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# Genome Editing and Defense against Its Misuse

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**Highlights**
Human genome editing methods ("genetic scissors") may be possibly used for biological attacks of various biological targets, including humans.
We should set up an international surveillance scheme to control the use of this method and to detect cases of hidden use of these destructive agents created on its basis.
***Relevance.*** Human genome editing has been implemented into clinical practice since 2021 to cure different, mainly hereditary diseases. This method is quite promising and in the long run it can be used to cure contagious and somatic diseases as well.
***The purpose of the study*** is to evaluate possible risks of misuse for this method.
***The source base of the study***. Scientific publications and research papers, available in PubMed.
***Research method.*** Analytical.
***Results.*** It has been shown that genome editing systems can block, remove or restore its own genes or human genome fragments. They can be employed to destroy whole ecosystems, to develop brand-new massive biological weapons to kill the population of certain countries. They are even able to change an evolutionary path of our species, and this may lead to its total extinction within several generations. Defense Advanced Research Projects Agency, DARPA has a particular focus on the development of tools that can identify the human genome editing, block possible changes and eliminate the consequences.
***Conclusions***. The fact that DARPA is so interested in tools mentioned above proves that genome editing is anymore not an experimental tool. Experts who realize the state of things in terms of biological warfare nowadays are concerned with the possible misuse of this tool. The use of genome editing should be regulated by a special Protocol to Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction. Because this protocol doesn't exist yet, the national regulatory authorities are obliged to establish limits for use of products that are based on these methods. They also should be able to prevent its misuse.

***Key words:*** *biological destruction; biological warfare; Cas endonuclease; CRISPR; CRISPR-Cas; genetic scissors; genome editing; palindromic repeats; protospacer; spacer*

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# Редактирование генома и защита от его враждебного использования

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**Основные моменты**
Технологии редактирования генома человека («генетические ножницы») могут и возможно уже используются для целей биологического поражения различных биологических объектов, включая людей.
Необходимо разработать способы международного контроля за использованием данной технологии и для обнаружения скрытого применения поражающих агентов, созданных на ее основе.
***Актуальность***. Технология редактирования генома активно внедряется в клиническую практику с 2021 г. для лечения наследственных болезней, в перспективе ее применение будет расширено для лечения инфекционных и соматических болезней.
***Цель работы*** – оценить возможность ее враждебного использования.
***Источниковая база исследования.*** Научные работы, представленные в базе данных медицинских и биологических публикаций PubMed.
***Метод исследования.*** Аналитический.
***Результаты.*** Системы редактирования генома позволяют осуществлять блокирование, удаление или восстановление собственных генов и фрагментов генома человека. Они могут использоваться для уничтожения экосистем, используемых человеком; разработки принципиально новых средств массового биологического поражения населения выбранных для уничтожения стран; и, даже, изменение эволюционной траектории нашего вида вплоть до его полного вымирания в течение нескольких поколений. Обращено внимание на повышенный интерес Агентства перспективных исследовательских проектов министерства обороны США (Defense Advanced Research Projects Agency, DARPA) на разработку технологий выявления и блокирования последствий редактирования генома.
***Заключение.*** Интерес DARPA к технологиям выявления и блокирования последствий редактирования генома человека свидетельствует, что ее освоение уже перешло экспериментальный рубеж, и ее бесконтрольное использование вызывает серьезные опасения со стороны тех, кто понимает реалии современной биологической войны. Использование технологии редактирования генома должно быть урегулировано в рамках особого Протокола к Конвенции о запрещении разработки, производства и накопления запасов бактериологического (биологического) и токсинного оружия и об их уничтожении. Пока его нет, то на уровне национальных регуляторов необходимо определить границы применения продуктов, созданных на основе такой технологии, и быть готовым к их враждебному использованию.

***Ключевые слова:*** *биологическая война; биологическое поражение; генетические ножницы; палиндромные повторы; протоспейсер; редактирование генома; спейсер; эндонуклеаза Cas; CRISPR*

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## Introduction

On November 15, 2024, Defense Advanced Research Projects Agency (DARPA), Biological Technologies Office (BTO) announced a PROGRAM SOLICITATION OVERVIEW INFORMATION about the Rapid Inhibitor Discovery and Development pipeLine (RIDDL)[1]. DARPA is soliciting innovative proposals to develop and demonstrate rapid methods to identify and optimize novel molecules that exhibit inhibitory effects on gene editing technologies[2]. Of particular interest are commonly used gene editors such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated proteins (CRISPR-Cas) nucleases; gene editing technologies beyond CRISPR-Cas systems are also of interest to keep pace with the rapidly advancing field and promote the safe, controlled use of these technologies.

*Objective.* Understanding of the dangers of the use of human genome editing technologies for military purposes.

*Source base of the study.* Scientific publications and research papers, available in PubMed. The searched keywords are [CRISPR/Cas9] AND [gene editing] OR [gene therapy].

*Research method.* Analytic.

## Main

**The Rapid Inhibitor Discovery and Development pipeLine (RIDDL) program** explicitly seeks transformative approaches that enable the rapid discovery, design, and development of novel inhibitors with enhanced activity, specificity, utility, and potency for gene editing technologies. **These approaches could serve as a rapid response to counteract the accidental or intentional misuse of gene editing technologies.** Novel inhibitor activity will be assessed in vitro over the course of the program to demonstrate the efficacy of the prototype discovery and development pipelines. The pipelines, as well as a subset of top-performing molecules at scaled-up quantities, will be transitioned for testing and evaluation by Department of Defense (DoD) stakeholders. Research that generates incremental improvements to the existing state-of-the-art is specifically excluded. Total Funding – DARPA has approximately $17M total for performer awards and anticipates making multiple awards.

From the background: "The rapidly evolving field of advanced genome editing tools has created the ability to modify genetic material in a manner that is precise, rapid, cost-effective, and broadly accessible. CRISPR-Cas technologies represent one of the most widely adopted tools in the genome engineering toolkit, which already consists of a diverse set of molecules, including meganucleases, transposons, recombinases, protein nucleic acids, zinc-finger nucleases, and Transcription Activator-Like (TAL) effector nucleases. From the initial discovery and demonstration of CRISPR-Cas gene editing technologies, the field has rapidly expanded both in the number and types of CRISPR-Cas systems via advanced computational discovery pipelines [1]. The advancement of CRISPR-based genome editing technologies has revolutionized the field of biotechnology and genetic engineering. However, concerns regarding the precision, specificity, and control of CRISPR-Cas systems remain. One promising avenue to address these concerns is the discovery or design of novel inhibitors. These molecules have the potential to inhibit and tune regulation of CRISPR-mediated genome editing by limiting unintended, off-target edits and enabling spatiotemporal control of gene editing activity, thereby enhancing its safety, efficacy, and utility. Previous DARPA investments in the Safe Genes program demonstrated discovery of potent protein inhibitors for a wide array [2] of CRISPR-Cas technologies, including enzymatic inhibitors capable of acting at sub-stoichiometric levels [3]. Safe Genes performers also developed platforms for discovery of small molecule inhibitors of CRISPR-Cas systems [4, 5]. Taken together with work from other groups in the literature describing nucleic acid-based inhibitors [6, 7, 8], multiple classes of molecules that exhibit anti-CRISPR activity have been demonstrated, providing significant depth and breadth for novel inhibitor discovery. The RIDDL program seeks to leverage these prior efforts to develop tools for rapid discovery, optimization, and validation of potent inhibitors for gene editing technologies. Beyond CRISPR-Cas technologies, some recent discoveries, such as Obligate Mobile Element Guided Activity (OMEGA) effector TnpB [9] and Fanzor [10], have further broadened the menu of RNA-guided DNA endonucleases that can be programmed for gene editing purposes. These new editor systems provide further opportunity to explore development of platform technologies for discovery of inhibitors to emerging gene editing technologies. Specifically, RIDDL will develop platform technologies for highly potent inhibitors of gene editors capable of arresting

[1] Rapid Inhibitor Discovery and Development pipeLine (RIDDL). URL: https://sam.gov/opp/d04ec5d6949b435083f6f582300aca27/view (date: 11.12.2024).

[2] Program Solicitation Rapid Inhibitor Discovery and Development pipeLine. URL: https://research-authority.tau.ac.il/sites/resauth.tau.ac.il/files/DARPA-RIDDL-PS-25-03.pdf (date: 11.12.2024).

nuclease activity for multiple classes, types, and species of editors. By harnessing advanced computational discovery capabilities such as clustering [11] and deep learning, RIDDL will develop a platform for 24-hour turnaround discovery and development of inhibitors of novel, emergent gene editor technologies. If successful, the RIDDL program will develop a pipeline capable of fielding validated inhibitors in less than 24 hours, **enhancing the safety of gene editing technologies and providing rapid response capabilities in the event of accidental or intentional misuse of gene editing technologies**"[3].

Here, "The RIDDL program is agnostic to the methods and approaches employed for discovery or design of novel inhibitors as long as they are potentially transformative. Proposals should focus on selecting diverse CRISPR-Cas systems, updating the CRISPR-Cas system space as novel variants are discovered or designed to keep pace with the state of the art, suitable for demonstrating inhibition and potency. Proposers are highly encouraged to include recently discovered CRISPR-Cas orthologs. Proposals must include inhibitors for CRISPR-Cas systems, specifically Cas9 and Cas12; however, molecules that can inhibit other nucleases are encouraged. Potential approaches to development of novel inhibitors include, but are not limited to:

- Bioinformatic, biochemical, computational, or genetic methods to discover new inhibitors;
- High-throughput biochemical, chemical, and/or genetic screens;
- Directed evolution;
- Multivalent molecules;
- Hybrid synthetic-biological materials;
- Fusion proteins with enzymatic activity;
- Small molecules;
- Modified nucleic acids and mimetics;
- Peptide nucleic acids.

The RIDDL program is also agnostic to the method(s) by which inhibitors arrest genome editing activity. Novel inhibitors may utilize a wide variety of mechanisms of action, including but not limited to the following:

- Inhibiting DNA binding;
- Inhibiting cutting activity;
- Inhibiting conformational changes required to initiate nuclease activity;
- Enzymatic degradation of CRISPR-Cas complexes or components;
- Cleaving crRNA;
- Inhibiting CRISPR-Cas RNA biogenesis;
- Inhibiting formation of CRISPR-Cas complexes with guide RNA".

Moreover, "Proposals should focus on the development of a rapid discovery platform for inhibitors of novel, emerging gene editor systems, including Cas9 and Cas12, and including those generated by deep learning language models. Objectives include (a) discovery pipeline for inhibitors of novel editing systems; (b) in vitro models and assays to test and validate candidate molecules; and (c) demonstration of potent inhibition of novel gene editing systems" [12].

**CRISPR-Cas system.** The CRISPR-Cas system is an adaptive "immune" system identified in bacteria and archaea [13].

It is an analogue of the adaptive immune system in humans. Its key feature is the generation of memory of past infections. This allows to rapidly build a more efficient and robust response to recurrent infections. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. The full form (CRISPR-Cas) talks about two main parts of the system. First, there are repetitive sequences, which are short DNA segments, 20–40 bp long. These repeats are palindromic. I.e. they are sequences of "letters" that read the same from the left or right. Like an example "NEVER ODD OR EVEN". In the palindromic repeat sequences, each DNA repeat is arranged in the palindromic fashion. This means that the repeat's sequence on one DNA strand is identical to the opposite strand's sequence when both are read in their respective 5' to 3' direction. (As 5'-GGATCC-3' and 3'-CCATGG-5'. When these palindromic sequences are transcribed to form mRNA, they create a hairpin structure (due to "internal" pairing – (GC)(UA)(GC) where UA "sits" on the loop of the hairpin). The repeat sequences are highly conserved within the CRISPR locus. This means that they are all identical, one after another after another and so on. These Short Palindromic Repeats are Regularly Interspaced. This means that spacer DNA or spacers are present between the repeat sequences. These spacers are not identical. Each segment of the spacer is unique. These unique sequences match with the DNA found in the viruses (bacteriophages). So, these spacer sequences are some kind of "memory" of the immune system in bacteria that protects the bacteria from viruses. The bacteria use these spacers as recognition elements to find matching DNA from virus genomes. This "immunological memory" bank stores the sequences from previous encounters with invading organisms. The number of spacers within a CRISOR array can range from as few as one to several hundreds. Another part of CRISPR

[3] The text in bold has been highlighted by the author.

is a region rich in adenine (A) and thymine (T). It is known as the leader sequence and is located upstream to CRISPR loci. The CRISPR array also consists of CRISPR-associated genes (*cas* genes). These *cas* genes code Cas proteins. They can be helicases (they unwind DNA) or nucleases (they cut the target DNA. These Cas proteins constitute the backbone of the CRISPR-Cas system (*Figure 1*).

How does the bacterial cell protect itself from the infection? To encounter the infection, the bacterial CRISPR-Cas plays a vital role. The CRISPR-Cas mediated defense process occurs in three steps [15].

i) The first step is known as adaptation or acquisition. During this step the genetic material of the invading phage is integrated into the CRISPR locus. When a virus infects a bacterial cell proteins Cas1 and Cas2 identify the invading viral DNA and excise a segment of specific length from the viral DNA [16].

This segment is known as the protospacer or spacer. Then the protospacer is added to the front of the CRISPR array between the two repeat sequences. By this mechanism the bacteria generate the "immunological" memory of the invaded virus.

ii) The second step is known as expression. A long primary transcript, the precursor-CRISPR RNA (pre-crRNA) is generated. This RNA contains a series of secondary structures or hairpins because of palindromic sequences. The pre-crRNA is subsequently processed into smaller crRNAs by Cas proteins. Each crRNA consists of a spacer flanked by partial repeats (*Figure 2A*).

iii) The third step is interference. In this step the mature crRNA forms a complex with one or more Cas proteins. This "search complex" patrols inside the cell and looks for invading phages or other foreign material that matches the crRNA sequence. Once the match is found the spacer sequence of crRNA pairs with the virus nucleic acid and the specific Cas proteins chop up the invading genetic material (*Figure 2B*). The crRNA is also called guide RNA or gRNA. Thus, the CRISPR-Cas system is also called RNA-guided targeting of the viral genome.

