9	3.16 kb	NNGRRT
Neisseria meningitidis	NmCas9	3.25 kb	NNNNGATT
Streptococcus thermophilus	St1Cas9	3.3 kb	NNAGAAW
Campylobacter jejuni	CjCas9	2.95 kb	NNNNRYAC
Geobacillus stearothermophilus	GeoCas9	3.26 kb	NNNNCRAA

Table 1. *Cas9 orthologs. A sample of Cas9 orthologs from various species with unique PAM specificities and sizes, expanding possibilities for basic and translational CRISPR research.*

Another Cas9 ortholog, this one from the bacterium *Neisseria meningitidis*, has drawn attention from biomedical researchers. The enzyme, termed NmCas9, has already been used to edit pluripotent stem cells (Hou, Zhang et al. 2013) and has helped drive study into anti-CRISPRs which act as off-switches for editing activity (Pawluk et al. 2016; Harrington, Doxzen et al. 2017). Like SaCas9, NmCas9 is a smaller nuclease (3.25 kb) which comes with the added benefit of being potentially much more specific than its counterparts (Amrani, Gao et al. 2017). The increased specificity of NmCas9 may be in part due to the fact that it recognizes a longer PAM (**Table 1**), or that it supports a longer protospacer (24 nucleotides instead of 20).

St1Cas9, derived from *Streptococcus thermophilus*, offers another alternative to SpCas9 (Gasiunas et al. 2012; Cong, Ran et al. 2013; Karvelis, Gasiunas et al. 2013) and was shown to successfully edit human cells in 2014 (Xu et al. 2014). In 2016, it was characterized as a more specific nuclease due in part to the fact that it recognizes longer

PAM sites than SpCas9 (**Table 1**) (Müller et al. 2016). Although St1Cas9 is smaller than SpCas9, it's still a bit larger than SaCas9 and NmCas9.

In 2017, Harrington et al. reported an interesting alternative to SpCas9 called GeoCas9 (from thermophilic bacterium *Geobacillus stearothermophilus*). While it is notable for being about the same size as NmCas9, what makes it particularly unique is that the enzyme remains active up to a much higher temperature (70 degrees) than other orthologs (45 degrees). The authors demonstrated that the robustness of GeoCas9 also allows it to remain stable and active in ribonucleoprotein form in human plasma for longer than SpCas9 (Harrington et al. 2017).

One ortholog that has also captured the focus of some CRISPR researchers is CjCas9, derived from *Campylobacter jejuni*. First introduced by Kim, Koo, Park and authors, this new enzyme is a mere 2.95 kb in size and can induce genome edits *in vivo* (Kim, Koo, Park et al. 2017). Like NmCas9 and GeoCas9, CjCas9 was found to be susceptible to anti-CRISPR off-switches which further expands safety options for therapeutic applications (Harrington, Doxzen et al. 2017).

Cpf1 As An Alternative to Cas9

Cpf1 (also known as Cas12a) is a Class II CRISPR nuclease that was also discovered in bacteria. Out of eight Cpf1-family proteins initially reported, only two (7-AsCpf1 from *Acidaminococcus* and 13-LbCpf1 from *Lachnospiraceae*) seem to have highly-specific cutting ability in mammalian (HEK293FT) cells. Cpf1 uses a single, much shorter 42 base pair CRISPR RNA (crRNA) as opposed to a full 100 nucleotide single guide RNA (sgRNA) used by most Cas9 orthologs (Zetsche, Gootenberg et al. 2015).

Cpf1 is unique in that it induces a staggered break (5 bp overhang) in target DNA while SpCas9 and its orthologs generate a blunt cut (**Fig. 1**). It's also a bit smaller nuclease than SpCas9 at 3.7-3.9 kb which, combined with the shorter crRNA, may make it easier to package into viral vectors. Finally, Cpf1's ability to recognize AT-rich PAM sites (TTTV) suggest the enzyme will prove useful in targeting repetitive, NGG-poor loci in the genome (Zetsche, Gootenberg et al. 2015).

