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CRISPR-Cas13 and its Applications in Human Therapeutics

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Abstract

CRISPR technology has revolutionized molecular biology, initiating large shifts in genetics and opening many opportunities to employ this technology for human advantage. However, its applications are as diverse as the family of CRISPR itself. In light of this diversity, this paper explores a specific type of CRISPR system— Cas13— and its various applications in human therapeutics ranging from past work involving cancer and the Zika virus, to the present SARS-CoV-2 pandemic. This paper reviews past, well-established research on Cas13 as well as recent, less-known discoveries and testing involving this nuclease. In the process, it highlights how most applications of Cas13 employ the Cas13a and Cas13b variants, while the newer Cas13d variant is under-used though it has arguably demonstrated superiority over its counterparts in all aspects including viral-packing compactness, RNA editing efficiency, and versatility.

Keywords

Cellular Biology, Genetics, Cellular Immunology, CRISPR, Cas13

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Introduction

Despite CRISPR-Cas13 systems' recent discovery, this family of CRISPR proteins has already proven to demonstrate immense potential in the fields of nucleic acid detection as well as gene therapies, including therapies that are already being tested and implemented to tackle global problems including the SARS-CoV-2 pandemic. Considering most information regarding this protein family is quite new, this review aims to amalgamate the recently gained knowledge and recent applications of the Cas13 family thus far, while reflecting on how the preference of certain types of Cas13 may have impacted efficiency as well as the family's demonstrated potential for nucleic acid editing and detecting technology.

Discussion

CRISPR-Cas13

CRISPR

Clustered, regularly interspaced, short palindromic repeats or CRISPR are essentially DNA loci with short DNA repeats that are separated by spacers of foreign origin (9). CRISPR regions are transcribed into CRISPR-RNAs (crRNAs) which are associated with Cas nucleases to locate and cleave complementary nucleic acid sequences. Together, the RNA-guided Cas protein and CRISPR region provide bacterial and Archean hosts protection from viruses and other mobile genetic elements through adaptive immunity, as described in Figure 1.

Researchers have identified two classes of CRISPR-Cas systems according to their "Cas gene content" and "mechanism of immunity" (9); Class 1 systems include crRNA with multiple Cas proteins in a complex and are encoded by 3-6 Cas genes (17). Types I, II and IV fall under this class. On the other hand, Class 2 systems include crRNA and a single Cas protein with multiple domains. Systems under this class are rarer, and include types II, V and VI, where type VI refers to Cas13 systems. Most Class 2 systems are DNA cleaving, including the well-known Cas9 and Cas12, however, Cas13 is RNA cleaving (10). Figure 2 describes some differences and similarities between Cas13 and Cas9 systems.

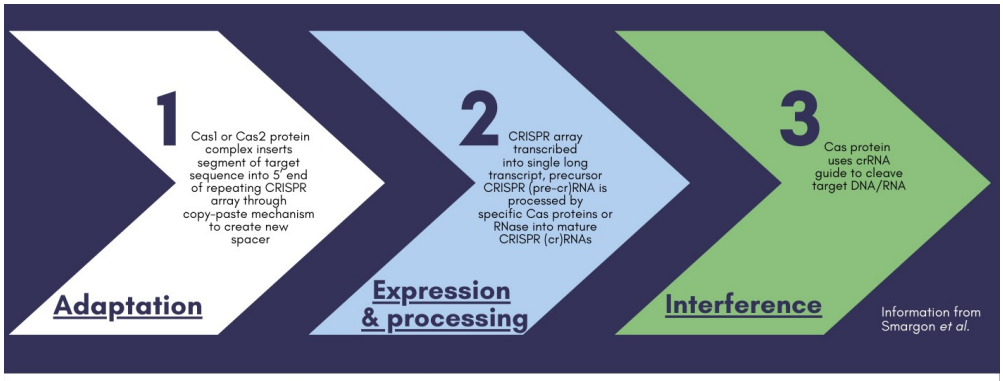


Figure 1: CRISPR Mediated Defence

Figure 2: Comparing Cas13 and Cas9

Information from:
O' Connell, Granados-Riveron and Arquino-Jarquin, Freije *et al.*

Cas13 systems

Formerly known as C2c2, Cas13 systems perform nonspecific RNA degradation using *trans*-RNase activity to arrest the growth of RNA and hence halt virus propagation in bacterial host cells, while allowing uninfected cells to survive (9). While the genome of the phage is not directly affected, the Cas nuclease prevents target transcripts from being produced. The discovery of type V Cas systems introduced the possibility that more types of Cas may exist, and indeed they did; Cas13 has more than four subtypes of A, B (B1 and B2), C and D, which were later characterized. All four types lack adaptation genes that facilitate evolution by cleaving DNA sequences in the host's genome. Though the reasons they lack these genes are unclear, it is most likely because they use modules from other loci, or perhaps they have lost the adaptation genes and do not acquire new protospacers (10).

Structure

The Cas13 nuclease domain is organized into two lobes: the crRNA recognition lobe and the nuclease lobe. The crRNA recognition lobe contains an N-Terminal Domain (NTD) which provides structure to the protein, and a Helical-1 domain which holds the RNA by binding to the stem. On the other hand, the nuclease lobe contains the Helical-2, HEPN-1 (I and II) and HEPN-2 domains, along with a helical linker. Cas13a homologs demonstrated that the HEPN2 and Helical 1 residues play a role in sequence-specific crRNA processing, which illustrates a similarity between type V-A and VI systems as both have dedicated nuclease domains for crRNA processing. However, Cas13's RNA targeting function can help avoid unintended DNA binding and allow the observation and manipulation of RNA species.

Additionally, though Cas13 conducts crRNA processing which allows crRNA maturation to take place, maturation is not necessary for the activity of Cas13 (2), unlike other Cas systems which are "incompetent" (10) without pre-crRNA processing as they cannot otherwise perform *trans*-single-stranded RNA (ssRNA) cleavage. The pre-crRNA processing truncates the long CRISPR transcript, reduces folding constraints and helps avoid steric interference from neighboring Cas13s. Some Cas13 orthologs such as Cas13b have protospacer flanking sequences (PFSs), which are sequences on the Cas13 protein next to the RNA target that can affect Cas activity by influencing catalysis. However, PFSs are not necessary to ensure the catalytic activity of Cas13 (10).

Subtypes

The four subtypes of Cas13— Cas13a, Cas13b, Cas13c and Cas13d—are relatively recent discoveries amongst CRISPR-Cas systems, with Cas13d being the newest addition. These subtypes share a variety of differences; for example, type C has shorter Direct Repeat (DR) regions compared to the other subtypes. Additionally, subtypes A, B and C have "varying dependence" on PFSs, though subtype D proteins have no PFS requirements, which allows for lesser sequence constraints and the efficient targeting of "strongly depleted arrays" (17) Unlike the other orthologs, the nucleic activity of Cas13b is modulated by two proteins: Csx27 which acts as a repressor, and Cas28 which behaves as a stimulator to the nucleolytic activity of Cas13b (2, 14). Similarly, subtype D is also often associated with accessory proteins containing YWL domains, though it differs from all other subtypes in its size; it is the smallest Cas13 variant. This subtype's unique association with bivalent cations also suggests that it follows a distinct biochemical mechanism (10). Figure 3 presents a focused comparison between the most popular variant— Cas13b— and its new, arguably more efficient counterpart—Cas13d, while the differences and similarities between Cas13 variants are outlined in more detail in Table 1. Overall, the structure and variations of Cas13 lend themselves to diverse potential functions, posing a versatile tool for nucleic acid editing and detection.

