Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (1): 1–17 (2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Review Article

Advances in the CRISPR/Cas9 Mediated Genome Editing: A Review

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Abstract: The primary long-lasting objective of biomedical researcher's is the expansion of some superior and fruitful conducts in order to make some targeted alterations to the living cells genome. In modern times, a novel and up-to-date tool have emerged which is CRISPR-associated protein-9 nuclease (Cas9) from a bacteria *Streptococcus pyogenes*. This tool has gained a lot of focus and delivered a significant excitement. It has been sanctioned that the Biological Sciences have one of the best ever discoveries in the form of CRISPR-Cas9, which makes the integration of anticipated traits in eukaryotic cells very well-organized and easy. Furthermore, these traits have the potency of being target specific. They do not follow a random insertion of genes in the genome of any organism. This revolutionary technology is very user and environment friendly, easy to handle, and can be easily applicable in other offshoots of biology including, health, agriculture, genetic syndromes, and other microbial diseases. In this review, we will provide, brief insights on the history of CRISPR/Cas9 system from its discovery to the mechanism of action. The literature for this review has been obtained from accessible catalogs namely, PubMed, Science Direct, Google Scholar and Research gate. With this revolutionary technology, the modification, regulation and locating of genomic loci in cells of all organisms indeed is a remarkable development.

Keywords: CRISPR/Cas9 system, DNA, Genome editing, Revolution.

1. INTRODUCTION

The proper foundation of genetic engineering was started back in the1970s and very proficient and dynamic equipment's were used and as a result this field has got very rapid progress[1]. 1970 was the starting novel period of biology in which the progress of rapid diagnostic tests (RDT) was at its peak [2]. This is because, scientists for the very first time were able to edit or change the molecules of DNA making it easy to analyze genes and control them differently to create new drugs [2]. Currently, the efficient of all diagnostic tools used for genome editing is CRISPR/Cas9, which is reliable and can be directed to any sequence in the genome embedded with a short RNA [2]. In *E. coli,* the CRISPR system was firstly discovered in 1987 and this system is newly developed for genetic engineering as a microbial adaptive immune system [1, 3].

In 2010, the CRSPR/Cas type-2 system was recognized well, that how could be gRNA endonuclease build for making desired changes in the genome [1]. By RNA-guided nuclease enzyme, Cas9 enzyme utilize Cas gene for the destruction of nucleus of external DNA or RNA [1]. The source of Cas9 is from *Streptococcus pyogenes* and it is used with a guide-RNA to excise strands of DNA from a specific region [2].

In bacteria and archaea, there is no other adaptive immune system [3] while the study on physiology and chemistry confirms that the CRISPR-Cas9

Received: February 2018; Accepted: March 2019
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provides adaptive immunity to bacteria. CRISPR basically stands for "clustered regularly interspaced short palindromic repeat associated 9 (Cas9) [5]. It allows very easy editing of agenome that has a lot of advantages but, it also has some moral concerns and biosafety issues [6]. The components of this system are present in a large number of archea as well as in bacteria comprises of immune systems (adaptive type) and these components comprises of specialized sequences that give defense versus external DNA or RNA [7]. CRISPR/Cas can be utilized for high-throughput screening in drug design because it contains "CRISPR array" which is composed of short repeats, divided by spacers which are short DNA sequences and Cas genes are present at the edge [8]. It holds the ability to develop and a 20 nucleotide RNA called gRNA guides to perform its action [7]. As the scientific applications needs to be accurate, so in order to achieve accuracy and correcting mutations, CRISPR/Cas9 cuts DNA sites which are defective or mutant [9]. It is very simple system, for the production of chimeric RNA that makes it easy to generate gRNA with greater proficiency for editing specific type of genome [1].

Cas9 stand for "CRISPR/associated protein" which is an endonuclease guided by RNA called the RNA-guided endonuclease [8]. Cas9 is used to direct exact position by a small RNA chain [10]. The function of Cas9 in this system is to cleave the nucleic acid [4]. For gene therapy and other diagnostic uses, the accuracy of Cas9 protein could be a serious matter for the reason that genetic manipulation cause long lasting alterations [10]. For the repair of damaged DNA, it activates different mechanisms (homologous recombination and non-homologous end joining) that create double strand breaks [11]. Genome modification facilitated by Cas-9 is a measurable and modest tool allowing scientists to explain the purpose of genes at different levels and it also creates relationship among genes and their physical characters [10].