CRISPR systems in bacteria had evolved to defend bacteria against viruses. However, the viral DNA targeted by the search complex has the same sequence as the protospacer DNA in the CRISPR array. (The protospacers are the excised segments of the invading viral DNA.) How is Cas protein able to distinguish between itself and "nonself"? Here comes PAM. PAM stands for Protospacer Adjacent Motif. PAM is a specific sequence of nucleotides, around 2–6 bp, that follows the protospacer genome in a viral genome. It is not a component of the bacterial locus. In the interference step of the CRISPR system mediated defense, specific Cas proteins recognize and bind to the PAM sequence. This binding facilitates unwinding the DNA target and allows the base pairing between the crRNA and the foreign DNA. The DNA is cleaved ("choped up") by the Cas proteins [17].

There are six major types of CRISPR-Cas systems. The classification is based on the differences in the pre-crRNA processing,

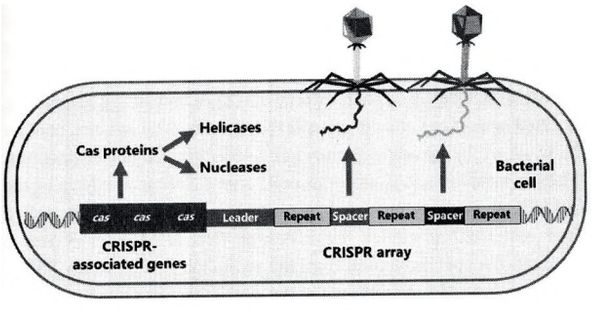



**Figure 1:** *The components of the CRISPR-Cas system. The figure is taken from [14]*

**Рисунок 1** – *Компоненты системы CRISPR-Cas. Рисунок взят из [14]*

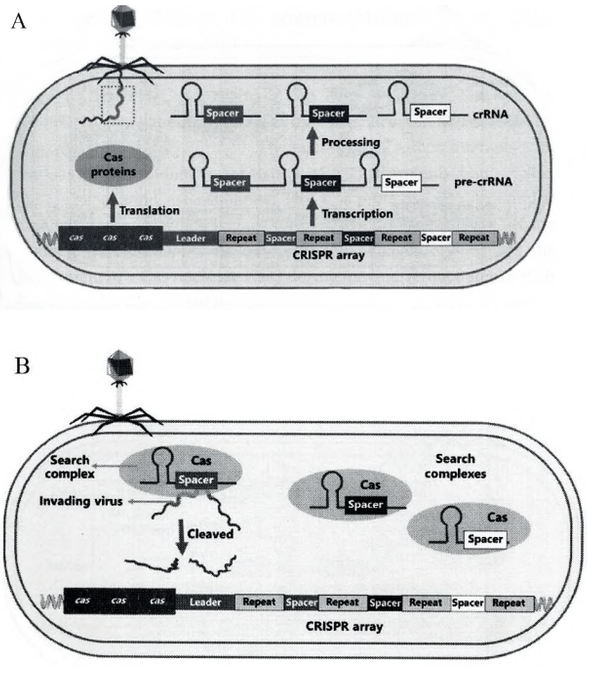



**Figure 2:** *A, CRISPR RNA (crRNA) expression; B, crRNA-Cas complex chops up the foreign DNA. The figure is taken from [14]*

**Рисунок 2** – *Экспрессия CRISPR РНК (crRNA), А; crРНК-Cas комплекс разрезает чужеродную ДНК, В. Рисунок взят из [14]*

the interference step, and the requirement of different Cas proteins [18, 19].

For further explaining we will proceed with the type 2 i.e. CRISPR-Cas9 system. This system gained significant attention and is widely used as a tool in genome editing. It follows from the fact that:

i) Type 1, 3, and 4 CRISPR-Cas systems require several Cas proteins for cleaving the target viral genome.

ii) The Cas9 protein, the part of the search effector complex, participates in the processing of pre-crRNA to form mature crRNAs.

iii) Thus, due to the simplicity and requiring of only the Cas9 enzyme for processing crRNA and forming search effector complex the CRISPR-Cas9 has been widely adopted as a gene adopting tool.

The type 5 CRISPR-Cas12 system uses the Cas12 enzyme. Cas12 differs from Cas9. It causes a "staggered" cut in double-stranded DNA, producing single-stranded overhang ends in contrast to "blunt" cut made by Cas9. Cas12 requires only a crRNA for successful targeting. On the other hand, Cas9 requires both crRNA and a transactivating rRNA to target the invading virus' DNA (see below). Type 6 CRISPR-Cas13 utilizes the Cas13 enzyme. Cas13 is an RNA-guided RNA endonuclease. It does not cleave DNA but only single-stranded RNA. An additional feature of Cas12 and Cas13 enzymes is that they show trans- or collateral cutting activity. This means that their cleavage activity is not restricted targeting DNA or RNA, but they can also cut any single stranded non-targeted nucleic acid molecule in the vicinity.

The simplified CRISPR-Cas9 system consists only of three components. Cas9, crRNA and tracrRNA. This makes this system easy to be used for genome editing [20].

The simplified engineered CRISPR-Cas9 system was developed linking the two RNAs – crRNA and tracrRNA to form a single guide RNA (gRNA) (*Figure 3*).

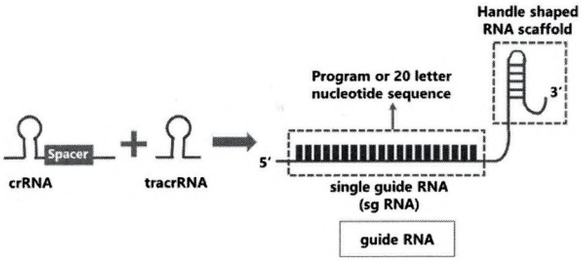



**Figure 3:** *Guide RNA. The figure is taken from [14]*
**Рисунок 3** – *Направляющая РНК. Рисунок взят из [14]*

Thus, the gRNA consists of two regions. One is a "program" – 20 letter nucleotide sequence. The second part is a handle-shaped RNA scaffold. The "program" of the 20-letter nucleotide sequence is complementary to the short sequence of the target DNA of the host which is "required" to be edited. For instance, if the target DNA has a sequence ATGCGC (5' to 3' direction), then the gRNA complementary sequence reads UACGCG (3' to 5' direction). The other region of gRNA is the handle-shaped RNA scaffold which interacts with the Cas9 protein. The gRNA-Cas9 complex then binds to the target sequence in the host genome. After binding to the desired position in the genome, the nuclease domains of the Cas9 introduce double strand breaks (DSB) in the desired DNA. The Cas9 protein has two lobes. One lobe is for target recognition, the other lobe contains nuclease activity. The recognition lobe is essential for binding gRNA (and target DNA). The nuclease lobe cleaves the target DNA by generating double-strand breaks. Additionally, the nuclease lobe contains HNH (His-Me finger) and RuvC nuclease domains [21].

The HNH nuclease domain cleaves the DNA strand that is complementary (i.e. paired) to the 20-letter nucleotide sequence of the gRNA. The RuvC nuclease domain cleaves the non-complementary (i.e. coding) DNA strand (*Figure 4*).

When Cas9 creates DSB the cell's inherent repair mechanisms can fix them via two pathways. The non-homologous end-joining repair (NHEJ) or homology-directed repair (HDR) [22, 23].

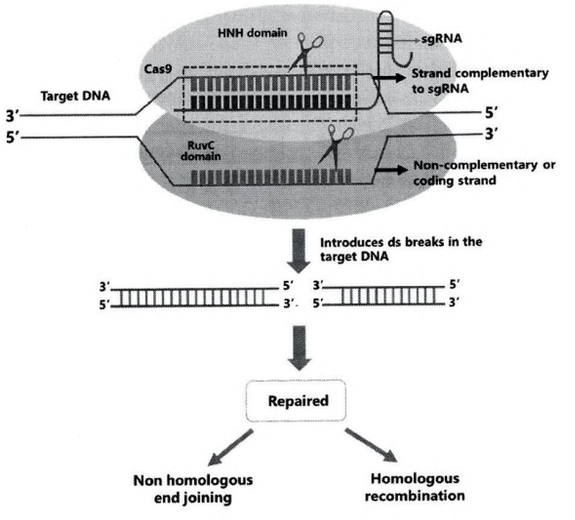



**Figure 4:** *CRISPR-Cas9 system for gene editing. The figure is taken from [14]*
**Рисунок 4** – *Система редактирования генома CRISPR-Cas9. Рисунок взят из [14]*

NHEJ-mediated repair of Cas9 generated breaks makes the gene inactive or inoperative. On the other hand, HDR pathway is highly accurate and can be used to introduce specific nucleotide changes into the target gene by inserting or replacing a DNA sequence near the break site using the donor repair template DNA. Thus, HDR pathway can be used for gene knockout, gene tagging, introducing specific mutations in the (desired) gene, knock-in, or for promotor studies. In general, HDR pathways are typically less efficient than the NHEJ pathways. To increase the HDR efficiency it is necessary to suppress some of the key molecules involved in the NHEJ [24].

Among the first bacteria harboring the CRISPR-Cas9 system, it was the bacteria *Streptococcus pyogenes* where the CRISPR type loci were characterized. Although SpCas9 revolutionized the field of genome editing and gene therapy it does have some limitations. The first limitation is its off-target activity which leads to modifications at 'non-target" sequences in the genome [25, 26].

This occurs when the guide sequence of gRNA pairs with another closely matched sequence in the genome. The second limitation of the SpCas9 enzyme is the stringent requirement for the PAM sequence "NGG" (N – stands for any nucleotide A, T, C, G). With the objective to overcome these limitations several variants of the SpCas9 enzyme have been developed [27].

The use of different (Cas9, Cas12, Cas13, Cas14) proteins utilized for genome engineering are reviewed here [28].

The *first* variant of SpCas9 enzyme used for gene editing is Cas9 nickase. Inactivation of one of the nuclease domains of the Cas9 enzyme results in the formation of SpCas9 nickase mutants that cleave or, in other words, introduce a single nick in one strand of the target dsDNA. For generating two nicks in the target DNA, two guide RNAs are required, one for each Cas9 nickase mutant. These two guided RNAs target the opposite strands of the target DNA., thus guiding the two Cas9 nickase mutants to generate nicks in both strands. The nicks in the genome are repaired with high fidelity as compared with blunt-ended DSBs (*Figure 5*). Thus, the double-nicking strategy of Cas9 nickase significantly reduces the cleavage and editing of unwanted off-targets in the genome. The *second* variant of SpCas9 is Fok1 fused catalytically inactive Cas9 [29].

To achieve this the SpCas9 is rendered catalytically inert by mutating both its nuclease domains, HNH and RuvC. The resulting mutated spCas9 nuclease is called dead-Cas9 (dCas9) [30, 31].

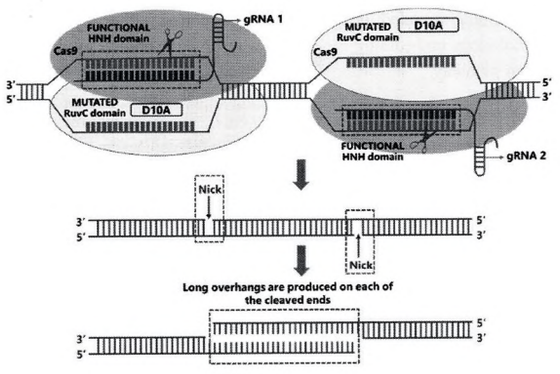



**Figure 5:** *Two nicks produced in the target DNA by the two paired mutated Cas nickases and two gRNAs. The figure is taken from [14]*

**Рисунок 5** – *Два надреза, сделанные в целевой ДНК двумя парными Cas-никазами, подвергшимся мутации и двумя направляющими РНК. Рисунок взят из [14]*

To achieve this the SpCas9 is rendered catalytically inert by mutating both its nuclease domains, HNH and RuvC. The resulting mutated spCas9 nuclease is called dead-Cas9 (dCas9) [30, 31].

This dCas9 is fused to the Fok1 nuclease domain to generate the fCas9 enzyme, The FokI domain on fCas9 becomes active only after dimerization. Therefore, it requires two monomers to bind to the target site simultaneously (*Figure 6*). The *third* type represent the SpCas9 nucleases with new PAM (Protospacer Adjacent Motif) specificities [32].

By introducing mutation into the PAM-interacting domains engineered SpCas9 variants can recognize the PAM sequences NGCG or NGAG. Additionally, when aspartic acid at the 1135th residue of SpCas9 is replaced by glutamic acid it obtains a much greater DNA specificity and it shows substantially lower off-

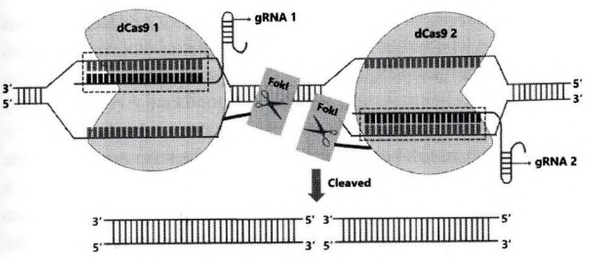



**Figure 6:** *Cleavage of DNA by two fCas9 enzymes with two gRNAs. The figure is taken from [14]*

**Рисунок 6** – *Процесс расщепления ДНК двумя энзимами fCas9 с двумя направляющими РНК. Рисунок взят из [14]*

target activity. The *fourth* type of SpCas9 are High Fidelity SpCas9s (SpCas9-HF1, Enhanced SpCas9, Hyper-accurate Cas9, and HiFiCas9). For these high-fidelity variants, certain mutations are introduced into the SpCas9 enzyme to reduce off-target interactions [33–35].