Cas13 vs. Cas9

The Cas9 CRISPR system is perhaps the most well-known and widely implemented in genetic engineering. Its DNA-targeting function has demonstrated great potential in disease therapeutics (21) with ongoing research indicating promising results in the treatment of certain blood and lung cancer (19), urinary tract infections (20) and sickle cell anemia (18) amongst other genetic diseases. As it binds to DNA, it can carry out gene silencing and activating functions that Cas13 cannot perform. Additionally, Cas9 arguably provides more accurate and safer base editing than Cas13, as it can avoid double-strand breaks in DNA and any resulting indels (3). Considering these factors, it appears that Cas9 will likely assume dominance over Cas13 when it comes to therapeutics. After all, Cas13 cannot modify the host's DNA, hence Cas13-based treatments

would require life-long administration and management.

However, one must note that DNA editors such as Cas9 are limited by the PAM sequence, while Cas13 has no such restriction. In addition, while Cas9 has demonstrated varying efficiency across gRNAs, Cas13 analogues have been found to consistently provide high levels of efficiency in editing (more than 80%) (5), while simultaneously being a more compact effector than Cas9, allowing for easier introduction into target cells (8). Moreover, with Cas9's high rewards come high risks—the possibilities of off-target DNA editing and unintended genomic alterations. In light of these risks, Cas13 and its RNA-based targeting may be a safer gene-modulating method. In a clinical setting, some patients may prefer Cas13-based treatments over the more permanent Cas9-based DNA modification for additional assurance and safety.

Considering these factors, Cas13-based interventions may be more likely to be approved by the FDA. One might disagree, considering that Cas9 is already in clinical trials. However, Cas9's faster advancement is unsurprising considering Cas9 was discovered significantly earlier and studied more extensively than Cas13. Hence, assuming equivalent clinical efficacy, Cas13 may yet prove to be more effective and safe than Cas9.

Altogether, Cas9 and Cas13 have some similarities as CRISPR enzymes—both, for instance, have greater specificity when compared to older targeting methods such as RNAi. Nevertheless, they have varying applications and which enzyme is deemed 'better' conceivably depends on which is better suited to a particular situation or application.

	Cas13b	Cas13d
HEPN dependent?	Yes	No
Positively Regulated by	Csx28 (small accessory protein)	Presence of divalent cations (pre-crRNA) and WYL1
PFS?	Yes, 5′ PFS of D (A, U or G), 3′ PFS of NNA or NAN	No
Negatively Regulated by	Csx27 (small accessory protein)	(pre-crRNA) EDTA
Subtypes?	Yes, V-B1 has Csx27 (less consistently), V-B2 has Csx28	No
WYL domain?	No	No

Figure 3: Cas13b Versus Cas13d

Information from: Yan *et al.*, 2018; Smargon *et al.*, 2017

Table 1: Cas13 Variants

	A	B	C	D
crRNA recognition	NTD + Helical-1	NTD + Helical-1	NTD + Helical-1	NTD
DR stem loop location with respect to spacer	5'	3'	5'	5'
DR lengths	Longer	Longer	Shorter (30nt)	Longer (36nt)
Stem lengths (bp)	Shortest, 5-6	Longest, 9-14	9	8-10
Loop lengths (nt)	Longest, 7-9	3-6	4-5	4-6
Presence of bulge	Yes	No	Perhaps	Yes
Presence of Flank	Yes	No	Yes	Yes
5' Flank Length (nt)	1-5	-	3	-
3' Flank Length (nt)	3-5	-	3	5
DR Formation	Watson-Crick interactions	Watson-Crick interactions	Watson-Crick interactions	Non Watson-Crick interactions
Interaction with upper portions of crRNA	Yes	Unknown	Unknown	No
Overall size comparison	Bigger	Bigger	Bigger	Smallest, 300aa
Exhibits crRNA processing activity?	Yes	Yes	Unknown	Yes
Pre-crRNA processing metal involvement	Independent	Independent	Independent	Dependent
Percentage (%) of potential target sites amongst 396 ssRNA HAVs	94.6	86.3% (different PFS)	Unknown	Unknown
Optimal minimal crRNA spacer Length	20-22	28-30	Unknown	20-22
Ease of inserting into AAV	-	-	-	Likely Easiest
Structure-specific contact for stabilisation	More contact with multiple domains: Stem loop bound between NTD & Helical I cleft + non-covalent stabilisation, Interactions between 2' hydroxyl groups of crRNA and amino acid side chains on NTD, Helical 1 and HEPN2	-	-	More compact, fewer contacts with HEPN domains, largely NTD: NTD contacts crRNA DR, Chelates MG 2+ ion w bulge, with combination of contacts from D and crRNA Unique to D

Information from: See References

Key:
- = inapplicable/ no information

REPAIR

REPAIR technology development

The REPAIR system (**R**NA **E**diting for **P**rogrammable **A** to **I** **R**eplacement), developed by Cox *et al*., was amongst the first Cas13-based technologies to be developed. It paved the way for the use of this ribonuclease in the field of RNA editing for mammalian cells with significant implications for human therapeutics. REPAIR version 1 (REPAIRv1) utilized a catalytically inactive ortholog of Cas13b (dCas13b) fused with the human ADAR2 system. The ADAR2 (adenosine deaminase that acts on RNA type 2) enzyme is one of two human ADAR orthologs and uses hydrolytic deamination to convert adenosine to *inosine*—a functional equivalent to guanosine in terms of translation and splicing which allows the editing of endogenous transcripts. In contrast to its counterpart ADAR1, ADAR2 mainly targets non-repetitive coding regions, enabling the endogenous editing of transcripts without protein assistance. A mutant form of ADAR2 — ADAR(E488Q)—allows for more flexible targeting. REPAIR utilized ADAR2(E488Q) fused to the catalytically inactive *Prevotella sp.* ortholog of Cas13b (PspCas13b) to demonstrate the editing of disease-relevant mutations introduced into cells, along with reporter and endogenous transcripts (3).

Tests were conducted using Human Embryonic Kidney (HEK) 293FT cells. First, plasmids were transfected into HEK293FT to compare the interference activity of multiple Cas13 orthologs—21 Cas13a, 15 Cas13b orthologs, and 7 Cas13C orthologs. Next, based on this test, five Cas13b and two Cas13a orthologs were selected whose interference levels were assayed, ultimately resulting in the selection of PspCas13b and LwaCas13b for their high levels of interference. Next, the interference specificities of both were compared, following which PspCas13b was utilized, though the reason for this selection was unclear—an example of the multiple instances where the use of Cas13b overshadowed other variants that may have been as effective, if not more effective in DNA modulation technology. Nevertheless, tests proceeded using Cas13b and were attributed to its "consistent, robust, and specific knockdown" (3). In order to deactivate PspCas13b, conserved areas within the HEPN domain were mutated, and the resultant dCAS13b was fused to the deaminase domain of $ADAR1_{DD}$(E1008Q) and $ADAR2_{DD}$(E488Q). Testing followed with $ADAR2_{DD}$(E488Q) due to its higher functionality and editing efficiency. The complex of pspCas13b fused with $ADAR2_{DD}$(E488Q) formed REPAIR version 1 (REPAIRv1).