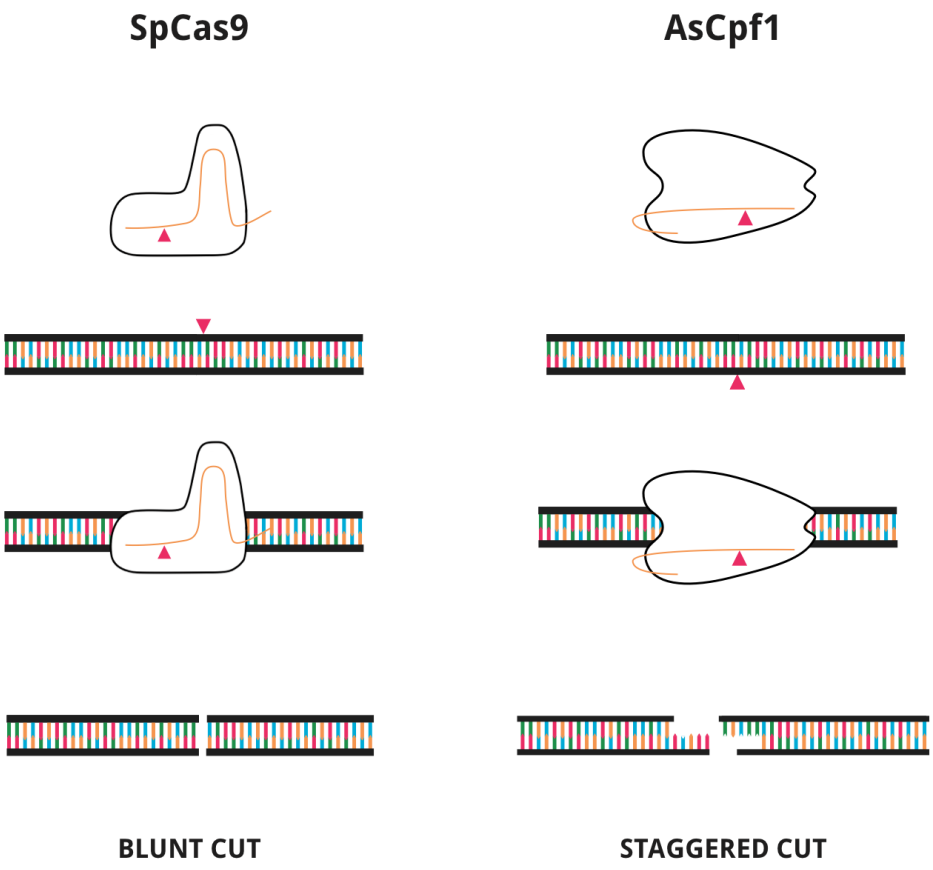


Figure 1. *SpCas9 and Cpf1. In contrast to SpCas9, AsCpf1 generates a staggered cut in target DNA with 5 base pair overhangs. AsCpf1 also uses a smaller crRNA guide rather than a single guide RNA (sgRNA).*

For these reasons, Cpf1 has attracted the attention of both basic and translational researchers. Cpf1 was used by Kim and coworkers to generate *Trp53* knockout mice in 2016 (Kim et al. 2016). Other studies have demonstrated the utility of catalytically deactivated AsCpf1 as an epigenetic regulator in prokaryotic (Zhang, Wang, Cheng et al. 2017) and eukaryotic (Liu, Han, Chen et al. 2017) cells. Cpf1 has also been shown to work well with dual-guide CRISPR approaches, allowing for for easier co-knockout experiments (Zetsche, Heidenreich, Mohanraju et al. 2017; Zhong et al. 2017).

Yang, Wei and co-authors demonstrated the orthogonality of Cpf1 by showing that it could uniquely disrupt oncogenic mutant *BRAF* in A375 cells while avoiding the gene's healthy wild-type counterpart. By contrast, wild-type Cas9 could not discern between the two. The reengineered Cas9 variant EQR yielded no detectable editing events (Yang, Wei et al. 2017).

Cpf1 has been used in other biomedical contexts, as well. Zhang and Long and coauthors edited induced pluripotent stem cells (iPSCs) for transplantation into mice with Duchenne muscular dystrophy, providing a potential *ex vivo* therapeutic strategy for the genetic disorder (Zhang, Long et al. 2017). In another study, Gaj and coworkers delivered Cpf1 to mice with amyotrophic lateral sclerosis (ALS) *in vivo* using AAV. The authors targeted

a *SOD1* mutation which reduced mortality and improved symptoms in the treated mice (Gaj et al. 2017).