While the commonly used *Streptococcus pyogenes* SpCas9 can tolerate multiple mismatches in the target sequence other naturally occurring Cas9 orthologs from *Staphylococcus aureus* (SaCas9), *Neisseria meningitidis* (NmCas9), *Campylobacter jejuni* (CjCas9) were reported to have higher specificity in genome editing compared to SpCas9. Additionally, these orthologous enzymes show different substrate specificities and recognize different PAM sequences [36].

**Delivery of CRISPR components to the target cells.** In principle the Cas9-gRNA construct cannot be directly inserted into the target cell. It is necessary to used carriers which are called vectors. The vectors can be viral or non-viral. We are not coming into the details here. The interested reader can find scholar description in the book cited mainly under the figure texts [book CRISPR-Cas]. Briefly, among used viral vectors there are adenoviral, adeno-associated viral (AAV), and lentiviral vectors. Non-viral methods offer few advantages over the viral vectors like reduced pathogenicity, low cost and ease of production [37].

For gene editing by CRISPR-Cas9, three types of cargo can be delivered to the target cell:

i) DNA plasmid encoding both the Cas9 protein and the guide DNA;

ii) mRNA for the translation of Cas9 protein and a guide RNA;

iii) or a ribonucleoprotein (RNP) complex composed of Cas9 protein and a guide RNA.

Non-viral delivery methods can be divided into physical or chemical carrier-mediated methods. Some of the physical methods include microinjection, electroporation, gene gun, sonoporation, optoporation or laser beam-mediated delivery. The chemical vectors include cationic lipids/lipoplexes [38], polyplexes polymeric nanoparticles, and magnetofection [39].

**Therapeutic applications of CRISPR-Cas.** In general, there are currently three main fields where the CRISPR-Cas9 is used in therapeutic applications. They include:

i) CRISPR-Cas in adoptive T-cell immunotherapy for cancer. This includes tumor-infiltrating lymphocyte (TIL) therapy, Engineered T-cell receptor (TCR) therapy, chimeric antigen receptor T (CAR-T) cell therapy.

ii) CRISPR-Cas9 as antiviral therapy.

iii) CRISPR-Cas9 in the treatment of genetic diseases.

**CRISPR-Cas system applications in gene editing**

CRISPR-based gene drive. Gene drives are self-propagating genetic elements that rapidly promote the inheritance and spread of the desired gene variant through a population[4]. In the traditional Mendelian inheritance each allele has a 50% chance of being passed on to an offspring. However, through gene drivers a desired genetic variant has a >50% chance of being passed onto the offspring thus can be spread through a population faster. Gene drivers contain molecular tool that targets other versions of the target gene, cut is out and replaces it with DNA using the gene drive as a template resulting in the homogenous presence of this modification. Therefore, the gene variant will be present in both copies on the chromosome pair (homozygote), and it is inherited in 100% of the offsprings. Over the successive generations this gene variant spreads through a population until all (the vast majority) individuals possess it. Synthetic gene drive (GD) systems constitute a form of novel invasive environmental biotechnology with far-reaching consequences beyond those of other known genetically modified organisms (GMOs). Recently developed CRISPR–Cas9 based gene-drive systems are highly efficient in laboratory settings, offering the potential to reduce the prevalence of vector-borne diseases, crop pests and non-native invasive species [40].

The bipartite nature and flexible programmability of CRISPR led to the rapid development of a variety of gene-drive systems. One of the strategies for deploying low threshold gene-drive systems to reduce the disease impacts of insect-borne pathogens is often referred to as “population suppression”. It is the genetic equivalent of insecticides (*Figure 7*). The application of gene drivers can address some real problems such as eradicating malaria, controlling invasive species, and protecting endangered species that are in danger of becoming extinct (for instance frogs and other amphibians worldwide which are under severe threat from pathogenic chytrid fungus). Here, in the last group through gene drives, a protective gene could be introduced and spread. Adding useful genes to endangered plants, such as drought tolerance or disease-

[4] Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values | The National Academies Press. URL: https://nap.nationalacademies.org/catalog/23405/gene-drives-on-the-horizon-advancing-science-navigating-uncertainty-and (date: 10.12.2024).

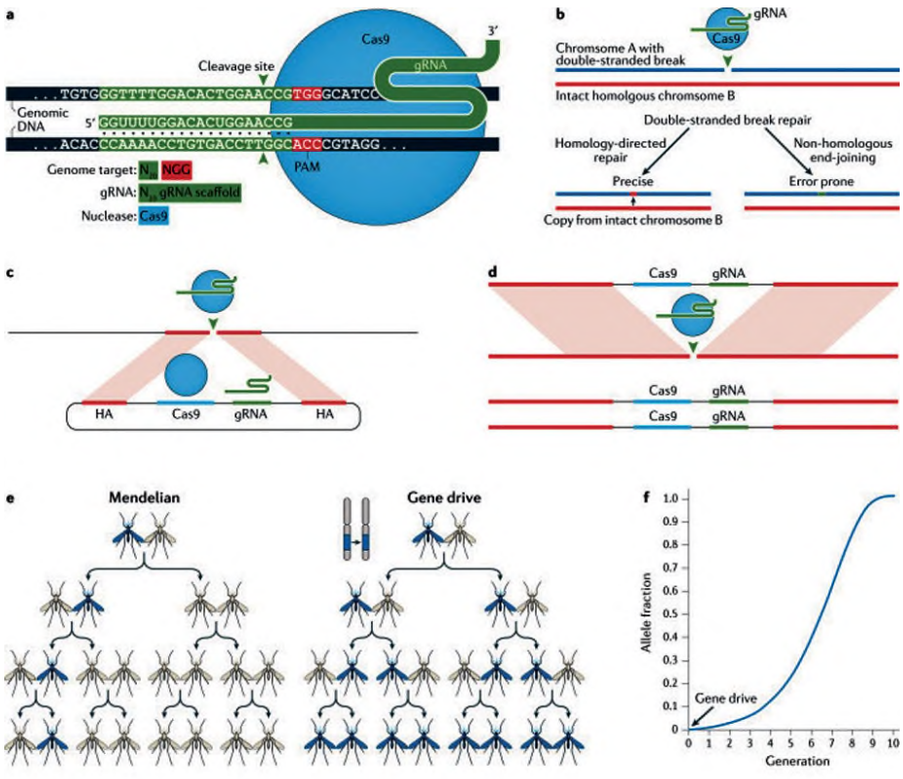



**Figure 7:** *Design and spread of CRISPR-based gene drives. Gene-drive scheme. The bipartite synthetic CRISPR system (a). A guide RNA (gRNA; green) binds Cas9 (cyan) directing it to bind and cleave DNA at complementary sites 20 nucleotides in length. The protospacer-adjacent motif (PAM) site (NGG; red) is required for Cas9 binding to genomic targets. In eukaryotic cells, double-stranded breaks are repaired either by the error-prone non-homologous end-joining or by homology-directed repair (HDR), the pathway acting in the germline (b). Insertion of a cassette encoding Cas9 (cyan), and a gRNA (green) flanked by homology arms (HAs) results in HDR-mediated copying of the cassette from the plasmid into the genomic cut site (c). The HAs directly flank the gRNA-directed cleavage site. Once inserted into the genome, the Cas9 + gRNA cassette directs cleavage of the homologous chromosome in the germline and is copied into the DNA break by HDR resulting in nearly all progeny (~99%) inheriting the 'gene-drive' cassette (d). Comparison of Mendelian versus gene-drive inheritance patterns. In each case, a few transgenic individuals (blue) are introduced to a large wild-type (WT) population (white) (e). Predicted logistic growth curve for seeding 1% gene-drive individuals into a WT population (f) (the figure was taken from [40])*

**Рисунок 7** – *Создание и распространение генных драйвов на основе CRISPR. Схема генного драйва. Двусторонняя синтетическая система CRISPR (a). Направляющая РНК (gRNA; зеленого цвета) связывает комплекс Cas9 (светло-голубого цвета), направляя его на установление связи с ДНК и разрезание ее на комплементарные участки длиной в 20 нуклеотидов. Область последовательности, примыкающей к протоспейсеру (NGG; красного цвета) необходимое условие для связывания геномных целей с помощью Cas9. В эукариотических клетках двойные разрывы цепочек восстанавливаются либо посредством ненадежного и негомологичного соединения концов, либо посредством репарации с участием гомологичной рекомбинации (HDR), путь находится в зародыше (b). Вставка кассеты, кодирующей Cas9 (светло-голубого цвета) и направляющей РНК (зеленого цвета), находящихся по обе стороны от гомологичных плеч (HAs), приводит к созданию копии кассеты посредством репарации с участием гомологичной рекомбинации. Копия создается из плазмиды и помещается на место разреза в геноме (c). Гомологичные плечи располагаются по бокам от места расщепления ДНК. Как только кассета, содержащая Cas9 + нРНК попадает в геном, она начинает управлять процессом расщепления гомологичных хромосом в зародышевой линии, копируется в место разрыва ДНК, происходит восстановление посредством репарации с участием гомологичной рекомбинации. В результате этого все последующие гены (~99 %) наследуют кассету с 'генным драйвом' (d). Сравнение Менделеевского типа наследования с наследованием по типу генного драйва. В каждом из этих случаев несколько трансгенных образцов (выделены синим цветом) внедряют в большое скопление генов, не имеющих никаких мутаций (wild-type (WT) population, выделено белым цветом) (e). Прогнозируемая логистическая кривая для посева 1 % отдельных генов с генным драйвом в скопление немутантных генов (f) (рисунок взят из [40])*

resistance genes can potentially ensure their long-term survival. However, the application of gene drive technology to eradicate a species can have direct effects such as the loss of species, or indirect effects. These indirect effects could lead to unforeseen impact on human and environmental health. The selective protection or eradication of certain species might lead to the elimination of other species with a lower status. One of the biggest concerns is the potential spread of gene drivers to unintended species. This could happen if crossbreeding of horizontal gene transfer takes place between the genetically modified *Anopheles gambiae* and closely related species of mosquitoes [41, 42]. This could lead to extinction or significant alternation of such species, endangering the food webs and whole ecosystems that depend on them.

Furthermore, there are situations where the Cas9 enzyme cuts the target site, but instead of being repaired by HDR-mediated by HDR-mediated pathway, it is repaired through an NHEJ pathway. This process may produce gene drive resistant insertion/deletion alleles. Moreover, the Cas9 enzyme and gRNA may unintentionally cause mutations in non-targeted areas of the DNA. This can lead to unpredictable effects. We will omit the use of the CRISPR in agriculture (mutagenesis/mutation breeding, transgene-free genome editing in plants, resistance development, crop improvement) and in animals (models of human diseases, xenotransplantation, in livestock). The interested reader can find more in the textbook [CRISPR-Cas] used and cited a couple times in this article.

**Prime editing.** There is no doubt that the CRISPR-Cas is not fool-proof. Its practical use in curing human diseases has been limited by challenges with the delivery and precision. The off-target effects can alter DNA at "unintended" loci in the genome. The CRISPR-Cas9 genome editing depends on the DNA repair mechanisms. It includes NHEJ or HDR to fix DNA breaks. However, these repair processes can lead to the undesired and random insertions or deletions (INDELs) in unintended sequences of DNA which may harm or disrupt the cell function. Prime editing is a rather new genome-editing technique with a greater precision and efficiency, while limiting the (possible) negative effects of the CRISPR-Cas system [43].

Prime editing enables targeted editing without generating double-strand DNA breaks. Like CRISPR, prime editing requires the presence of an endonuclease (Cas) and a single guide (sg) RNA. The prime editing utilizes Cas nickase, a variant of Cas9 endonuclease. The replacement of histidine by alanine at the position 840 (H840A) inactivates the Cas9 nuclease HNH domain. With the RuvC functioning Cas9 nuclease domain introduces only a single-strand breaks (hence the name Cas9 nickase). Moreover, Cas9 nickase is fused to a reverse transcriptase (RT) enzyme. The function of the RT is to synthetize DNA from a single-stranded RNA template. This construct is referred as prime editor or PE (*Figure 8*). The second component, sg RNA is also modified in the prime editing method [44].

Here, the sgRNA is fused with a primer binding sequence (PBS). The sgRNA also carries the template RNA sequence for the change that one wants to make the genomic target DNA (*Figure 8*). These sequences are added at the 3' end of the sgRNA. This sgRNA is called prime editing guide RNA (pegRNA) [45, 46].

This pegRNA is performing two tasks simultaneously. First, it identifies the target site with the help of sgRNA and second, it provides a new template (i.e. genetic information) to replace the target DNA nucleotides. Thus, the prime editing method can mediate target insertions, deletions and base-to-base corrections without the need for DSBs.