Application

REPAIRv1 was tested successfully to correct the disease-relevant human mutations of 878G → A(AVPR2 W93X) and 1517G → A (FANCC W506X)—diabetes and Fanconi anaemia respectively—with 35% correction of AVPR2 and 23% correction of FANCC. However, there existed some off-target edits. In order to decrease the off-target edits caused by $ADAR_{DD}$(E488Q) used in REPAIRv1, REPAIR version 2 (REPAIRv2) was developed, offering increased specificity. With the restrictions of a PAM site that were otherwise imposed by Cas9 systems eliminated, and Protospacer flanking sequences (PFS) constraints minimised by Cas13b, REPAIR exhibited a more flexible and versatile system compared to other nucleic acid editing systems to edit pathogenic mutations within full length transcripts. REPAIRv2 was also utilized in tandem with SHERLOCK to form a "Multiplexed and Portable Nucleic acid detection platform"(6) as a component of SHERLOCK version 2 (SHERLOCKv2), further discussed in the SHERLOCK section below.

Comparison to Type II Cas9 systems

Unlike the popular Cas9 systems, Cas13 does not have any Protospacer Adjacent Motif

(PAM) which lead to targeting sequence constraints. Hence, due to the lack of motif preference surrounding the target of inosine (3), the REPAIR system has greater editing flexibility than Cas9 technologies. This also allows Cas13 systems such as REPAIR to edit more human-relevant genes such as those within ClinVar (3) compared to Cas9 DNA editors. Another advantage specific to REPAIR is that the ADAR domain specifically deaminates adenosines into inosines, not relying on endogenous repair pathways to perform the desired edits, as Cas9's inherently does.

Additionally, while DNA-targeting Cas9 performs edits on sense and antisense DNA strands, Cas13 technologies only target transcribed mRNA, reducing the risk of permanent damage, as the transient nature of RNA editing allows it to be reversed. This also allows Cas13 technology including REPAIR to prevent the progression of diseases such as Alzheimer's which involve transient changes such as inflammation, phosphorylation and other modifications of proteins related to disease-related signal transduction. At the very least, Cas13 systems such as REPAIR expand the potential for CRISPR Cas mediated therapeutics which Cas9 systems initially brought into the mainstream.

Opportunity Cost of Cas13 orthologs used

During the development of REPAIR, all testing, including assessments and comparisons of gene knockdown efficiency amongst Cas13 orthologs, were limited to Cas13b, Cas13a and Cas13c—in decreasing order of usage and testing— before the announcement (12) of the new Cas13d variant. The results indicated that PspCas13b had "similar or increased" interference and knockdown compared to other orthologs of Cas13b and multiple other Cas13a and Cas13c variants including *Leptotrichia wadei* Cas 13a. It is likely that these results not only impacted the development and engineering of REPAIRv1 and REPAIRv2, but also initiated the widespread and long-term reliance on Cas13b for Type VI system technologies. Papers published after Cox *et al*.'s findings focused mainly on Cas13b, often citing Cox *et al*. in their research. This idea shall be revisited in following sections. In this manner, the scope and efficiency of CRISPR Cas13 systems may have unintentionally been limited as most research into the Type VI systems for RNA editing and recognition turned to Cas13b.

Later as well, limited experiments investigated the exact differences in activity and efficiency between 13a, 13b, 13c and 13d. The replacement of Cas13b with Cas13d may have led to improved functionality of REPAIRv1, as the size of REPAIRv1 had indeed been a limitation to its packaging in Adeno-associated viral (AAV) vectors with the capacity of 4.7kb and could not initially "accommodate the large size of dCas13b-$ADAR_{DD}$" (3). This led to the need for C-terminal and N-terminal truncations of REPAIR. The usage of Cas13d may have reduced the need for such truncations as it is 26% smaller than all other Cas13 variants, with an average size of 300 amino acids (10). As the sections removed from Cas13b were not parts that Cas13d inherently lacks, the usage of Cas13d may potentially have resulted in negligible difference in efficacy while significantly reducing the size.

Admittedly, the late discovery of Cas13d would not have allowed for such a possibility during the initial designing of REPAIRv1 and REPAIRv2, though upon the integration of REPAIR with SHERLOCK technology in SHERLOCKv2 (6), using Cas13d instead of Cas13b would have certainly been achievable and may have been advantageous. Yet, as a pioneering study, the focus on Cas13b and Cas13a was understandably justifiable and followed by subsequent research. An example illustrating this discrepancy in the usage and preference of subtype B compared to other variants in the RNA editing field includes DUX4 mRNA silencing, involving the

treatment of Facioscapulohumeral muscular dystrophy.

DUX4

Facioscapulohumeral muscular dystrophy

Cas13b was found to successfully silence the Double homeobox 4 (DUX4) gene by 90% (11). DUX4 in *Homo sapiens* codes for a transcription factor in skeletal muscle. It is also expressed in the testes and involved in embryonic development. Those affected experience muscle wasting and weakness, resulting in loss of ambulatory function. When the gene is "properly silenced" DUX4 is not expressed in adult skeletal muscle (13). For patients who have lost 1-3 repeating units, the *D4Z4* locus of DUX4 is de-repressed, leading to Facioscapulohumeral muscular dystrophy (FSHD).

Cas13 usage

In this case, Cas13 proves to be a safer approach than the use of Cas9 which leaves the potential for DNA damage as Cas9 is primarily DNA cleaving and is also limited in its effectiveness as there exist "hundreds of copies" of the DUX4 gene (11). Taking advantage of Cas13's RNA-specific functionality, there were reduced risks of permanent DNA alterations and damage to the host cell. Yet, only one Cas13 variant was used in the designing and testing of DUX4 suppression both in vitro and in vivo: Cas13b. Several Cas13b guide RNAs (gRNAs) delivered by AAV vectors were tested for their ability to "suppress DUX4 and prevent cell death" (11) first in vitro and next in both adult and new-born FSHD mice models. Results reflected more than 90% success in cells and almost 100% reduction in tibialis anterior muscles of TIC-DUX4. Yet again, we see the preferential usage of not only Cas13 over the more well-known Cas9, but also Cas13b over Cas13d while using the same method for gene therapy delivery—AAV vectors—where the Cas13d ortholog may have been a more appropriate choice.

Oncology

Another approach to the usage of Cas13 is in oncology, an application which was explored in 2018 but was met with little follow up research and implementation. Yet, the direction proposed by Granados-Riveron and Aquino-Jarquin (8) for the extrapolation of Cas13's RNA binding ability to push the boundaries of cancer research is worth noting as an example of the potential for the use of non-mainstream CRISPR technology to further human therapeutics and disease-prevention.