Some CRISPR Nucleases Target RNA, Not DNA

Cas13a, formerly known as LsC2c2, was discovered in the bacterium *Leptotrichia shahii* and is functionally different from most other CRISPR nucleases. Instead of targeting DNA, Cas13a binds and destroys target messenger RNA (mRNA) (**Fig. 2**) (Abudayyeh, Gootenberg, Konermann et al. 2016). This is similar in mechanism to RNAi, which uses an small hairpin/interfering RNA (sh/siRNA) to guide a protein complex to degrade gene transcripts.

Both RNAi and Cas13a generate a similar transient phenotypic outcome to CRISPR interference (CRISPRi), a technique you can read more about in our Resource. However, unlike RNAi, Cas13a relies on an additional protospacer flanking site (PFS) binding event which, like a PAM in traditional CRISPR experiment, is requisite for nuclease activity (Abudayyeh, Gootenberg, Konermann et al. 2016). This may explain why Cas13a seems more specific than promiscuous RNAi methods (Abudayyeh, Gootenberg et al. 2017).

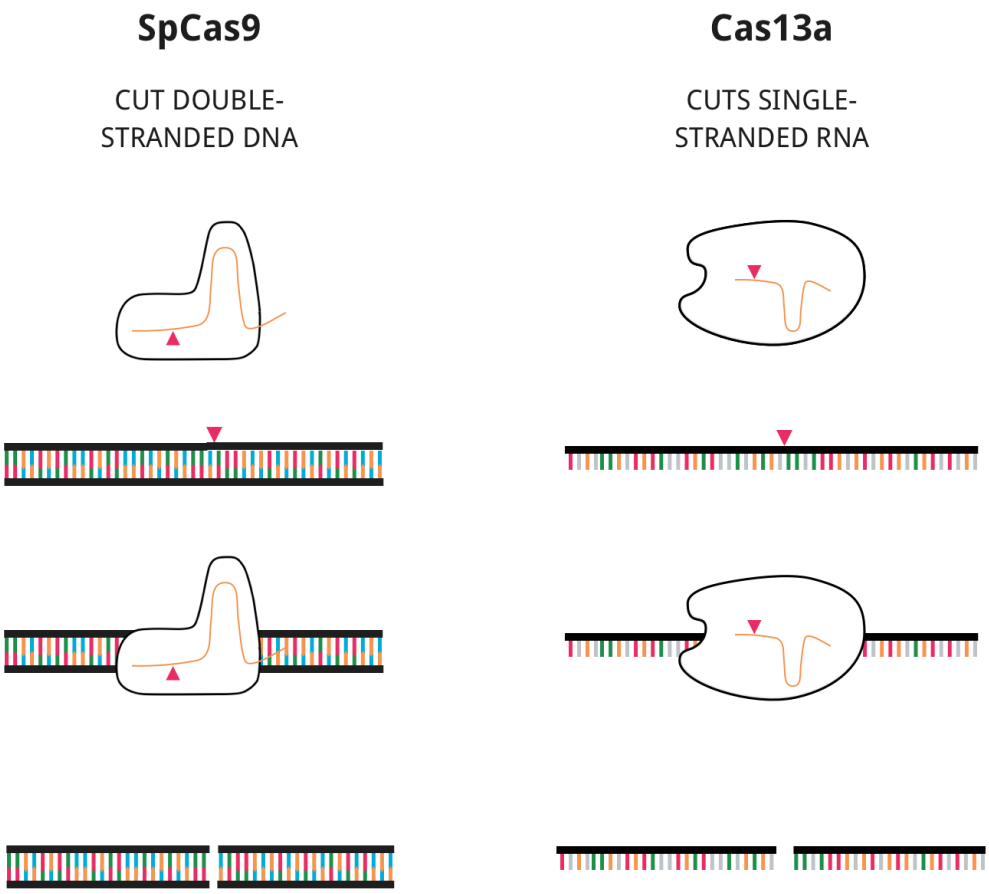


Figure 2. *SpCas9 and Cas13a. Cas13a, also known as C2c2, differentiates itself from SpCas9 in that it cleaves single-stranded RNA as opposed to double-stranded DNA. Figure adapted from Abudayyeh, Gootenberg, Konermann et al. 2016.*

Since its characterization, Cas13a has been used in several different contexts. In one example, Abudayyeh, Gootenberg et al. demonstrated how an ortholog of LsC2c2 from *Leptotrichia wadei* (LwaCas13a) can be used in mammalian and plant cells (Abudayyeh, Gootenberg et al. 2017). A follow-up report suggested that LwaCas13a can sensitively detect RNA viruses like Dengue and Zika, genotype human cells and more (Gootenberg, Abudayyeh et al. 2017). Subsequent work has been done to repurpose C2c2 as a base editor, the mechanism of which we cover in our Resource.