The mechanism how the prime editing works is rather complicated (*Figure 9*) and we are not

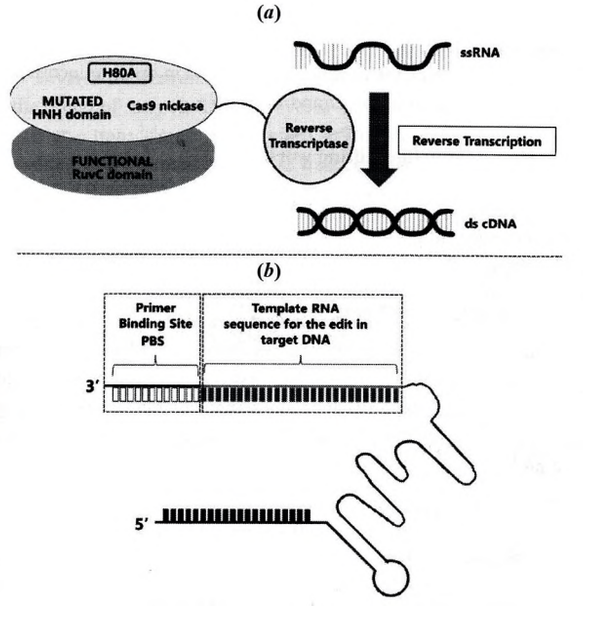



**Figure 8:** *Components of prime gene editing. (a) prime editor, (b) pegRNA. The figure is taken from [14]*
**Рисунок 8** – *Компоненты системы улучшенного редактирования генов. a – улучшенный редактор, b – pegРНК. Рисунок взят из [14]*

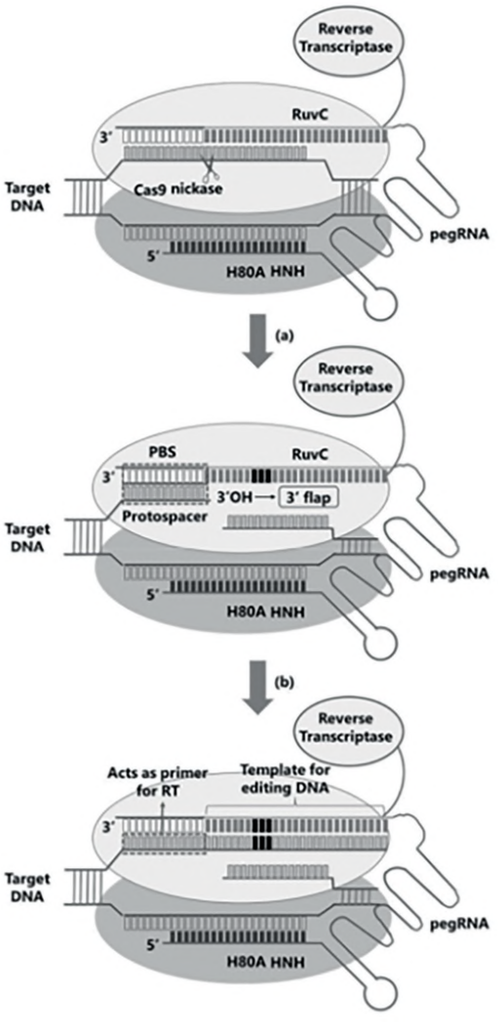



**Figure 9:** *Mechanism of prime gene editing. The figure is taken from [14]*
**Рисунок 9** – *Улучшенный механизм редактирования. Рисунок взят из [14]*

coming into details here. The interested reader can find the details elsewhere [47, 48].

**CRISPR-mediated base editing.** For base editing two major classes of base editors have been developed. Cytidine base editors (CBE) that allow conversion of CG to TA base pair, and the adenosine base editors (ABE) which convert AT to GC base pair. CBEs are formed by fusing Cas9 nickase with cytidine deaminase. On the other hand, ABEs are composed of a Cas9 nickase fused to E. coli tRNA adenosine deaminase (TadA) [49–51].

The schema of CRISPR-mediated base editing is shown in *Figure 10*. Precision editing system like base editing exhibit significantly lower INDEL rates. They may also have an important role in agriculture [52, 53].

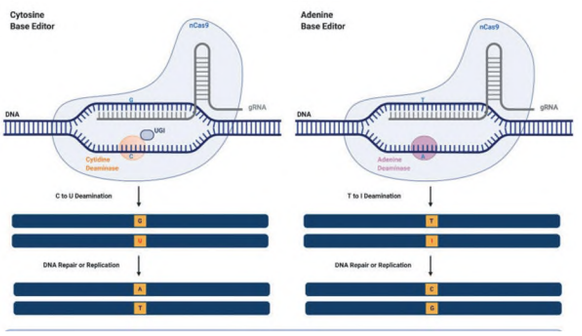



**Figure 10:** *DNA Base-editing. DNA base-editors encompass two key components: a Cas enzyme for programmable DNA binding and a single-stranded DNA modifying enzyme for targeted nucleotide alteration. Two classes of DNA base-editors have been described: cytosine base-editors and adenine base-editors. Cytosine deamination generates uracil, which base pairs as thymidine in DNA. Fusion of uracil DNA glycosylase inhibitor (UGI) inhibits the activity of uracil N-glycosylate (UNG), thus increasing the editing efficiency of cytosine base-editing in human cells. Adenosine deamination generates inosine, which has the same base pairing preferences as a guanosine in DNA. Collectively, cytosine and adenine base-editing can install all four transition mutations (C→T, T→C, A→G, and G→A). The figure and the text are taken from [49]*
**Рисунок 10** – *Базовое редактирование ДНК. Базовые редакторы ДНК содержат два ключевых компонента: энзим Cas для управления процессом связывания ДНК и фермент, модифицирующий одноцепочную ДНК для точечных изменений нуклеотидов. Были описаны 2 класса базовых редакторов ДНК: редакторы на основе цитозина и редакторы на основе аденина. Дезаминирование цитозина приводит к образованию урацила, который является комплементарной парой тимидина в ДНК. Слияние ингибитора урацил-ДНК-гликозилаза тормозит активность урацил N-гликозилата, что, в свою очередь, повышает эффективность цитозина при редактировании клеток человека. Дезаминирование аденозина приводит к образованию инозина, имеющего такие же комплементарные пары в ДНК, как и гуанозин. Вместе цитозин и аденин в процессе редактирования могут образовать 4 переходные мутации (C→T, T→C, A→G и G→A). Рисунок и текст взяты из [49]*

**CRISPR-mediated gene regulation.** CRISPR-Cas system has the potential to reversibly activate or silence genes with the dead Cas9 enzyme. The dead Cas9 (dCaS9) mutant is a Cas9 where both cleavage domains (HNH and RuvC) are inactivated. Although dCas9 can no longer cleave DNA, it can still bind target DNA with the same precision when guided by sg RNA. When transcriptional activators are fused to dCas9, the resulting complex can activate the

expression of the desired gene. This activation is called CRISPR activation (CRISPRa). For the activation of gene expression in eucaryotic cells the transcriptional activator used are VP64 or p64. VP64 is a synthetic tetramer of the activation of *Herpes simplex* viral protein 16. On the other hand, the p65 is a subunit of the NF-kB transcription factor (Nuclear Factor kappa-light-chain-enhancer of activated B cells) [54].

To enhance the transcription activation power of dCas9 some CRISPR activation systems have been developed and are expressed in viral vectors such as adeno-associated virus or lentiviral vectors. The first CRISPR activation system is the dCas9-VP64-p65-Rta (dCas9-VPR) [55, 56].

To enhance the expression of multiple genes within a single cell utilizing the dCas9-VP64 system, multiple sgRNAs are introduced into the cell [57].

The second way to enhance the transcription activation power of dCas9 is by developing the synergistic activation mediator (SAM). Such a system is built upon the basic dCas9-VPR structure but includes a modified sgRNA that incorporates two 138 nucleotide hairpin aptamers [58].

The RNA aptamers form the binding sites for the dimers of the bacteriophage MS2 coat proteins (*Figure 11*). MS2 coat proteins can further recruit additional activators such as p65 and the human heat shock factor 1 (HSF1). Through synergistic interactions among the VP64, p65, and HSF1 activation domains the dCas9-Sam system can enhance the gene expression from 10 to multiple thousand-fold.

The third way of enhancing the transcription activation of dCas9 is by using Supernova Tagging (SunTag) system. The SunTag is a repeating polypeptide array with multiple copies of GCN4 peptide [59], which can recruit multiple copies of antibodies that are attached to transcriptional factors like VP64 or p65 [60, 61].

Such SunTag-dCas9 activating complex has the potential to amplify the gene expression by more than 50-fold.

The CRISPR systems can be used in gene therapy [62, 63].

Moreover, it can be used a gene repression, i.e. gene silencing [64–66] and in epigenetic editing such as DNA methylation and histone modification [67–69].

**Inhibition of CRISPR-Cas systems.** In the **Introduction** we have introduced the approximately $17M DARPA program. The Rapid Inhibitor Discovery and Development pipeLine (RIDDL) program explicitly seeks transformative approaches that enable the rapid discovery, design, and development of novel inhibitors with enhanced activity, specificity, utility, and potency for gene editing technologies. Officially, the main reason is that "these approaches could serve as a rapid response to counteract the accidental or intentional misuse of gene editing technologies". It should be noted that this approach is not new. In 2021 Christopher L. Barkau and colleagues published a paper "Small Nucleic Acids and the Path to the Clinic for Anti-CRISPR" [70].

We will follow this paper to show the reader the basic features of the research. The authors state "CRISPR-based therapeutics have entered clinical trials but no methods to inhibit Cas enzymes have been demonstrated in a clinical setting. The ability to inhibit CRISPR-based gene editing or gene targeting drugs should be considered a critical step in establishing safety standards for many CRISPR-Cas therapeutics. Inhibitors can act as a failsafe or as an adjuvant to reduce off-target effects in patients". In this review the authors discuss the need for clinical inhibition of CRISPR-Cas systems and three existing inhibitor technologies: anti-CRISPR (Acr) proteins, small molecule Cas inhibitors, and small nucleic acid-based CRISPR inhibitors (*Figure 12*).

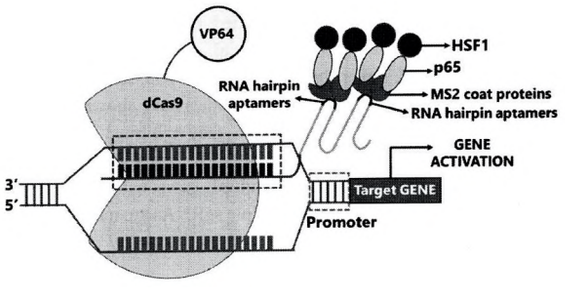
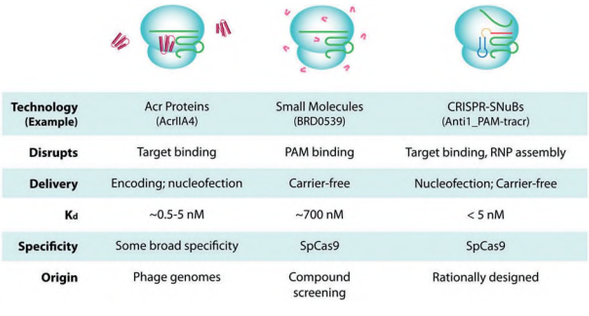



**Figure 11:** *Synergetic Activation Mediator (SAM) system. The figure is taken from [14]*
**Рисунок 11** – *Система медиаторов синергической активации. Рисунок взят из [14]*



| Technology (Example) | Acr Proteins (AcrIIA4) | Small Molecules (BRD0539) | CRISPR-SNuBs (Anti1_PAM-tracr) |
|---|---|---|---|
| Disrupts | Target binding | PAM binding | Target binding, RNP assembly |
| Delivery | Encoding; nucleofection | Carrier-free | Nucleofection; Carrier-free |
| $K_d$ | ~0.5-5 nM | ~700 nM | < 5 nM |
| Specificity | Some broad specificity | SpCas9 | SpCas9 |
| Origin | Phage genomes | Compound screening | Rationally designed |

**Figure 12:** *Comparison of anti-CRISPR technologies. The figure is taken from [70]*
**Рисунок 12** – *Сравнение технологий анти-CRISPR. Рисунок взят из [70]*

Why Inhibit CRISPR-Cas? "Amid the excitement and progress in CRISPR research and therapeutic development it may not be immediately obvious why inhibiting Cas proteins is desirable. However, inhibition is vital to the responsible use of CRISPR. It is becoming increasingly clear that CRISPR will impact disparate and possibly unforeseen aspects of our day-to-day life, including our environment, our food, and our health. Thus, there emerges a potential need for kill-switch inhibitors that can directly and completely disable CRISPR-Cas systems in a variety of contexts. For therapeutic development, possibly even FDA approval of certain CRISPR-based drugs, the development of an easily deliverable inhibitor to stop activity may become essential. Many approved drugs have an antidote that can be administered in the event of accidental misuse or to alleviate side effects, such as vitamin K and prothrombin complex concentrate for anti-coagulants like warfarin or protamine sulfate for heparin. Importantly, these drugs have relatively short-lived effects on the body, whereas the effect of CRISPR is permanent, making the availability of a kill-switch potentially even more vital. Among the proposed applications of CRISPR is the development of gene drives to amplify a trait (for example malaria resistance in mosquitoes) throughout a population or cause wild populations of organisms to crash entirely. These methods, and others yet to be developed, constitute a form of environmental engineering that could affect ecosystems, human health, economies, and power structures on a global scale. The production of widely applicable CRISPR inhibitors to counteract instances of accidental or intentional misuse of gene drives, or the weaponization of CRISPR against human populations, may become an urgent global security priority" [70].

Further: "A practical rationale for inhibiting CRISPR is also the prevention of off-target effects, defined as the unintended cleavage and mutation of sequences other than the target locus. In an extreme example of off-target effects, a recent study utilizing CRISPR in human embryos discovered that unrepaired cleavage products can persist through cell division resulting in allele-specific loss of entire chromosomes. For off-target reduction, inhibitors might function by two methods. The first is prevention of significant off-target cleavage by timed inhibition. This method is built on the hypothesis that on-target cleavage, being more energetically favorable due to full guide-target complementarity, occurs rapidly while off target activity is less favorable and accumulates primarily after the on-target locus has been cut and edited. For example, it has been shown that temporally limiting Cas9 and sgRNA persistence in cells raises the ratio of on-target to off-target editing. Encoding a sgRNA that targets the gene for Cas9 itself has been demonstrated to cause a self-restriction of functional Cas9 expression, reducing off-target editing in human liver cells. This method was further refined by the addition of an L7Ae:K-turn repression system to simultaneously attenuate Cas9 transcription and translation. Similar results have also been achieved using timed delivery of the anti-CRISPR protein AcrIIA45" [70].