Mostly, CRISPR/Cas9 comprises of two basic parts,which can be transfer by one specific plasmid i.e., sgRNA(protospacer which is 20 base pairs) and Cas9 protein [2]. To device the CRISPR-Cas9, different methods are used where the model of sgRNA is very significant and simple. To enhance the Cas9 efficiency and to change the protospacer adjacent motif (PAM) designs, there have been many CRISPR/Cas9 alternates were developed [2]. To estimate the proficiency of constructed gRNA, several bioinformatics systems were utilized [6]. Many of these tools have been used to check effectiveness of CRISPR/Cas9 including the deep sequencing and Surveyor assay [6]. From various bacterial strains, PAM sequences are discovered with different size and nucleotide structure and for the specific gene, the endonuclease activity of CRISPR/Cas9 is triggered by various PAM sequences [9]. CRISPR/Cas9 can alter or modify the genome of any organism including animals, plants and bacteria [10]. It has been predicted that this tool will be completely able to improve genetic mutations permanently in the near future. This tool is helpful in inducing or reducing the total loss of specific gene function in plants, animals and bacterial genome [12]. The extensive utilization of this system in the genome editing of living systems comprising cell cultures, secondary cell lines, fungi, pests and vertebrates is a great advantage [9]. The ability to cure human disorders including cancer and HIV is also a greatest benefit of this technology [9]. Therefore, keeping all these applications of CRISPR/Cas9 in mind, this review was written to set out the state of art of recent advancements in the CRISPR/Cas9 system from its discovery to the mechanism of action.

2. MECHANISM OF CRISPR/Cas SYSTEM

CRISPR/Cas9 system comprises of many steps, which gives a protospacer from foreign DNA and saves it as a spacer in CRISPR array [13]. Cas9 protein is basically an RNA guided endonuclease that cuts the dsDNA comprising complementary sequences to guide RNA 20 nucleotide's sequence [14]. By modifying only the gRNA sequence, Cas9 can be encoded for the new target sites. The Cas9 performs its function by identifying the target DNA by 20 nucleotides bps interaction between gRNA and the target strand [15].

Cas9 has two lobes, one is nuclease lobe (NUC) and the other is recognition lobe (REC). The particular portion of Cas9 which is responsible for its function and to link with different repeats and anti-repeats is the recognition lobe (REC) which is a lengthy chain and it has further two domains named as REC1 and REC 2 [6]. The CRISPR/Cas9 guided genetic manipulation by first attachment