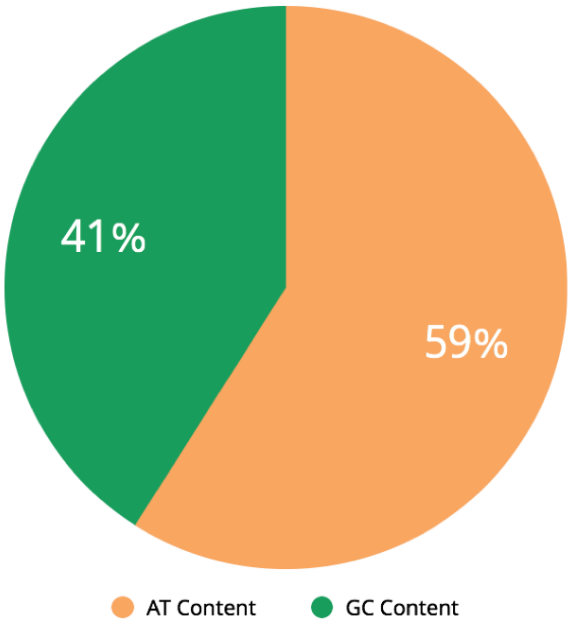
Excitingly, a report by Strutt and co-authors indicates that even some Cas9 orthologs have single-stranded RNA-targeting capability. The authors report that while SpCas9 does not cut RNA in the absence of a PAM or similar site, SaCas9 and CjCas9 are PAM-independent. The report focuses on the more robustly characterized SaCas9 and indicates that its RNA-cutting ability seems to depend on the target being complementary single-stranded RNA (Strutt et al. 2018).

Strutt and coworkers suggest that RNA-targeting CRISPR nucleases are an evolutionarily useful defense against RNA viruses. They demonstrated this by expressing SaCas9 in *E. coli* and measuring successful resistance against MS2 phage. The authors then showed that dSaCas9 can bind to RNA without degrading it as an alternative strategy to gene knockdown (Strutt et al. 2018).

Bringing CRISPR Alternatives Into the Lab

SpCas9 remains a gold standard enzyme which will forever hold historical importance for demonstrating the power of CRISPR genome editing to the world. However, it is critical to the progression of the field to acknowledge the depth and breadth of SpCas9 alternatives. We have endeavored to cover many of the most exciting examples in this Resource, but more are bound to be discovered, characterized and utilized in the coming years.

The potential for SpCas9 alternatives to improve the targeting range of CRISPR screens alone is transformative. Expanding the number of potential PAM sites that a library can target is key when looking at difficult-to-target loci, particularly in repetitive, AT-rich noncoding DNA for which unique SpCas9 guide RNAs are difficult to design (**Fig. 3**). Supporting this kind of library with novel predictive algorithms will be an essential step in increasing the efficiency and reliability of screens. Right now, most of these algorithms are catered to SpCas9, indicating a need for more large-scale experiments using alternative nucleases.



ADAPTED FROM LANDER ET AL. 2001

Figure 3. *AT and GC content of the human genome. The human genome is mostly AT (59%), and repetitive noncoding regulatory regions are often AT-rich. Targeting these regions with standard SpCas9, which recognizes NGG PAM sites, can be difficult. Data adapted from from Lander et al. 2001.*

Beyond the lab, therapeutic development of CRISPR depends heavily on improving the specificity of CRISPR to ensure safety. Without going through the trouble of developing specially mutated SpCas9 variants or fusion nucleases, naturally occurring options like NmCas9 may serve the same purpose. Moreover, Cas9 orthologs may induce different immune responses in the host than SpCas9. Reaching beyond the standard CRISPR nuclease will help researchers develop safer translational applications for their work both *ex vivo* through cell therapy and *in vivo*.

Increased specificity, broader delivery options and robust nuclease activity under adverse conditions are all exciting features of alternative nucleases. Where we take these enzymes remains to be seen, but the need and opportunity for executing studies with these proteins is clear. A world of possibilities opens up when we look beyond the basics of genome editing.

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