And finally: "The second mechanism by which inhibitors can decrease off-target editing is by inhibiting excess enzyme. While this is similar to timed inhibition, it typically involves simultaneous delivery of the effector and inhibitor. Another related concept is "off-tissue" editing. In this case, it is not an incorrect genetic locus or target that is edited, but an on-target site in a tissue or organ where editing is not desired. Unrestricted CRISPR-mediated editing exposes diverse tissues and cell types, which may not be disease relevant, to potentially dangerous off-target mutations, including deletion of long genomic tracts or chromosomal rearrangements. Off-tissue editing should thus be avoided if possible. While some methods such as tissue-specific expression of Cas9 and sgRNA and modular LNP formulation for Cas9 ribonucleoprotein (RNP) delivery have been described, the ability to inhibit Cas effector enzymes in non-target tissues would be a valuable alternative or supplement to other approaches. In fact, many of these findings may aid the development of tissue-specific inhibitor delivery or restricted CRISPR activity. Thus, the inhibitors of interest are molecules that can largely act independently from enzyme engineering approaches and be added directly to an in vitro reaction, a cell, or potentially a living animal to block CRISPR-Cas endonuclease or gene targeting activity" [70].

*anti-CRISPR (Acr) proteins.* Anti-CRISPR (Acr) proteins are encoded by phages to help evade bacterial and archaeal CRISPR systems. They are also found in certain bacteria and encoded by mobile genetic elements [71]. Acrs were first discovered as five genes encoded in phages of *Pseudomonas aeruginosa* [72].

They inhibited the bacterium's type I-F CRISPR-Cas defense system, allowing them to infect *P. aeruginosa* cultures. Experimenting with translationally incompetent versions of the genes revealed that inhibition was translation-dependent and therefore likely to be protein-based.

*Small molecules.* Despite the attractiveness of small molecules as CRISPR-Cas inhibitor drugs, relatively little work has been done on the discovery and characterization of small molecules as inhibitors. An early small molecule investigation screened a library of 189,606 compounds for their ability to inhibit either RuvC or HNH nuclease activity and found six compounds that exhibited greater than 30% inhibition of SpCas9 in their system [73].

Unfortunately, these molecules were found to be prohibitively toxic to cells at 10 μM and were thus not considered to be candidates for animal studies. The authors speculate that the high number of interactions, as well as interaction strength, between Cas9, sgRNA, and its target DNA make it difficult to target with small molecules. This is similar to known challenges faced with disrupting protein–protein interactions [74].

Although this initial small molecule screen was not successful in finding immediately useful compounds, it provided a potentially useful platform for quickly and efficiently screening other possible inhibitors [75].

*Small nucleic acid-based inhibitors (SNuBs).* Inspired by the discovery of Acrs and previous work on chemically modified CRISPR guide RNAs [76, 77], small nucleic acids as potential inhibitors of Cas9 have been explored. The Cas9 RNP is a prime target for rational design of inhibitors that can mimic RNA and DNA binding, which are natural interactions for Cas enzymes. Nucleic acids can utilize multiple points of sequence-specific and sequence nonspecific contact that can be exploited to disrupt RNP assembly or target binding. Oligonucleotides designed to have two key points of contact, Watson-Crick pairing to the guide RNA repeat region and binding to the PAM-interacting (PI) domain of Cas9, act as strong inhibitors of SpCas9 [78].

These designs comprised a DNA hairpin containing an NGG sequence (anti-PAM), which mimics a PAM motif, that was tethered via a polyethylene glycol (PEG) linker to a 2'-O-methyl oligonucleotide for guide RNA base-pairing (anti-tracr) (*Figure 13*). These two linked modules, anti-PAM and anti-tracr, function synergistically to stably bind the Cas9-guide RNA complex and sterically block target binding. Initial designs produced an inhibitor with a Kd of ~ 25 nM while successive generations have since produced binding affinities at least an order of magnitude better, in the very low nanomolar range.

One of the major challenges CRISPR-Cas inhibitors will face on their path to clinical application lies in efficient delivery. While the obvious application of inhibitors is to reduce off-target editing across the genome, they could also be used to suppress editing in non-target tissues and organs. The former would necessitate delivery of an inhibitor with the same target distribution as the CRISPR therapeutic itself, either later point or simultaneously at a finely tuned ratio to the effector. The latter, however, would involve inhibitor delivery with an inverse distribution before or at the same time as CRISPR delivery. The distinct nature of each inhibitor technology discussed above—Acr proteins, small molecules, and nucleic acid brings unique advantages and difficulties for their delivery. Potential avenues of delivery for each inhibitor type are shown in *Figure 14.* Currently newer approaches of the "cargo delivery" have been developed [79–84].

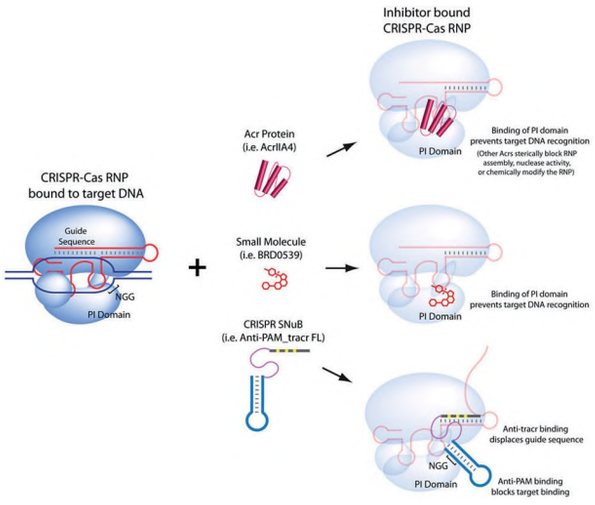



**Figure 13:** *Mechanism of Three Anti-CRISPR Technologies. The figure is taken from [70]*
**Рисунок 13** – *Механизм работы трех технологий анти-CRISPR. Рисунок взят из [70]*

**Clinical signs that make it possible to establish the fact of human health damage by CRISPR-Cas systems.** This crucial and fundamental problem has, in the author's opinion, no straightforward answer. However, in the recent article [85] *"Safely balancing a double-edged blade: identifying and mitigating emerging biosecurity risks in precision medicine"* the authors claim: "Tools and methods of precision medicine are developing rapidly, through both iterative discoveries enabled by innovations in biomedical research (e.g., genome editing, synthetic biology, bioengineered devices). These are strengthened by advancements in information technology and the increasing body of data as assimilated, analyzed, and made accessible and affectable through current and emerging cyber and systems technologies. Taken together, these approaches afford ever greater volume and availability of individual and collective human data. Machine

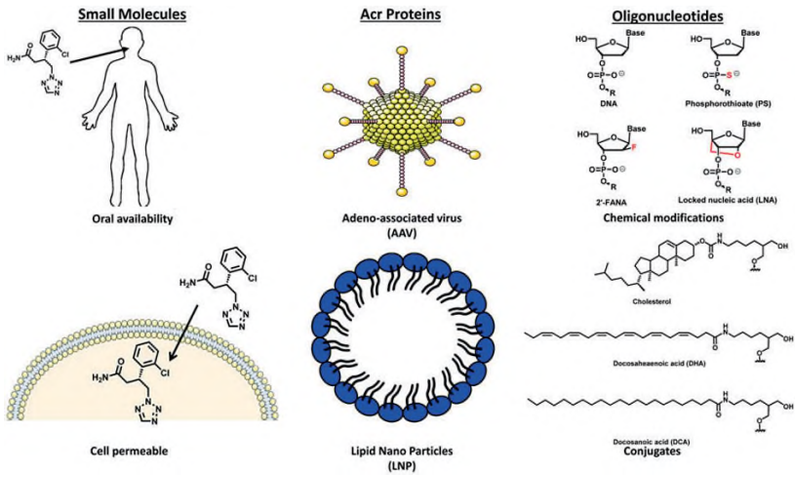



**Figure 14:** *Methods of Anti-CRISPR Delivery. Small molecules can be taken orally and are cell permeable. Acr proteins must be encoded in an AAV vector or packaged in lipid nanoparticles (LNPs) and delivered by injection. Oligonucleotides (SNuBs) can be injected directly or via LNP and exhibit uptake and distribution patterns modulated by chemical modifications and conjugates. The figure is taken from [70]*

**Рисунок 14** – *Методы доставки анти-CRISPR. Небольшие молекулы могут доставляться орально, они также являются клеточно-проницаемыми. Белки Acr должны быть закодированы в вектор аденоассоциированного вируса или должны находиться в липидных наночастицах и попадают в организм при инъекции. Олигонуклеотиды могут попадать в организм непосредственно при инъекции или через липидные наночастицы, при этом процессы усвоения и распределения регулируются посредством химических изменений и сложных соединений. Рисунок взят из [70]*

learning and/or artificial intelligence approaches are broadening this dual use risk; and in the aftermath of COVID-19, there is growing incentive and impetus to gather more biological data from individuals and their environments on a routine basis. By engaging these data and the interventions that are based upon them, precision medicine offer promise of highly individualized treatments for disease and injury, optimization of structure and function, and concomitantly, the potential for (mis)using data to incur harm". Both authors (DiEuliis & Giordano) are widely known because of their 2017 paper "*Why Gene Editors Like CRISPR/Cas May Be a Game-Changer for Neuroweapons*" [86]. As a possible example we consider the Huntington's disease (HD). HD is a neurodegenerative autosomal dominant disorder, which is characterized by involuntary choreatic movements with cognitive and behavioral disturbances. It occurs because of cytosine, adenine, and guanine (CAG) trinucleotide repeats on the short arm of chromosome 4p16.3 in the Huntingtin (HTT) gene. This mutation leads to an abnormally long expansion of the polyglutamine in the HTT protein, which leads to neurodegeneration. The expansion also causes the HTT protein to be more prone to aggregation and accumulation that mitigates protein folding. HD commonly affects patients between the ages of 30 to 50 years. However, the longer the CAG repeats, the earlier the onset of symptoms. Diagnosis can be made clinically in a patient with motor and or cognitive and behavioral disturbances with a parent diagnosed with HD and can be confirmed by DNA determination. In those patients who are at-risk for the disease, pre-manifest diagnosis can determine if they carry the gene. There is no cure for the disease, and affected patients tend to be entirely dependent on their caregiver as the disease progresses. Therefore, treatment is aimed at improving the quality of life and decreasing complications. Pneumonia is a common cause of death, followed by suicide [87]. Nevertheless, the attempts to treat HD with CRISPR-Cas systems are coming to age. The selective silencing of mutant HTT produces robust therapeutic benefits. In [88] the authors developed an allele-specific CRISPR/Cas9 strategy to permanently inactivate mutant HTT through nonsense-mediated decay (NMD). Comprehensive sequence/haplotype analysis identified SNP-generated NGG PAM sites on exons of common HTT haplotypes in HD subjects, revealing a clinically relevant PAS-based mutant-specific CRISPR/Cas9 strategy. Alternative allele of rs363099 (29th exon) eliminates the NGG PAM site on the most frequent normal HTT haplotype in HD, permitting mutant-specific CRISPR/Cas9 therapeutics in a predicted ~20% of HD subjects with European ancestry. Their rs363099-based CRISPR/Cas9 showed perfect allele specificity and good targeting efficiencies

in patient-derived cells. They were able to show a dramatically reduced mutant HTT mRNA and complete loss of mutant protein. The summary of the latest attempts to treat HD can be found in a review [89]. The HD can be picked up as a model for the misuse of the CRISPR-Cas technology. Instead of silencing the HTT gene one can imagine the introduction of the mutant HTT gene by the same CRISPR-Cas technology. This would enhance the number of CAG repeats in the brain cells of a healthy population. Now we can follow the "classical" path in the disease diagnosis. Firstly, form the medical point of view we can take the anamnesis. Secondly, we can recognize a (sudden) growth of the number of the persons with subtle problems with mood or mental/psychiatric abilities followed by general lack of coordination and an unsteady gait. The progression of the disease is accompanied with uncoordinated, involuntary body movements of chorea become more apparent. Physical abilities gradually worsen until coordinated movement becomes difficult and the person is unable to talk. Mental abilities generally decline into dementia, depression, apathy, and impulsivity at times. For an experienced neurologist this (sudden) enhancement of above-mentioned clinical symptoms must mandatorily lead to the suspicion of some "unnatural" origins of the disease. Thirdly, we are obliged to perform laboratory "helpful" tests. Here, the development of next-generation CRISPR/Cas-based ultrasensitive diagnostic tools can be helpful in the diagnosis of the (mis)use of CRISPR-Cas systems in humans [90] (*Figure 15*).

Further, and probably more important, the physicians can rely on the personalized medicine with the powerful techniques able to sequence the whole human genome[5]. Even the single base change, not to mention the whole insert or deleted DNA sequence can be easily detected by this "personalized, precision medicine" approach. It should be noted that the main issue from the diagnostic approach – the anamnesis and clinical features are the important factors which could point to the altered gene(s) necessary to be sequenced[6]. This could "spare" the sequencing of the whole genome and establish the diagnosis sooner. The employment of AI in these laboratory approaches seems to be mandatory.