REPAIR

Unsurprisingly, Granados-Riveron and Aquino-Jarquin considered the Cas13b-driven REPAIR technology in their discussion of future oncological implementation of CRISPR Cas13 (8). Citing the work of Cox and colleagues, they explored the potential of REPAIR's A-to-I editing in, for example, the *Alu*-repeat sequences with A-to-I editing sites which play roles in advanced leukemia and correlate with survival rates in head and neck, and liver and breast cancers. Additionally, other members of the same ADAR family utilized in REPAIR can modulate "RNA-based functionalities" by editing ncRNAs, and in doing so can impact the malignancy of a phenotype (8). Studies with ADAR 1 editing on chronic myelogenous leukemia and metastatic melanoma lines show ADAR1's ability to influence tumor-suppressing species, melanoma growth and metastasis. The ability of Cas13 to target and bind to ADAR substrates including a variety of RNA species lends itself to the versatile applicability of Cas13 technology such as REPAIR for the treatment of diseases such as cancer as well as for disease diagnosis, which is explored in the SHERLOCK section below.

Subcellular RNA localization and Cancer

Important components of gene regulation include intracellular trafficking—the movement of small molecules, mRNA, and associated proteins within the cell—as well as subcellular localization of RNA, which is the location of the RNA for a particular gene within the cell. Both exert significant influence on gene regulation through the control of asymmetric protein distribution related to various biological processes in different types of cells, including cancer cells. The main player of both components—mRNA—modulates this asymmetric distribution through localization. Any interruption in this mRNA localization may lead to tumorigenesis, causing cancer (8).

Cas13 for mRNA localization and advantages over Cas9

Although work on Cas13 therapeutics for cancer treatment is yet to reach the clinic, its RNA binding function demonstrates clear potential, as conveyed by Granados-Riveron and Aquino-Jarquin (8). For example, catalytically inactive Cas13—dCas13—could be used for real-time transcript localization, which involves the live tracking of different RNA species through the targeting and inactivation of RNA binding proteins. Such binding can also allow the identification of different splicing errors, one of many ways Cas13 can be taken advantage of to expand knowledge about the function of RNA regulators and their dysregulation in cancer. Besides live-cell identification and tracking of endogenous RNA species, Cas13 can also be utilized as an alternative to siRNA, and in RNA immunoprecipitation sequencing to identify RNA bound by RNA binding proteins (RBPs) of interest in cancer cell lysates. Both Cas13 editing and tracking could reveal more information on splicing errors, the functions of different regulators, and the dysregulation of RNA, helping expand our understanding of RNA regulation's role in cancer.

In this context of oncology-related transcriptome engineering, Granados-Riveron and Aquino-Jarquin attribute the advantage to Cas9 when it comes to offering "robust gene-silencing" (8). However, they acknowledge the then 'novel' protein Cas13d's superiority as "one of the most compact single-effector Cas enzymes compared with the Cas9 and Cas13a-Cas13c effectors" (8). Overall, they conclude that the varying applications of Cas9 and Cas13 render them incomparable; dCas9 can visualize DNA loci, whereas dCas13 can visualize RNA. Though they recognize CRISPR-Cas13 as a pioneering technology in its natural affinity for RNA manipulation, their comparison of the Cas proteins is arguably inadequate as it fails to address the specific structural differences between Cas9 and Cas13 drastic differences in functionality, and consequently, differences in efficiency and usability for human therapeutics.

Cas13 and cancer diagnosis

From the researchers of REPAIR and *Nucleic Acid detection with CRISPR-Cas13a/C2c2* came the nucleic acid detecting platform SHERLOCK (**S**pecific **H**igh-sensitivity **E**nzymatic **R**eporter Un**lock**ing). With attomolar sensitivity, SHERLOCK—as demonstrated by Gootenberg and colleagues—was able to detect low-frequency "cancer somatic mutations in cfDNA fragments" (8), demonstrated through its detection of EGFR L858R and B-Raf proto oncogenes, as well as serine/threonine kinase (BRAF) V600E mutations in mock cfDNA. This provided an initial impetus for CRISPR-Cas13's prospective usage for nucleic acid detection in fields including oncology, though it is only the tip of the iceberg when it comes to SHERLOCK's demonstrated performance and potential.

SHERLOCK

SHERLOCK, or **S**pecific **H**igh Enzymatic **R**eporter **Unlock**ing is CRISPR-Cas13a based

nucleic acid detection platform which provides DNA and RNA detection with attomolar (aM) sensitivity; single-stranded (ss)DNA can be detected at 1aM concentration and RNA at 2 aM concentration (8). It combines Cas13a with isothermal amplification to form a CRISPR-based diagnostic platform (CRISPR-Dx) (7) SHERLOCK was found to detect strains of Zika virus (ZIKV) in chemical isolates of urine and ZIKV-positive blood serum. It could also discriminate between ZIKV and Dengue virus (DENV) and demonstrated the ability to identify single-nucleotide differences or single-base mismatches at low concentrations. To further investigate this ability, SHERLOCK was tested against health-related single nucleotide polymorphisms (SNPs) and "benchmarked" against the "genotypic data" from the company 23andMe (7). Researchers used purified samples of saliva, with results indicating that SHERLOCK distinguished genotypes with high significance to the extent that it could differentiate between homozygous and heterozygous genes. This illustrated SHERLOCK's vast potential for use in rapid human genotyping.

Another application that was demonstrated by Gootenberg and collogues (7) was the identification of bacterial genes and pathogens. They targeted the conserved flanking regions within the V3 region of the 16s rRNA gene. This region contains Recombinase Polymerase Amplification (RPA) primers used universally across bacterial species, as well as a variable internal region that varies between species, used by SHERLOCK to correctly genotype pathogenic strains from five possible targeting crRNAs. SHERLOCK was also successfully used to distinguish between two antibiotic resistance genes: *Kleebsiella penumoniae* carbapenemase (KPC) and New Delhi metallo-ß-lactamase 1 (NDM-1). Both applications auger well for the future use of SHERLOCK in epidemiology.

Moreover, SHERLOCK also showed promise in the field of oncology in its detections of low-frequency cancer mutations in cell-free (cf)DNA—particularly useful due to the large amount of wild-type DNA in blood (7). It could detect cancer mutations of EGFR L85R and B-Raf proto-oncogenes, as well as Serine/threonine kinase (BRAF) V600E mutations in mock cfDNA samples.

The first version of SHERLOCK faced a few limitations as it was non-quantitative instead relying on fluorescence detection. This led to improvements resulting in SHERLOCK version 2 (SHERLOCKv2).

SHERLOCK Version 2 (SHERLOCKv2)

The new version included four significant changes: the incorporation of additional orthogonal CRISPR enzymes multiplexed with a four-channel single-reaction; quantitative measurements for input concentrations "as low as 2 attomolar"; more than 3 times the signal sensitivity with added Csm6—a CRISPR associated enzyme; and lateral flow readout (6). SHERLOCKv2 also demonstrated the ability to detect Dengue and Zika single-stranded RNA, along with mutations in liquid biopsy samples.