**Establishing the use of CRISPR-Cas systems in humans.** The first CRISPR treatment for diseases was approved by the MHRA, UK on 16th November 2023. Following a thorough evaluation of its safety, efficacy, and quality, the Medicines and Healthcare Products Regulatory Agency (MHRA) has approved a novel medication for patients 12 years of age and older with sickle-cell

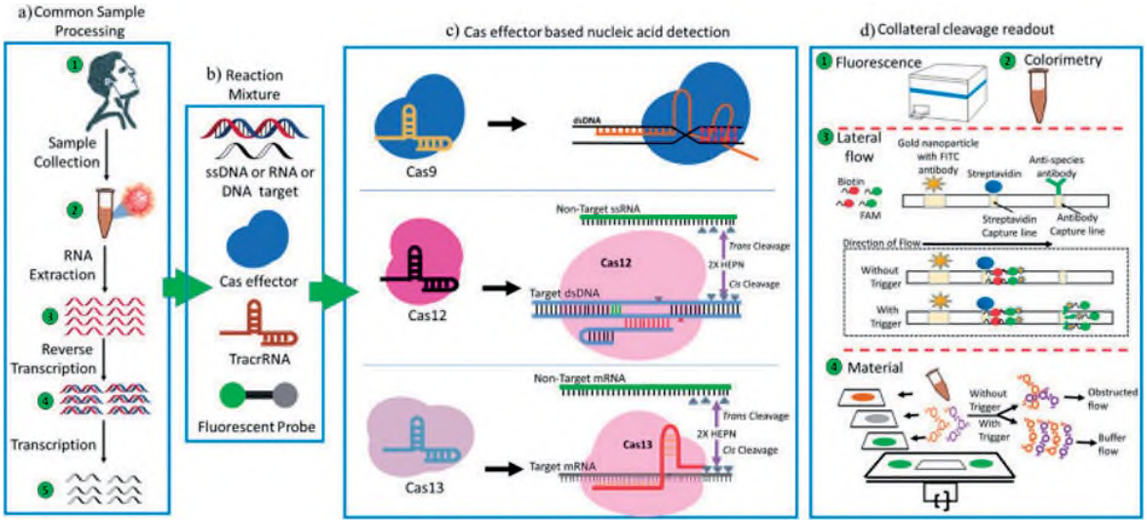



**Figure 15:** *Overview of the basic mechanism involved while adopting CRISPR as biosensors. (a) sample processing, (b) common reaction pool, (c) Cas effector-based cis–trans cleavage, and (d) post-cleavage detection. The figure is taken from [90]*

**Рисунок 15** – *Обзор основного механизма, включающегося при обработке CRISPR биосенорами. a – анализ образца, b – общий реакционный пул, c – цис–транс расщепление при участии эффекторов Cas, d – обнаружение после расщепления. Рисунок взят из [90]*

[5] Rentz A. Human genome sequencing powers personalized, precision medicine. URL: https://hub.jhu.edu/2025/02/28/nih-funding-human-genome-rajiv-mccoy/ (date: 10.12.2024).

[6] UCSC Genome Browser Home. URL: https://genome.ucsc.edu/ (date: 10.12.2024).

disease and transfusion-dependent β-thalassemia [91]. However, the application potential of first-generation CRISPR-based gene editing tools is limited by several key factors, the principal ones being specificity, targeting scope, and the need to rely on endogenous DSB repair mechanisms to achieve genomic edits. Moreover, the delivery of the CRISPR components is limited by specific constraints of the delivery vectors and target cells or organisms [92] (*Figure 16*).

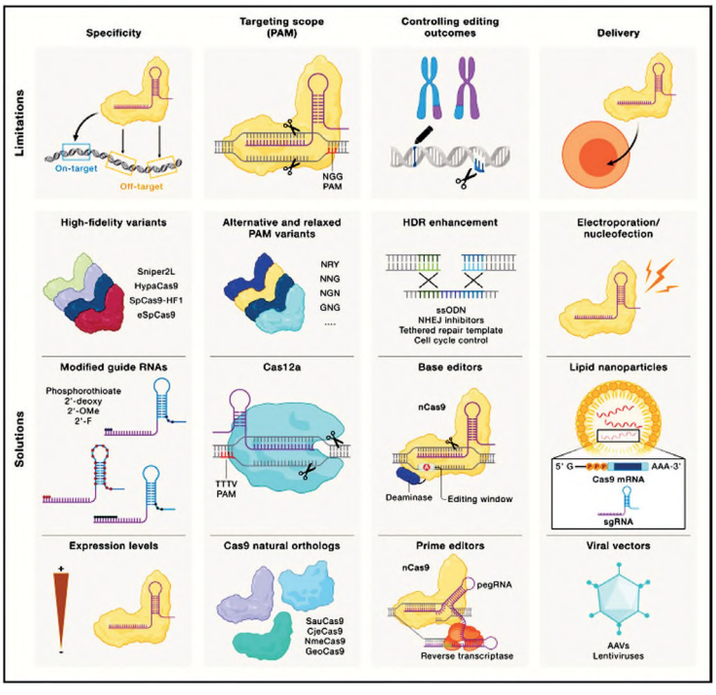



**Figure 16:** *Limitations of CRISPR genome editing. CRISPR genome editing faces four principal limitations, each addressed by specific technological solutions.* **Specificity:** *off-target activities of genome editors have been addressed by the development of high-fidelity nuclease variants, chemically modified guide RNAs, and controlled expression of genome editor nucleases.* **Targeting scope:** *the NGG PAM sequence requirement of SpCas9 restricts the scope of targetable genomic sites. This is addressed using engineered variants of Cas9 with alternative or relaxed PAM requirements, other naturally derived Cas9 orthologs with alternative PAM requirements, and Cas12a enzymes.* **Control of editing outcomes:** *various approaches, including asymmetric or tethered HDR repair templates, cell cycle synchronization, and NHEJ inhibitors, have been developed to enhance the efficiency of HDR and suppress the formation of indels by end-joining pathways. Second-generation technologies such as base or prime editing enable the introduction of precise modifications independently of HDR.* **Delivery:** *cellular delivery of genome editor components is facilitated by electroporation/nucleofection, lipid nanoparticles, and viral vectors. The figure and the text are taken from [92]*

**Рисунок 16 –** *Ограничения технологии редактирования генома с применением CRISP. В редактировании генома с применением CRISPR существуют четыре основных ограничения, каждое из которых связано с определенными технологическими решениями.* **Специфичность (Specificity):** *Нецелевая деятельность редакторов генома затрагивает развитие высококачественных вариантов нуклеазы, химические изменения направляющих РНК и контролируемую экспрессию нуклеаз редакторов генома.* **Целевой диапазон (Targeting scope):** *Требование области последовательности, примыкающей к протоспейсеру SpCas9 ограничивает диапазон целевых областей генома. Эта проблема решается использованием новых искусственно созданных вариантов Cas9 с абсолютно другими или более мягкими требованиями в отношении PAM, а также естественно полученными ортологами Cas9, которые имеют другие требования к PAM. Также возможно использование энзимов Cas12a.* **Контроль результатов редактирования (Control of editing outcomes):** *Существуют разные подходы позволяющие повысить эффективность репарации с участием гомологичной рекомбинации в том числе ассиметричная или привязанная репарация с участием гомологичной рекомбинации, синхронизация клеточного цикла, и ингибиторы негомологичного соединения концов. Эти методы также позволяют остановить образование вставок посредством соединения концов. Технологии нового поколения такие как базовое или улучшенное редактирование позволяют вносить необходимые точечные изменения вне зависимости от репарации с участием гомологичной рекомбинации.* **Доставка (Delivery):** *Доставка компонентов редакторов генома в клетки может осуществляться посредством электроимпульсного открытия клеточных пор / нуклеоинфекции, липидных наночастиц и вирусных векторов. Рисунок и текст взяты из [92]*

As the limitations of current CRISPR technologies have become increasingly clear over the past decade, novel approaches and methodologies continue to be developed and fine-tuned to address these constraints and improve the efficacy and versatility of CRISPR-based genome editing. These emergent, third-generation tools and technologies include recently discovered classes of compact RNA-guided nucleases that have been adapted for DSB-based editing and could also serve as RNA-guided DNA binding platforms for other genome editor modalities such as base editing (BEs) and prime editing (Pes). The insertion of long, gene-sized DNA sequences, particularly in post-mitotic cells lacking HDR, remains a major unmet need in the genome editing field. In this context, the development of CRISPR-guided recombinases and transposons presents a promising and potentially powerful avenue to fill this technology gap. New approaches have also emerged for genome editing technologies based on retrotransposons and for editing RNA transcripts. Finally, the creation of new genome editor tools continues to go hand in hand with advances in the development of delivery methods, which represent a major challenge for therapeutic applications [92] (*Figure 17*).

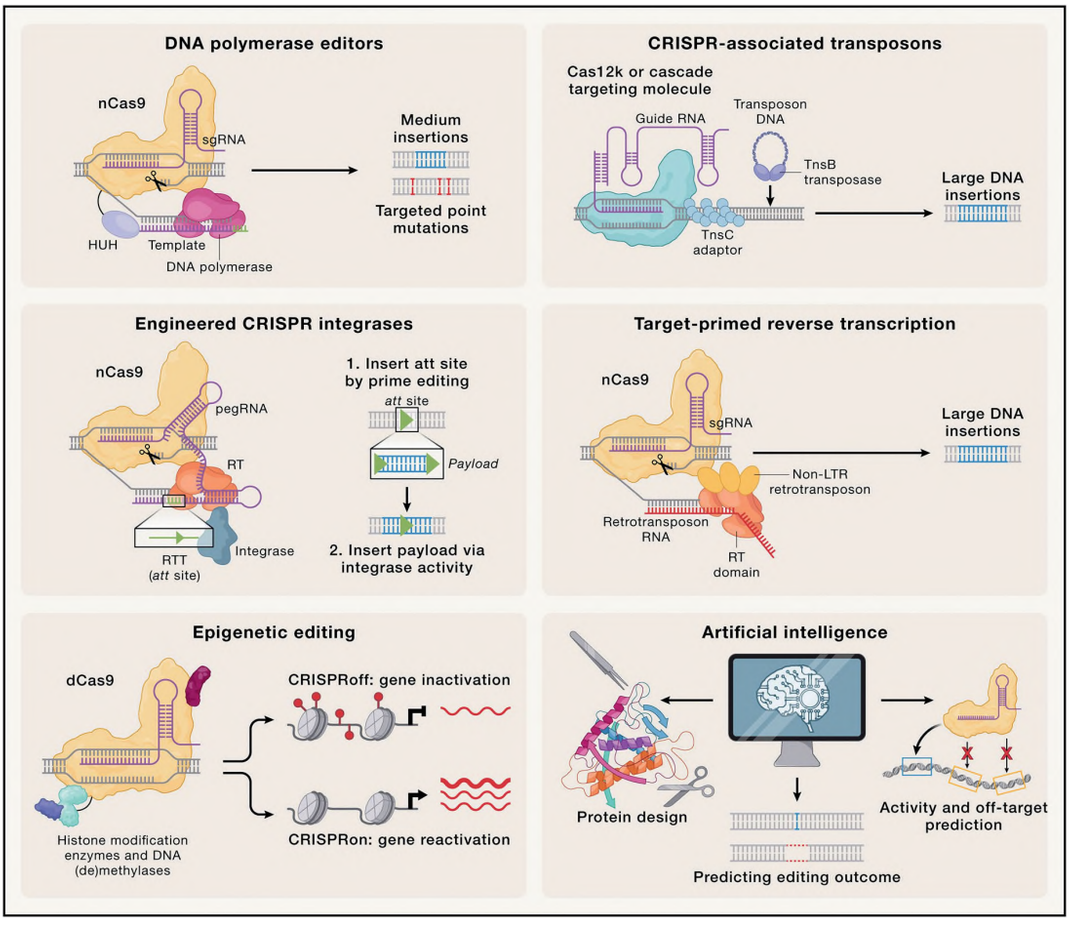



**Figure 17:** *Merging technologies in genome editing. A summary of emerging technologies in the genome editing field.* **DNA polymerase editors:** *This technology combines Cas9 nickases with DNA polymerases and tethering of a single-stranded DNA template, for example, using an HUH endonuclease. A key difference from prime editing lies in its use of DNA polymerase rather than reverse transcriptase and the delivery of the DNA template in trans.* **CRISPR-associated transposons:** *These naturally occurring mobile genetic elements utilize CRISPR effector complexes in conjunction with transposase proteins for RNA-guided transposition to insert long DNA sequences into specific genomic sites.* **Engineered CRISPR integrases:** *These technologies are based on combining prime editors with site-specific serine recombinases. The prime editing initially introduces a recombinase att the site at the target DNA location, subsequently enabling recombinase-catalyzed insertion of large DNA payloads.* **Target-primed reverse transcription:** *This process involves fusing nickase Cas9 with non-long terminal repeat (non-LTR) retrotransposon-derived reverse transcriptases and RNAs. It operates by nicking*

*the target DNA to generate a free 30 end to prime reverse transcription of the retrotransposon-associated RNA, resulting in targeted DNA insertion.* **Epigenetic editors:** *fusions of deactivated dCas9 with DNA methylases and histone-modification enzymes enable targeted chromatin modifications at specific genomic locations, leading to the heritable repression of gene expression (CRISPRoff) without altering the underlying DNA sequence. Gene reactivation (CRISPRon) involves targeting repressed genes using Cas9 fusions with DNA demethylases and transcriptional activator domains.* **Artificial intelligence in gene editing:** *AI is making significant inroads in de novo protein and guide design, as well as in computational prediction of off-target sites and editing outcomes. The figure is taken from [92]*