To increase sensitivity, Gootenberg and colleagues (6) compared the cleavage preferences of Lwa13a, Lba13a, Psm13b and Cca13b for collateral cleavage activity, and used them to detect synthetic ZIKV and DENV. The more active orthologs—Psm13b, Cca13b and Lwa13a—were combined with Cas12a to expand SHERLOCK's multiplexing. CRISPR type II effector nuclease Csm6 was also incorporated to improve detection. The collateral cleavage products of Lwa13A and Psm13b produce linear adenine homopolymers terminated with a 2',3'-cyclic phosphate, which activates Csm6 and allows for an amplified detection signal (6). Another element added to overcome the limitation of florescence detection was the incorporation of a "visual readout of activity" (6). Gootenberg and collogues first tested a colorimetric RNase reporter to provide a visual readout, but

discovered it required a higher RNase activity than Cas13 could provide. Following his, they designed and tested a lateral flow readout system—the kind found on commercial lateral flow strips—that resulted in a 2 aM sensitivity when tested on ZIIKV and DENV (6).

The updates to SHERLOCK allowed for the creation of a portable paper test for non-small cell lung cancer. SHERLOCK successfully detected the epidermal growth factor receptor (EGFR) L858R mutation and Exon 19 deletion from liquid biopsies from patients.

Another application of SHERLOCKv2 explored after the updates was the usage of the technology as a Cas13 diagnostic *and* therapy platform. This combines REPAIR—which uses Cas13b from *Prevotella* PspCas13b—and SHERLOCKv2 to not only detect, but to correct mutations. This combination was used to correct a mutation adenomatous polyposis coli tumor suppressor gene which is disrupted in the familial adenomatous polyposis 1 disease. Synthetic healthy and mutant cfDNAs were transfected into HEK cells, which showed results of detection by multiplexed SHERLOCK along with 43% editing by REPAIR (6).

Advantages as a Diagnostic Platform

SHERLOCK is compatible with long-term storage as its reagents can be lyophilized for cold-chain independence (6). Additionally, its components allow it to function in the form of a small paper test costing only $0.61 per test to produce. Moreover, it produces relatively fast diagnostic results; SHERLOCKv1 produced results on ZIKV and DENV testing in < 90 minutes, and rapid DNA extraction from saliva purification and insertion into SHERLOCK was possible in < 23 minutes. Considering that this was the time recorded *without* the need to purify the saliva certainly attests to SHERLOCK's great potential in providing rapid diagnostics. The technology provides "quantitative, visual and multiplexed readouts", with the added advantage of being instrument-free. Overall, SHERLOCK has broad field potential, with applicability in areas ranging from pharmacogenomic genotyping and field detection of GMOs to the identification of co-occurring pathogens, nucleic acid detection where portable or power readers are unavailable, and cases of rare DNA species such as circulating DNA (6).

Usage of Cas13a and Cas13b

Once again, we see the extensive usage of Cas13a and Cas13b variants for applications ranging from DNA modulation to disease diagnosis. Though the frequent reliance on these variants is understandably due to their earlier discovery and the researchers' previous familiarity with these Cas13 orthologs, updating at editing or diagnostic technology with replacement of Cas13a and Cas13b by Cas13d may have resulted in not only improved editing efficiency, but could also improve the range of nucleic acid sequences that can easily and successfully be detected, as 13d is HEPN independent and lacks protospacer flanking motifs (PFSs)—factors present in Cas13a and Cas13b. This may be limiting the performance of technology such as SHERLOCK, unbeknownst to the researchers as comparisons between A, B *and* D variants were not attempted. According to research by Konermann and collogues (5), CasRx—an engineered Cas13D effector—showed a 96% knockdown compared to 66-80% for Cas13a and b. These findings indicate a distinct difference at least in the modulating activity of 13D versus other variants, if not suggesting differences in their RNA target identification and binding capability (5). Hence, testing of modulation and detection technology based on Cas13D against other variants may potentially offer improvements and advantages that may increase the performance of platforms such as SHERLOCK.

CARVER

Technology development and testing

The disease detecting and editing applications of CRISPR editing were further explored by Freije *et al* (4). who tested Cas13 activity with three viruses: lymphocytic choriomeningitis virus (LCMV), influenza A virus (IAV), and vesicular stomatitis virus (VSV) (4). They developed the **C**as-13 **A**ssisted **R**estriction of **V**iral **E**xpression **R**eadout (CARVER) technology, used to detect and destroy viral RNA. First, the impacts of conservation and target RNA nucleotide content on Cas13's viral activity were tested using two Cas13 orthologs: LwaCas13a and PspCas13b. Results indicated that both orthologs had differing optimal target sites due to their different PFSs and cleavage activity. They also found approximately 350 potential target sites for human-associated viruses (HAVs), 94.6% of which were putative sites for Cas13a, and 86.3% were putative sites for Cas13b, indicating a wide range of antiviral applicability.

Freije and colleagues (4) first tested the activity of Cas13a with LCMV in HEK293FT cells. LCMV is one of many Mammarena viruses lacking FDA- approved vaccines, making it ideal for therapeutic testing. Results indicated that *Lwa*Cas13a efficiently reduced LCMV viral levels for most crRNAs tested, at low Multiplicity of Infection (MOI) as well as high MOI values > 5 (4). According to measurement by RT-qPCR 48 hours post infection (hpi), the activity of *Lwa*Cas13a resulted in a 2 to14-fold reduction in viral RNA. Testing on IAV followed, which differs with LCMV in cellular localization and replication. Freije and colleagues used *Psp*Cas13b—introduced through a plasmid via electroporation—against IAV in Madin-Darby Canine Kidney (MDCK) epithelial cells. This resulted in 7 to 22-fold reductions in viral levels (4). To broaden the observation of Cas13's targeting and editing capability, the possible inhibitory effects of *Psp*Cas13 on the neurotrophic RNA virus VSV were studied. crRNAs were designed for both main serotypes of VSV: Indiana and New Jersey and were tested within HEK293FT cells expressing PspCas13b (4). VSV Viral RNA levels decreased 7.8 to 43.3-fold at 48 hpi. To ensure Cas13 treatment did not lead to mutations at the crRNA target sites of viral populations, viral RNA was extracted 48 hours after infection. Targeting by Cas13 was found to have no mutation effect, and no significant differences in the amount of single nucleotide minority variants (SNVs) between cells with no Cas13, non-targeting crRNAs, and targeting crRNAs (4). This indicates CARVER acted as a reliable targeting and modulating platform with no observed negative effects on host DNA.