**Рисунок 17** – *Слияние технологий в редактировании генома. Краткий обзор новых методов в области редактирования генома.* **ДНК-полимеразы (DNA polymerase editors)** – *эта технология объединяет в себе никазы Cas9 и ДНК-полимеразы, к которым присоединяется образец одноцепочной ДНК, например, посредством эндонуклеазы HUH. Основное отличие от улучшенного редактирования заключается в том, что здесь используется ДНК-полимераза, а не обратная транскриптаза и доставка образца ДНК осуществляется в пути.* **CRISPR-ассоциированные транспозоны (CRISPR-associated transposons)** – *эти мобильные генетические элементы, появляющиеся естественным образом используют комплексы с CRISPR эффекторами в сочетании с белками транспозазы для перемещения направляющей РНК, чтобы иметь возможность вставлять последовательности ДНК в участки генома.* **Искусственно созданные CRISPR интегразы (Engineered CRISPR integrases)** – *эти технологии основаны на совмещении улучшенных редакторов с сериновыми рекомбиназами, которые являются специфичными элементами для конкретного участка генома. Улучшенное редактирование изначально создает рекомбиназу в том месте, где находится целевой участок ДНК, что в дальнейшем позволяет катализировать процесс и вставить большие цепочки ДНК в нужное место.* **Обратная транскрипция с фокусом на цель (Target-primed reverse transcription)** – *этот процесс подразумевает слияние никазы Cas9 с ретротранспозонными обратными транскриптазами с недлинным терминальным повтором и РНК. Выполняется надрез целевой ДНК, что приводит к образованию свободного 30 конца, затем происходит обратная транскрипция ретротранпозон-ассоциированной РНК и вставка целевой ДНК.* **Эпигенетические редакторы (Epigenetic editors):** *слияния деактивированной dCas9 с ДНК метилазами и ферментами модификации гистона позволяют проводить целевые модификации хроматина в отдельных участках генома, что приводит к наследственному подавлению экспрессии генов (CRISPRoff) без необходимости внесения изменений в исходную последовательность ДНК. Реактивация генов (CRISPRon) подразумевает поиск целевых подавленных генов при помощи слияния Cas9 с ДНК метилазами и доменами активации транскрипции* **Использование ИИ при редактировании генов (Artificial intelligence in gene editing)** – *ИИ позволяет достичь значительных успехов в разработке белков de novo и дизайна направления, а также в вычислительном прогнозировании нецелевых участков и результатах редактирования. Рисунок взят из [92]*

## Conclusion

The appeal of DARPA to "develop and demonstrate quick tools for identification and optimization of new molecules that exert an inhibitive impact on genome editing methods" is quite comprehensible from a logical point of view - any new technology can be used for dual purposes. However, it also proves that genome editing is not an experimental method anymore, and experts who nowadays realize the state of matter are concerned with the possible misuse of these tools in terms of new biological warfare development. From the medical point of view when the patient's history is properly taken, a clinical assessment is made, and all laboratory tests are conducted, including the sequencing of the „suspect" modified gene(s) or the whole human genome, the diagnosis can be determined quite easily. This approach is straightforward to find (the place) the attack starts and to prevent it or to stop it. This article discusses some approaches to optimization of methods that exert an inhibitive impact on "genetic scissors". Nevertheless, this is not enough. The genome editing use should be regulated by a special Protocol to Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction. As this protocol doesn't exist yet, then the national regulatory authorities are obliged to establish the borders for use of products that are based on these methods. They also should be able to prevent its misuse.

***Limitations of the study / Ограничения исследования***

All data were obtained from public sources; therefore the article is strictly limited on these public sources only. / Все данные получены из открытых источников, поэтому статья строго ограничена только этими открытыми источниками.

***References/Список источников***

1. Altae-Tran H, Kannan S, Suberski AJ, Mears KS, Demircioglu FE, Moeller L, et al. Uncovering the functional diversity of rare CRISPR-Cas systems with deep terascale clustering. *Science*. 2023;382(6673):eadi1910. https://doi.org/10.1126/science.adi19102

2. Marino ND, Pinilla-Redondo R, Csörgő B, Bondy-Denomy J. Anti-CRISPR protein applications: natural brakes for CRISPR-Cas technologies. *Nat Methods.* 2020;17(5):471–79. https://doi.org/10.1038/s41592-020-0771-6

3. Knott GJ, Thornton BW, Lobba MJ, Liu JJ, Al-Shayeb B, Watters KE, et al. Broad-spectrum enzymatic inhibition of CRISPR-Cas12a. *Nat Struct Mol Biol.* 2019;26(4):315–21. https://doi.org/10.1038/s41594-019-0208-z

4. Maji B, Gangopadhyay SA, Lee M, Shi M, Wu P, Heler R, et al. A High-Throughput Platform to Identify Small-Molecule Inhibitors of CRISPR-Cas9. *Cell.* 2019;177(4):1067–79.e19. https://doi.org/10.1016/j.cell.2019.04.009

5. Lim D, Zhou Q, Cox KJ, Law BK, Lee M, Kokkonda P, et al. A general approach to identify cell-permeable and synthetic anti-CRISPR small molecules. *Nat Cell Biol.* 2022;24(12):1766–75. https://doi.org/10.1038/s41556-022-01005-8

6. Zhao J, Inomata R, Kato Y, Miyagishi M. Development of aptamer-based inhibitors for CRISPR/Cas system. *Nucleic Acids Res.* 2021;49(3):1330-44. https://doi.org/10.1093/nar/gkaa865

7. Barkau CL, O'Reilly D, Rohilla KJ, Damha MJ, Gagnon KT. Rationally Designed Anti-CRISPR Nucleic Acid Inhibitors of CRISPR-Cas9. *Nucleic Acid Ther.* 2019;29(3):136–47. https://doi.org/10.1089/nat.2018.0758

8. Camara-Wilpert S, Mayo-Muñoz D, Russel J, Fagerlund RD, Madsen JS, Fineran PC, et al. Bacteriophages suppress CRISPR-Cas immunity using RNA-based anti-CRISPRs. *Nature.* 2023;623(7987):601–7. https://doi.org/10.1038/s41586-023-06612-5

9. Karvelis T, Druteika G, Bigelyte G, Budre K, Zedaveinyte R, Silanskas A, et al. Transposon-associated TnpB is a programmable RNA-guided DNA endonuclease. *Nature.* 2021;599(7886):692–6 https://doi.org/10.1038/s41586-021-04058-1

10. Saito M, Xu P, Faure G, Maguire S, Kannan S, Altae-Tran H, et al. Fanzor is a eukaryotic programmable RNA-guided endonuclease. *Nature*. 2023;620(7974):660–8. https://doi.org/10.1038/s41586-023-06356-2.

11. Pinilla-Redondo R, Shehreen S, Marino ND, Fagerlund RD, Brown CM, Sørensen SJ, et al. Discovery of multiple anti-CRISPRs highlights anti-defense gene clustering in mobile genetic elements. *Nat Commun.* 2020;11(1):5652. https://doi.org/10.1038/s41467-020-19415-3

12. Madani A, Krause B, Greene ER, Subramanian S, Mohr BP, Holton JM, et al. Large language models generate functional protein sequences across diverse families. *Nat Biotechnol.* 2023;41(8):1099–106. https://doi.org/10.1038/s41587-022-01618-2

13. Marraffini LA. CRISPR-Cas immunity in prokaryotes. *Nature.* 2015;526(7571):55–61. https://doi.org/10.1038/nature15386

14. Bansal R. *CRISPR-Cas: Applications in gene editing & beyond: CRISPR Cas System.* 1st ed. 2023. ISBN 978-9358912173.

15. Hille F, Richter H, Wong SP, Bratovič M, Ressel S, Charpentier E. The Biology of CRISPR-Cas: Backward and Forward. *Cell.* 2018;172(6):1239–59. https://doi.org/10.1016/j.cell.2017.11.032

16. Xiao Y, Ng S, Nam KH, Ke A. How type II CRISPR-Cas establish immunity through Cas1-Cas2-mediated spacer integration. *Nature.* 2017;550(7674):137-41. https://doi.org/10.1038/nature24020

17. Gleditzsch D, Pausch P, Müller-Esparza H, Özcan A, Guo X, Bange G, Randau L. PAM identification by CRISPR-Cas effector complexes: diversified mechanisms and structures. *RNA Biol*. 2019;16(4):504–17. https://doi.org/10.1080/15476286.2018.1504546

18. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, et al. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol.* 2011(6):467–77. https://doi.org/10.1038/nrmicro2577

19. Makarova KS, Koonin EV. Annotation and Classification of CRISPR-Cas Systems. *Methods Mol Biol.* 2015;1311:47–75. https://doi.org/10.1007/978-1-4939-2687-9_4

20. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science.* 2014;346(6213):1258096.
https://doi.org/10.1126/science.1258096

21. Babu K, Kathiresan V, Kumari P, Newsom S, Parameshwaran HP, Chen X, et al. Coordinated Actions of Cas9 HNH and RuvC Nuclease Domains Are Regulated by the Bridge Helix and the Target DNA Sequence. *Biochemistry.* 2021;60(49):3783–800.
https://doi.org/10.1021/acs.biochem.1c00354

22. Davis AJ, Chen DJ. DNA double strand break repair via non-homologous end-joining. *Transl Cancer Res.* 2013;2(3):130–43.
https://doi.org/10.3978/j.issn.2218-676X.2013.04.02

23. Yang H, Ren S, Yu S, Pan H, Li T, Ge S, et al. Methods Favoring Homology-Directed Repair Choice in Response to CRISPR/Cas9 Induced-Double Strand Breaks. *Int J Mol Sci.* 2020;21(18):6461.
https://doi.org/10.3390/ijms21186461

24. Song F, Stieger K. Optimizing the DNA Donor Template for Homology-Directed Repair of Double-Strand Breaks. *Mol Ther Nucleic Acids.* 2017;7:53–60.
https://doi.org/10.1016/j.omtn.2017.02.006

25. Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol Ther Nucleic Acids.* 2015;4(11):e264.
https://doi.org/10.1038/mtna.2015.37

26. Tycko J, Myer VE, Hsu PD. Methods for Optimizing CRISPR-Cas9 Genome Editing Specificity. *Mol Cell.* 2016;63(3):355–70.
https://doi.org/10.1016/j.molcel.2016.07.004

27. Cebrian-Serrano A, Davies B. CRISPR-Cas orthologues and variants: optimizing the repertoire, specificity and delivery of genome engineering tools. *Mamm Genome.* 2017;28(7–8):247–61.
https://doi.org/10.1007/s00335-017-9697-4

28. Hillary VE, Ceasar SA. A Review on the Mechanism and Applications of CRISPR/Cas9/Cas12/Cas13/Cas14 Proteins Utilized for Genome Engineering. *Mol Biotechnol.* 2023;65(3):311–25.
https://doi.org/10.1007/s12033-022-00567-0

29. Saifaldeen M, Al-Ansari DE, Ramotar D, Aouida M. CRISPR FokI Dead Cas9 System: Principles and Applications in Genome Engineering. *Cells.* 202021;9(11):2518.
https://doi.org/10.3390/cells9112518

30. Tadić V, Josipović G, Zoldoš V, Vojta A. CRISPR/Cas9-based epigenome editing: An overview of dCas9-based tools with special emphasis on off-target activity. *Methods.* 2019;164–165:109–19.
https://doi.org/10.1016/j.ymeth.2019.05.003

31. Karlson CKS, Mohd-Noor SN, Nolte N, Tan BC. CRISPR/dCas9-Based Systems: Mechanisms and Applications in Plant Sciences. *Plants (Basel).* 2021;10(10):2055.
https://doi.org/10.3390/plants10102055

32. Siksnys V, Gasiunas G. Rewiring Cas9 to Target New PAM Sequences. *Mol Cell.* 2016;61(6):793–4.
https://doi.org/10.1016/j.molcel.2016.03.002

33. Guo M, Ren K, Zhu Y, Tang Z, Wang Y, Zhang B, et al. Structural insights into a high-fidelity variant of SpCas9. *Cell Res.* 2019;29(3):183–92.
https://doi.org/10.1038/s41422-018-0131-6

34. Liu MS, Gong S, Yu HH, Jung K, Johnson KA, Taylor DW. Engineered CRISPR/Cas9 enzymes improve discrimination by slowing DNA cleavage to allow release of off-target DNA. *Nat Commun.* 2020;11(1):3576.
https://doi.org/10.1038/s41467-020-17411-1

35. Rabinowitz R, Offen D. Single-Base Resolution: Increasing the Specificity of the CRISPR-Cas System in Gene Editing. *Mol Ther.* 20213;29(3):937–48.
https://doi.org/10.1016/j.ymthe.2020.11.009

36. Gasiunas G, Young JK, Karvelis T, Kazlauskas D, Urbaitis T, Jasnauskaite M, et al. A catalogue of biochemically diverse CRISPR-Cas9 orthologs. *Nat Commun.* 2020;11(1):5512.
https://doi.org/10.1038/s41467-020-19344-1

37. Behr M, Zhou J, Xu B, Zhang H. In vivo delivery of CRISPR-Cas9 therapeutics: Progress and challenges. *Acta Pharm Sin B.* 2021;11(8):2150–71.
https://doi.org/10.1016/j.apsb.2021.05.020

38. Sousa DA, Gaspar R, Ferreira CJO, Baltazar F, Rodrigues LR, Silva BFB. In Vitro CRISPR/Cas9 Transfection and Gene-Editing Mediated by Multivalent Cationic Liposome-DNA Complexes. *Pharmaceutics.* 2022;14(5):1087.
https://doi.org/10.3390/pharmaceutics14051087

39. Hryhorowicz M, Grześkowiak B, Mazurkiewicz N, Śledziński P, Lipiński D, Słomski R. Improved Delivery of CRISPR/Cas9 System Using Magnetic Nanoparticles into Porcine Fibroblast. *Mol Biotechnol.* 2019;61(3):173–80. https://doi.org/10.1007/s12033-018-0145-9

40. Bier E. Gene drives gaining speed. *Nat Rev Gen*et. 2022;23(1):5–22. https://doi.org/10.1038/s41576-021-00386-0

41. Vogan AA, Higgs PG. The advantages and disadvantages of horizontal gene transfer and the emergence of the first species. *Biol Direct.* 2011;6:1. https://doi.org/10.1186/1745-6150-6-1