Usage of Cas13a

CARVER is yet another example of Cas13 technology taking advantage of one of the two most popular variants: Cas13a, instead of Cas13b. Freije *et al.* unsurprisingly cite Abudayyeh and colleagues—those who first explored Cas13a or '*C2c2'* and went on to explore its potential while developing REPAIR and SHERLOCK (4). There is also special mention of CARVER's advantages over "previous applications of Cas13-based targeting of mRNA" including its ability to function at high MOI, demonstrating high enough efficiency to overcome the challenge of high viral replication rates (4). During this description of CARVER's advantages, the authors implicitly make comparisons to *two* other Cas13a technologies including REPAIR —which is frequently referenced in the article — and only *one* non-Cas13a technology*:* CasRx that is based on Cas13d previously mentioned in the SHERLOCK section. One can argue that this demonstrates how the popularity of a particular protein such as Cas13a amongst certain researchers along with its earlier discovery makes it more likely to be tested, explored and utilized to create new technologies than newer proteins such as Cas13d despite their arguably higher potential. This is perhaps because research comparing, improving and competing with existing

technology is favored compared to research using recent technologies. This phenomenon may or may not be advantageous to the growth of CRISPR gene modulating technologies, though viewing a technology that does utilize the novel Cas13d and spearhead research into mainstream use such as Pac-Man can offer perspective on the issue.

COVID-19

The COVID-19 pandemic has created new interest into viral detection and anti-viral solutions, including CRISPR mediated nucleic acid and gene editing platforms such as STOP SHERLOCK and Pac-Man.

STOP COVID

The creators of SHERLOCK, have recently updated the technology, developing SHERLOCK Testing in One Pot COVID—STOP COVID. First versions are being tested within hospitals in Thailand for COVID-19 screening (15) and utilize amplification reagents complementary to SARS-CoV-2. The technology has been found to detect infection with 97% sensitivity and 100% specificity, presenting results using a lateral flow readout as used in SHERLOCKv2, demonstrating impressive accuracy and easy-to-determine results (15). Is ability to provide results in less than an hour and use of unsophisticated equipment makes faster, more wide-scale testing available in developing and rural affected sites (15). STOP COVID only involves a single reaction, as it utilizes an additional CAS12b variant which allows testing to take place "at a constant temperature", eliminating the need to "open tubes" halfway through the process and reducing risks of contamination and providing more accurate results (15). Though this technology is a more indirect use of Cas13 and is specific to diagnostics, research has also led to advancements in editing technology directed towards SARS-CoV-2 and other viruses pathogenic to humans.

PAC-MAN

Despite garnering recognition as "one of the most compact single-effector Cas enzymes" with advantages over Cas9 and Cas13a-Cas13c effectors (8), technology and research surrounding Cas13d has been relatively limited due to its recent discovery. Yet, its potential is increasingly being explored through research such as the in the work of Abbot and colleagues, specifically the development of the **P**rophylactic **A**ntiviral **C**RISPR in Hu**man** cells: PAC-MAN. Offering "pan-coronavirus protection" using Cas13d-mediated RNA cleavage, PAC-MAN targets and cleaves SARS-CoV-2 sequences and was also found to reduce H1N1 IAV load in respiratory epithelial cells (1).

SARS-CoV-2 Considerations

Keeping in mind the evolution of the L and S strains of SARS-CoV-2, PAC-MAN technology was developed to combat a broad range of Coronaviruses, including the viruses that caused Severe Acute Respiratory Syndrome (SARS) and Middle Eastern Respiratory Syndrome (MERS) to provide a reliable technology that may be utilized for future coronavirus variants as well. While the vaccine approach to SARS-CoV-2 relies on the human body's recognition of the coronavirus proteins and the subsequent reduction of viral entry into cells, PAC-MAN's CRISPR approach offers a more direct antiviral strategy of targeted viral cleavage and inhibition of viral replication while taking advantage of Cas13d's small size, high catalytic activity in human cells and high specificity (1). Additionally, vaccines rely on the highly mutating regions of surface proteins, while PAC-MAN targets highly conserved regions, reducing the likelihood that the viruses can escape inhibition through mutation. Recognizing the respiratory nature of SARS-CoV-2, PAC-MAN was created to be compatible with airway delivery

of the six PAC-MAN reagents, each containing variations of crRNAs used as a guide by Cas13D from *Ruminococcus flavefaciens* to cause viral genome degradation and prevent viral gene expression of more than 90% of coronaviruses.

Technology Development

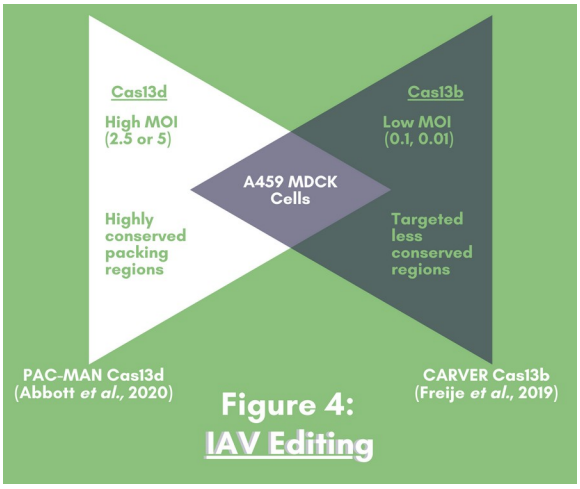
Abbott and colleagues first created a "bioinformatic pipeline" (1), where multiple viruses were aligned to find highly conserved regions which would become the targets of Cas13d. It was found that six crRNA sequences covered more than 91% of sequences coronaviruses, while 22 crRNAs covered *all* sequenced coronaviruses, offering ample breadth of usage (1). Computational analysis specifically for SARS-CoV and MERS-CoV genomes found the two most highly conserved regions to be of RNA-dependent RNA polymerase (RdRP) which functions to maintain the proliferation of all corona viruses, and the Nucleocapsid, which packaged the virus. Yet, the sample size of this testing was small, relying on data from 47 patients with SARS-CoV. Analysis resulted in 3802 possible crRNAs to use, shortlisted to 40 RNAs with 20 targeting RcRP and the Nucleocapsid each by excluding sequences with potential off-target binding and Poly Ts which would prevent crRNA expression. Synthesized fragments of SARS-CoV-2 fused to GFP were placed in human lung epithelial cells A549 with Cas13D and 4 crRNAs targeting either RdRP or the Nucleocapsid region. The use of multiple crRNAs served to avoid virus escape through mutations. Results demonstrated consistent and substantial repression of GFP, suggesting that the Cas13-d PAC-MAN could effectively target and degrade SARS-CoV sequences in human cells, and also reflected that crRNA design is important in determining efficacy (1).

Due to the lack of access to live SARS-CoV-2 virus, the researchers tested Cas13D activity in human lung epithelial cells A549 with live Influenza A virus (IAV), specifically the H1N1 strain due to its similar tropism compared to SARS-CoV-2. However, the viruses do share some differences: SARS-CoV-2 is positive sense and has single, continuous RNA whereas IAV is segmented and is negative sense. As before, the most conserved regions of influenza were analyzed, and were found to be the segment ends which are important for packaging. Once again, six crRNAs were found that could cover 92% of all sequenced IAV. PAC-MAN was tested and proven to inhibit IAV, with a possible trend of higher efficiency at lower titers such as those observed in new infections (1).