42. Emamalipour M, Seidi K, Zununi Vahed S, Jahanban-Esfahlan A, Jaymand M, Majdi H, et al. Horizontal Gene Transfer: From Evolutionary Flexibility to Disease Progression. *Front Cell Dev Biol.* 2020;8:229. https://doi.org/10.3389/fcell.2020.00229

43. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Raguram A, Liu DR. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*. 2019;576(7785):149–57. https://doi.org/10.1038/s41586-019-1711-4

44. Marzec M, Brąszewska-Zalewska A, Hensel G. Prime Editing: A New Way for Genome Editing. *Trends Cell Biol.* 2020;30(4):257–9. https://doi.org/10.1016/j.tcb.2020.01.004

45. Li X, Zhou L, Gao BQ, Li G, Wang X, Wang Y, et al. Highly efficient prime editing by introducing same-sense mutations in pegRNA or stabilizing its structure. *Nat Commun.* 2022;13(1):1669. https://doi.org/10.1038/s41467-022-29339-9

46. Nelson JW, Randolph PB, Shen SP, Everette KA, Chen PJ, Anzalone AV, et al. Engineered pegRNAs improve prime editing efficiency. *Nat Biotechnol.* 2022;40(3):402–10 https://doi.org/10.1038/s41587-021-01039-7

Epub 2021 Oct 4. Erratum in: *Nat Biotechnol.* 2022;40(3):432. https://doi.org/10.1038/s41587-021-01175-0

47. Chen PJ, Liu DR. Prime editing for precise and highly versatile genome manipulation. *Nat Rev Genet.* 2023;24(3):161–77. https://doi.org/10.1038/s41576-022-00541-1

48. Hillary VE, Ceasar SA. Prime editing in plants and mammalian cells: Mechanism, achievements, limitations, and future prospects. *Bioessays*. 2022;44(9):e2200032. https://doi.org/10.1002/bies.202200032

49. Kantor A, McClements ME, MacLaren RE. CRISPR-Cas9 DNA Base-Editing and Prime-Editing. I*nt J Mol Sci.* 2020;21(17):6240. https://doi.org/10.3390/ijms21176240

50. Huang TP, Newby GA, Liu DR. Precision genome editing using cytosine and adenine base editors in mammalian cells. *Nat Protoc.* 2021;16(2):1089–128. https://doi.org/10.1038/s41596-021-00525-1

51. Chen S, Jiao Y, Pan F, Guan Z, Cheng SH, Sun D. Knock-In of a Large Reporter Gene via the High-Throughput Microinjection of the CRISPR/Cas9 System. *IEEE Trans Biomed Eng.* 2022;69(8):2524–32. https://doi.org/10.1109/TBME.2022.3149530

52. Li Y, Li W, Li J. The CRISPR/Cas9 revolution continues: From base editing to prime editing in plant science. *J Genet Genomics.* 2021;48(8):661–70. https://doi.org/10.1016/j.jgg.2021.05.001

53. Molla KA, Sretenovic S, Bansal KC, Qi Y. Precise plant genome editing using base editors and prime editors. *Nat Plants.* 2021;7(9):1166–87. https://doi.org/10.1038/s41477-021-00991-1

54. Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene.* 2006;25(51):6680–4. https://doi.org/10.1038/sj.onc.1209954

55. Böhm S, Splith V, Riedmayr LM, Rötzer RD, Gasparoni G, Nordström KJV, et al. A gene therapy for inherited blindness using dCas9-VPR-mediated transcriptional activation. *Sci Adv.* 2020;6(34):eaba5614. https://doi.org/10.1126/sciadv.aba5614

56. Riedmayr LM, Hinrichsmeyer KS, Karguth N, Böhm S, Splith V, Michalakis S, et al. dCas9-VPR-mediated transcriptional activation of functionally equivalent genes for gene therapy. *Nat Protoc.* 2022;17(3):781–18. https://doi.org/10.1038/s41596-021-00666-3

57. Omachi K, Miner JH. Comparative analysis of dCas9-VP64 variants and multiplexed guide RNAs mediating CRISPR activation. *PLoS One*. 2022;17(6):e0270008. https://doi.org/10.1371/journal.pone.0270008

58. Hunt C, Hartford SA, White D, Pefanis E, Hanna T, Herman C, et al. Tissue-specific activation of gene expression by the Synergistic Activation Mediator (SAM) CRISPRa system in mice. *Nat Commun.* 2021;12(1):2770. https://doi.org/10.1038/s41467-021-22932-4

59. Mittal N, Guimaraes JC, Gross T, Schmidt A, Vina-Vilaseca A, Nedialkova DD, et al. The Gcn4 transcription factor reduces protein synthesis capacity and extends yeast lifespan. *Nat Commun.* 2017;8(1):457. https://doi.org/10.1038/s41467-017-00539-y

60. Heckl D, Charpentier E. Toward Whole-Transcriptome Editing with CRISPR-Cas9. *Mol Cell.* 2015;58(4):560-2. https://doi.org/10.1016/j.molcel.2015.05.016

61. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature.* 2015;517(7536):583–8. https://doi.org/10.1038/nature14136

62. Montefiori LE, Nobrega MA. Gene therapy for pathologic gene expression. *Science.* 2019;363(6424):231–2. https://doi.org/10.1126/science.aaw0635

63. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov.* 2019;18(5):358–78. https://doi.org/10.1038/s41573-019-0012-9

64. Alerasool N, Segal D, Lee H, Taipale M. An efficient KRAB domain for CRISPRi applications in human cells. *Nat Methods.* 2020;17(11):1093–96. https://doi.org/10.1038/s41592-020-0966-x

65. Stoll GA, Pandiloski N, Douse CH, Modis Y. Structure and functional mapping of the KRAB-KAP1 repressor complex. *EMBO J.* 2022;41(24):e111179. https://doi.org/10.15252/embj.2022111179

66. Zhang X, Blumenthal RM, Cheng X. Keep Fingers on the CpG Islands. *Epigenomes.* 2024;8(2):23. https://doi.org/10.3390/epigenomes8020023

67. Ueda J, Yamazaki T, Funakoshi H. Toward the Development of Epigenome Editing-Based Therapeutics: Potentials and Challenges. *Int J Mol Sci.* 2023;24(5):4778. https://doi.org/10.3390/ijms24054778

68. Kuscu C, Mammadov R, Czikora A, Unlu H, Tufan T, Fischer NL, et asl. Temporal and Spatial Epigenome Editing Allows Precise Gene Regulation in Mammalian Cells. *J Mol Biol.* 2019;431(1):111–21. https://doi.org/10.1016/j.jmb.2018.08.001

69. Cai R, Lv R, Shi X, Yang G, Jin J. CRISPR/dCas9 Tools: Epigenetic Mechanism and Application in Gene Transcriptional Regulation. *Int J Mol Sci.* 2023;24(19):14865. https://doi.org/10.3390/ijms241914865

70. Barkau CL, O'Reilly D, Eddington SB, Damha MJ, Gagnon KT. Small nucleic acids and the path to the clinic for anti-CRISPR. *Biochem Pharmacol.* 2021;189:114492. https://doi.org/10.1016/j.bcp.2021.114492

71. Pawluk A, Bondy-Denomy J, Cheung VH, Maxwell KL, Davidson AR. A new group of phage anti-CRISPR genes inhibits the type I-E CRISPR-Cas system of Pseudomonas aeruginosa. mBio. 2014;5(2):e00896. https://doi.org/10.1128/mBio.00896-14

72. Bondy-Denomy J, Pawluk A, Maxwell KL, Davidson AR. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature.* 2013;493(7432):429–32. https://doi.org/10.1038/nature11723

73. Seamon KJ, Light YK, Saada EA, Schoeniger JS, Harmon B. Versatile High-Throughput Fluorescence Assay for Monitoring Cas9 Activity. *Anal Chem.* 2018;90(11):6913–21. https://doi.org/10.1021/acs.analchem.8b01155

74. Jin L, Wang W, Fang G. Targeting protein-protein interaction by small molecules. *Annu Rev Pharmacol Toxicol.* 2014;54:435–56. https://doi.org/10.1146/annurev-pharmtox-011613-140028

75. Maji B, Gangopadhyay SA, Lee M, Shi M, Wu P, Heler R, et al. A High-Throughput Platform to Identify Small-Molecule Inhibitors of CRISPR-Cas9. *Cell.* 2019;177(4):1067–79.e19. https://doi.org/10.1016/j.cell.2019.04.009

76. O'Reilly D, Kartje ZJ, Ageely EA, Malek-Adamian E, Habibian M, Schofield A, et al. Extensive CRISPR RNA modification reveals chemical compatibility and structure-activity relationships for Cas9 biochemical activity. *Nucleic Acids Res.* 2019;47(2):546–58. https://doi.org/10.1093/nar/gky1214

77. Kartje ZJ, Barkau CL, Rohilla KJ, Ageely EA, Gagnon KT. Chimeric Guides Probe and Enhance Cas9 Biochemical Activity. *Biochemistry.* 2018;57(21):3027-31. https://doi.org/10.1021/acs.biochem.8b00107

78. Barkau CL, O'Reilly D, Rohilla KJ, Damha MJ, Gagnon KT. Rationally Designed Anti-CRISPR Nucleic Acid Inhibitors of CRISPR-Cas9. *Nucleic Acid Ther.* 2019;29(3):136–47.
https://doi.org/10.1089/nat.2018.0758

79. Zeng C, Zhang C, Walker PG, Dong Y. Formulation and Delivery Technologies for mRNA Vaccines. *Curr Top Microbiol Immunol.* 2022;440:71–110.
https://doi.org/10.1007/82_2020_217

80. Qiu M, Li Y, Bloomer H, Xu Q. Developing Biodegradable Lipid Nanoparticles for Intracellular mRNA Delivery and Genome Editing. *Acc Chem Res.* 2021;54(21):4001–11.
https://doi.org/10.1021/acs.accounts.1c00500

81. Kim J, Eygeris Y, Gupta M, Sahay G. Self-assembled mRNA vaccines. *Adv Drug Deliv Rev.* 2021;170:83–112.
https://doi.org/10.1016/j.addr.2020.12.014

82. Wang X, Liu S, Sun Y, Yu X, Lee SM, Cheng Q, et al. Preparation of selective organ-targeting (SORT) lipid nanoparticles (LNPs) using multiple technical methods for tissue-specific mRNA delivery. *Nat Protoc.* 2023;18(1):265–91.
https://doi.org/10.1038/s41596-022-00755-x

83. Parhiz H, Atochina-Vasserman EN, Weissman D. mRNA-based therapeutics: looking beyond COVID-19 vaccines. *Lancet.* 2024;403(10432):1192–204.
https://doi.org/10.1016/S0140-6736(23)02444-3

84. Iqbal Z, Rehman K, Mahmood A, Shabbir M, Liang Y, Duan L, Zeng H. Exosome for mRNA delivery: strategies and therapeutic applications. *J Nanobiotechnology.* 2024;22(1):395.
https://doi.org/10.1186/s12951-024-02634-x

85. DiEuliis D, Giordano JJ. Safely balancing a double-edged blade: identifying and mitigating emerging biosecurity risks in precision medicine. *Front Med (Lausanne).* 2024;11:1364703.
https://doi.org/10.3389/fmed.2024.1364703

86. DiEuliis D, Giordano J. Why Gene Editors Like CRISPR/Cas May Be a Game-Changer for Neuroweapons. *Health Secur.* 2017;15(3):296–302.
https://doi.org/10.1089/hs.2016.0120

87. Ajitkumar A, De Jesus O. Huntington Disease. 2023 Aug 23. In: *StatPearls* [Internet]. Treasure Island (FL): StatPearls Publishing; 2025. PMID: 32644592

88. Shin JW, Hong EP, Park SS, Choi DE, Seong IS, Whittaker MN, Kleinstiver BP, Chen RZ, Lee JM. Allele-specific silencing of the gain-of-function mutation in Huntington's disease using CRISPR/Cas9. *JCI Insight.* 2022;7(19):e141042.
https://doi.org/10.1172/jci.insight.141042

89. Cheng Y, Zhang S, Shang H. Latest advances on new promising molecular-based therapeutic approaches for Huntington's disease. *J Transl Int Med.* 2024;12(2):134–47.
https://doi.org/10.2478/jtim-2023-0142

90. Sahel DK, Giriprasad G, Jatyan R, Guha S, Korde A, Mittal A, Bhand S, Chitkara D. Next-generation CRISPR/Cas-based ultrasensitive diagnostic tools: current progress and prospects. *RSC Adv.* 2024;14(44):32411–35.
https://doi.org/10.1039/d4ra04838e

91. Wong C. UK first to approve CRISPR treatment for diseases: what you need to know. *Nature.* 2023;623(7988):676–77.
https://doi.org/10.1038/d41586-023-03590-6

92. Pacesa M, Pelea O, Jinek M. Past, present, and future of CRISPR genome editing technologies. *Cell.* 2024;187(5):1076–100.
https://doi.org/10.1016/j.cell.2024.01.042

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Elaboration of the concept of the paper; collection, analysis, and systematization of scientific literature; writing and edition of paper / Разработка концепции статьи; сбор, анализ и систематизация научной литературы; написание статьи.

***Author's statement / Заявление автора***

I am declaring that I prepared the article from sources freely available on the Internet and free available publications, figures, and other possible legal sources. I, as a sole author declare that the research was conducted in

the absence of any commercial or financial relationship that could be construed as a potential conflict of interest / Я заявляю, что подготовил статью из источников, находящихся в свободном доступе в Интернете, а также свободно доступных публикаций, рисунков и других возможных легальных источников. Я, как единственный автор, заявляю, что исследование проводилось при отсутствии каких-либо коммерческих или финансовых отношений, которые могли бы быть истолкованы как потенциальный конфликт интересов.

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