Cas13b vs. Cas13d IAV editing

As described in the CARVER section, Freije and colleagues also performed tests of Cas13 editing on IAV, using the 13b variant PspCas13b (4). Both CARVER and PAC-MAN tests were performed on A549 MDCK cells. Yet, there are some notable differences between these studies, as depicted in Figure 4; Abbott and colleagues used high MOIs (2.5 or 5), whereas the previously conducted CARVER study was performed at lower MOIs of 0.1 or 0.01, perhaps offering a more effective method of observing Cas13 cleavage during high-titer infection conditions (1, 4). The crRNAs for CARVER were designed with spacers near PspCas13b's 5' GK PFS. Since Cas13d used in PAC-MAN has no PFS requirements, this suggests that editing in CARVER's "7-to 22-fold" (4) reduction in IAV may have been restricted by Cas13b's PFS as editing sites were required to be "filtered" in order to ensure the presence of the PFS specific to each Cas13 ortholog (4). PAC-MAN may hence be more effective under the same conditions. Additionally, CARVER targeted moderately, or 'somewhat' conserved regions with "up to 2 bases with <95% allele frequency per crRNA" (4). On the other hand. PAC-MAN was designed to target "highly conserved packaging regions" from "all eight IAV segments" (1). This demonstrates that PAC-MAN, in its current state, is likely to show

more effectiveness and specificity compared to CARVER. Lastly, Freije and colleagues used spacers that were 30nt in length in CARVER, whereas 22nt crRNAs were used in PAC-MAN (1, 4). This highlights the more compact nature of Cas13d and PAC-MAN. Overall, the differences between PAC-MAN and CARVER technologies indicate higher performance by the Cas13d-driven PAC-MAN when it comes to available editing Cas13 therapies.



Future Potential

PAC-MAN was demonstrated to be a prospective approach to combating diseases caused by Beta coronaviruses including COVID19, SARS and MERS, mostly targeted by the first three of the six crRNAs tested by Abbott and colleagues. The next steps in the technology likely involve its development in efficiency and specificity for testing with *live* SARS-CoV-2, finding safe methods for in vivo delivery and making sure the Cas13d and crRNAs are expressed in a certain percentage of patient cells to demonstrate that PAC-MAN can be successfully utilized as an anti-viral strategy. Moreover, since viral genomes may be protected with protein, the crRNAs that are most effective against the live virus need to be screened. Overall, the PAC-MAN technology illustrates Cas13d's wide potential for usage in gene modulating therapeutics where antiviral strategies against RNA viruses are valuable and essential. This specific variant's advantages of "small size (967 amino acids), high specificity, and strong catalytic activity" (1), and relative ease of cell introduction as recognized and addressed by Abbott *et al.* highlights the significant differences between Cas13d and the other Cas variants, as well as the potential implications of these differences of allowing more effective viral targeting and destruction when introduced in human cells. Admittedly, as a single example of Cas13d's application, using only this variant in PAC-MAN may be premature. Further studies exploring Cas13d's uses in modulating and RNA localization or tracking should provide a better understanding of the protein's potential in different fields including those of Genetics and Oncology. However, despite the optimism brought by the CRISPR-Cas systems' ability to combat viral infection, new research indicates that viruses are quickly adapting to combat CRISPR systems (16), leading to more uncertainty in Cas13's future.

Cas13 Inhibition

Though inevitable, viral mechanisms against CRISPR-Cas inhibition have evolved to include the recent discovery of phage-encoded Anti-CRISPR (Acr) regions which produce small proteins (<150 aa) that intervene in host

CRISPR-mediated immunity allowing for successful viral invasion and lysis.(9, 16) Though previously not found for Cas13 (Type VI) effectors, bioinformatic screening revealed coding for a protein that blocks Cas13a's "guide-exposed face", therefore inhibiting its access and interaction with the target RNA (16). Type VI-A Acr genes (AcrVIAs) were found to inhibit Cas13a targeting not only in bacteria, but in human cells as well (16). Research by Lin and colleagues (16) uncovered a series of 7 AcrVIA genes which significantly reduced the single nucleic acid modulating ability of the dCas13a-based toolkit. AcrVIAs 1, 4, 5 and 6 binds to LwaCas13a, while AcrVIAs 2 and 3 can only bind to the crRNA complex of Lwa13.

Similar findings were discussed in Meeske *et al.* (9), where a Listeriaphage (LS46) was discovered to encode a protein that intercepts Cas13a's access to the target RNA by blocking the guide-exposed face of the protein, therefore preventing proper binding and hence inhibiting the conformational changes required to activate the nuclease. To investigate the precise molecular mechanism of the inhibition, phages from *Listeria* spp. were collected as it commonly expresses Cas13a. Lysogens that were isolated from the phages were infected with *L. seeligeri* and tested for their ability to disarm "Cas13a-mediated immunity against plasmid conjugation" (9). The lysogen fLS46 demonstrated highly efficient plasmid transfer, indicating that this prophage harbors a Cas13a inhibitor (9). The region containing the Acr sequence contains four genes, amongst which gp2 seems to be responsible for the Anti-CRISPR phenotype AcrVIA1. Gene gp3 demonstrates similarities with AcrIIA6 (Anti-CRISPR for Type II A systems) in its helix-turn-helix domain (HTH), suggesting that it may inhibit type II CRISPR-Cas systems. Testing also showed that AcrVIA1 can neutralize Cas13a at a low viral load and other unfavorable conditions in natural hosts, likely due to is RNA-targeting nature which allows continuous transcription (9).

Altogether, though Acr genes may be a roadblock when it comes to Cas13-driven antiviral therapies, as phages such as LS46 would be able to combat the Cas13 and avoid RNA cleavage, evidence does not yet indicate that Anti-CRISPRs can interfere with Cas13 RNA modulation. In fact, they offer potential benefits of allowing more precision and control with Cas13 modulation, knockdown and visualization in heterologous hosts, and may facilitate the study of "bacterium-phage interaction" (9, 16).

Conclusion

Cas13 has proven to be a versatile RNA targeting protein, with its multiple variants allowing for a variety of applications ranging from RNA knockdown, RNA targeting, antiviral technology, nucleic acid detection, imaging, tracking and localization (24). The protein has also been utilized in areas not discussed in this paper, including splicing alteration, the induction of apoptosis and the regulation of gene expression (23). Considering current circumstances it is likely that we shall see much more of Cas13 in the fields of immunology, pathology, diagnostics and human therapeutics as it has demonstrated significant potential in aiding the human immune defense system.

However, a considerable drawback of the Cas13 system is its ineffectiveness in neurodegenerative conditions including Motor Neuron Disease, where the amount of RNA is a critical factor in disease progression (21). Further exploration of Cas13 is required to determine whether it is possible to overcome this barrier.

Though understandably there is sparse implementation of the Cas13d variant due to its recent discovery, the advantages it confers over

other variants must be acknowledged, studied and taken advantage of. Incorporating the Cas13d variant may increase the effectiveness, efficiency and success of Cas13 technologies. t Overall, Cas13 occupies a unique niche in CRISPR and gene editing as its RNA targeting allows it to be used across organisms and kingdoms(25, 26), with significant potential for research in human biology.

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References

1. Abbott, Timothy R., et al. "Development of CRISPR as an Antiviral Strategy to Combat SARS-CoV-2 and Influenza." *Cell*, vol. 181, no. 4, Elsevier, May 2020, pp. 865-876.e12, doi:10.1016/j.cell.2020.04.020.

2. Burmistrz, Michal, et al. "RNA-Targeting CRISPR–Cas Systems and Their Applications." *International Journal of Molecular Sciences*, vol. 21, no. 3, MDPI AG, 1 Feb. 2020, doi:10.3390/ijms21031122.

3. Cox, David B. T., et al. *RNA Editing with CRISPR-Cas13*. 2017, http://science.sciencemag.org/.

4. Freije, Catherine A., et al. "Programmable Inhibition and Detection of RNA Viruses Using Cas13." *Molecular Cell*, vol. 76, no. 5, Cell Press, Dec. 2019, pp. 826-837.e11, doi:10.1016/j.molcel.2019.09.013.

5. Konermann, Silvana, et al. "Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors." *Cell*, vol. 173, no. 3, Elsevier, Apr. 2018, pp. 665-676.e14, doi:10.1016/j.cell.2018.02.033.

6. Gootenberg, Jonathan S., et al. "Multiplexed and Portable Nucleic Acid Detection Platform with Cas13, Cas12a, and Csm6." *Science*, vol. 360, no. 6387, Apr. 2018, pp. 439 LP – 444, doi:10.1126/science.aaq0179.

7. Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., Verdine, V., Donghia, N., Daringer, N. M., Freije, C. A., Myhrvold, C., Bhattacharyya, R. P., Livny, J., Regev, A., Koonin, E. V., Hung, D. T., Sabeti, P. C., Collins, J. J., & Zhang, F. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. Science (New York, N.Y.), 356(6336), 438–442. https://doi.org/10.1126/science.aam9321

8. Granados-Riveron, Javier T., and Guillermo Aquino-Jarquin. "CRISPR–Cas13 Precision Transcriptome Engineering in Cancer." *Cancer Research*, vol. 78, no. 15, Aug. 2018, pp. 4107 LP – 4113, doi:10.1158/0008-5472.CAN-18-0785.

9. Meeske, Alexander J., et al. "A Phage-Encoded Anti-CRISPR Enables Complete Evasion of Type VI-A CRISPR-Cas Immunity." *Science*, vol. 369, no. 6499, July 2020, pp. 54 LP – 59, doi:10.1126/science.abb6151.

10. O'Connell M. R. (2019). Molecular Mechanisms of RNA Targeting by Cas13-containing Type VI CRISPR-Cas Systems. Journal of molecular biology, 431(1), 66–87. https://doi.org/10.1016/j.jmb.2018.06.029

11. Rashnonejad, A., et al. "O.8DUX4 MRNA Silencing with CRISPR-Cas13 Gene Therapy as a Prospective Treatment for Facioscapulohumeral Muscular Dystrophy." *Neuromuscular Disorders*, vol. 29, 2019, p. S40, doi:https://doi.org/10.1016/j.nmd.2019.06.026.

12. Rizk, C. (2018, March 23). CRISPR Startup Arbor Biotechnologies, Salk Institute Concurrently Discover New Cas13d Enzyme. Retrieved from https://www.genomeweb.com/gene-silencinggene-editing/crispr-startup-arbor-biotechnologies-salk-institute-concurrently-discover

13. Rojas, L. Alejandro, et al. "P38α Regulates Expression of DUX4 in Facioscapulohumeral Muscular Dystrophy." BioRxiv, Jan. 2019, p. 700195, doi:10.1101/700195.

14. Smargon, A. A., Cox, D., Pyzocha, N. K., Zheng, K., Slaymaker, I. M., Gootenberg, J. S., Abudayyeh, O. A., Essletzbichler, P., Shmakov, S., Makarova, K. S., Koonin, E. V., & Zhang, F. (2017). Cas13b Is a Type VI-B CRISPR-Associated RNA-Guided RNase Differentially Regulated by Accessory Proteins Csx27 and Csx28. *Molecular cell,* 65(4), 618–630.e7. https://doi.org/10.1016/j.molcel.2016.12.023

15. Lall, S. (2020, May 5). SHERLOCK-based one-step test provides rapid and sensitive COVID-19 detection . Retrieved November 21, 2020, from https://mcgovern.mit.edu/2020/05/05/sherlock-based-one-step-test-provides-rapid-and-sensitive-covid-19-detection/

16. Lin, Ping, et al. "CRISPR-Cas13 Inhibitors Block RNA Editing in Bacteria and Mammalian Cells." *Molecular Cell*, vol. 78, no. 5, Elsevier, June 2020, pp. 850-861.e5, doi:10.1016/j.molcel.2020.03.033.

17. Yan, Winston, et al. "Cas13d Is a Compact RNA-Targeting Type VI CRISPR Effector Positively Modulated by a WYL Domain-Containing Accessory Protein." *Molecular Cell*, vol. 70, Mar. 2018, doi:10.1016/j.molcel.2018.02.028.

18. Ye, L. *et al.* (2016) 'Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and β-thalassemia', *Proceedings of the National Academy of Sciences*, 113(38), pp. 10661 LP – 10665. doi: 10.1073/pnas.1612075113.

19. Su, S. *et al.* (2016) 'CRISPR-Cas9 mediated efficient PD-1 disruption on human primary T cells from cancer patients', *Scientific Reports*, 6(1), p. 20070. doi: 10.1038/srep20070.

20. Gupta, S. *et al.* (2021) 'Targeting of Uropathogenic Escherichia coli papG gene using CRISPR-dot nanocomplex reduced virulence of UPEC', *Scientific Reports*, 11(1), p. 17801. doi: 10.1038/s41598-021-97224-4.

21. Guerrero, E. N. *et al.* (2016) 'TDP-43/FUS in motor neuron disease: Complexity and challenges', *Progress in neurobiology*. 2016/09/28, 145–146, pp. 78–97. doi: 10.1016/j.pneurobio.2016.09.004.

22. Ebina, H. *et al.* (2013) 'Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus.', *Scientific reports*, 3, p. 2510. doi: 10.1038/srep02510.

23. Li, Y. *et al.* (2019) 'CRISPR/Cas Systems towards Next-Generation Biosensing', *Trends in Biotechnology*. Elsevier Ltd, pp. 730–743. doi: 10.1016/j.tibtech.2018.12.005.

24. Burmistrz, M., Krakowski, K. and Krawczyk-Balska, A. (2020) 'RNA-targeting CRISPR–cas systems and their applications', *International Journal of Molecular Sciences*. MDPI AG. doi: 10.3390/ijms21031122.

25. Schindele, P., Wolter, F. and Puchta, H. (2018) 'Transforming plant biology and breeding with CRISPR/Cas9, Cas12 and Cas13', *FEBS Letters*. Wiley Blackwell, pp. 1954–1967. doi: 10.1002/1873-3468.13073.

26. Wolter, F. and Puchta, H. (2018) 'The CRISPR/Cas revolution reaches the RNA world: Cas13, a new Swiss Army knife for plant biologists', *Plant Journal*. Blackwell Publishing Ltd, pp. 767–775. doi: 10.1111/tpj.13899.