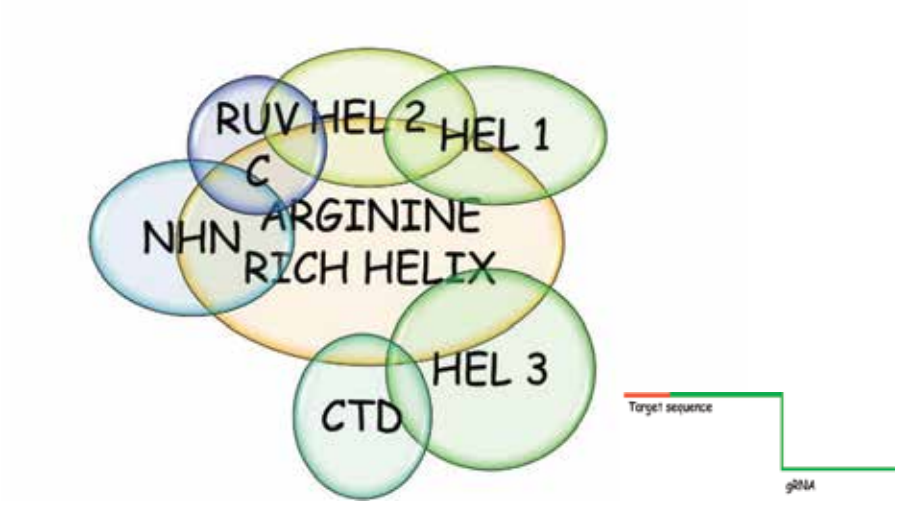

Fig. 1. Inactive cas-9 complex

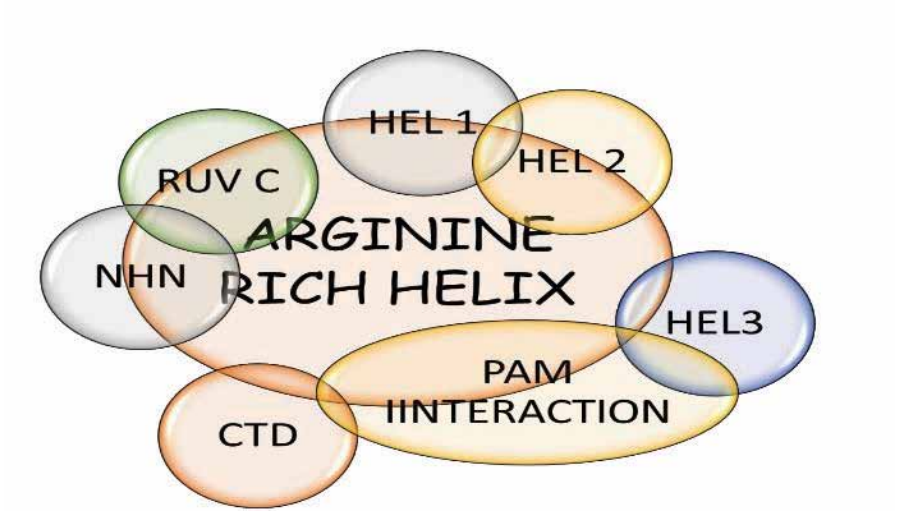

Fig. 2. Active Cas-9 complex

of single guide RNA (sgRNA) with the binding channel linking different domains and REC and NUC lobes [9]. The active and inactive forms of Cas9 enzyme are given in Figure 1 and 2.

To create a double stranded break, two nuclease domains of Cas9 are used like the HNH and Ruv-C domains. If mutation occurs in nuclease domain then only one strand of DNA will be cleaved by Cas9 [15]. Editing of genome starts when gRNA interacts with channels that are present between REC and NUC lobes because of which it turns into active form. When it finds target protospacer sequence, CRISPR/Cas9 and gRNA complex interact with protospacer DNA and then gRNA starts base pairing with the target DNA [9].

NUC lobe further contains the PAM interacting domains like Ruv-C, HNH and WED (Wedge domain). PAM region ensures the target DNA specificity. REC lobe recognizes gRNA that is linked with target DNA and it varies in different Cas9 systems [15]. Different enzymes are involved in DNA repair, including the polymerases, RNA binding proteins, gRNA and Cas9 having identifiable domains characteristic of helicases [16]. That's why, the Cas9 form double stranded breaks in target sequence with host-mediated DNA repair mechanisms.

If the repair template is not present, then NHEJ (Non-homologous end joining) causes mutations like insertions, substitutions and deletions with altered gene function. If donor template is present,

then the HDR (Error-free homology directed repair) pathway is started and a desired mutation can be induced [17]. An additional ability of CRISPR is that it can integrate spacers at specific locus into the host genome. Spacers translated into non-coding small RNAs which when interact with Cas9 binds to the foreign material. This helps in the destruction of various types of DNA [16]. Self-targeting can also lead to CRISPR/Cas autoimmunity and is important to prevent it otherwise the DNA itself will be degraded [13]. Although, CRISPR/Cas is very successful and efficient tool, but it's off target activity sometimes causes mismatches between gRNA and the target DNA resulting in sequence cut which is partially complementary to gRNA [9].

3. CLASSIFICATION OF CRISPR/Cas SYSTEM

Because of the huge diversity of CRISPR/Cas, the consistent annotation and classification of the Cas proteins are major tasks. CRISPR/Cas is divided into two major classes and five sub-types. Due to the complexity of the composition of the CRISPR/Cas systems the evidence from comparative genomic, phylogenetic and structural analysis provides the proper classification of Cas9 proteins. Three important types of CRISPR/Cas systems (Fig. 3) are recognized in the classification system. The three types could be easily distinguishable by the presence of three marker genes: Cas3 in type I systems, Cas9 in type II, and Cas10 in type III [6, 7,9]. For the genome engineering, CRISPR/Cas9 type 2 is extensively used system.

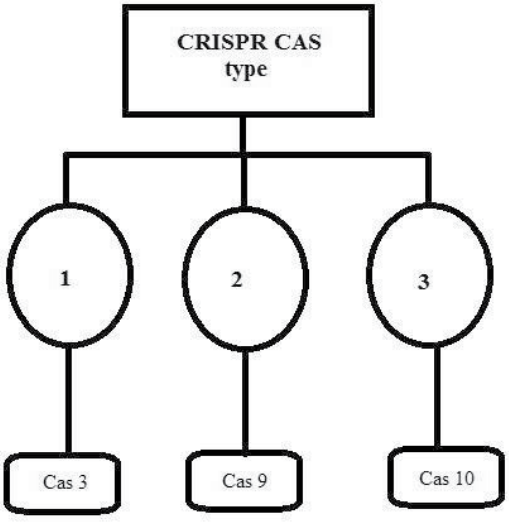

Fig. 3. Specific Cas proteins for specific type of CRISPR [6, 7, 9, 22]

The three CRISPR/Cas9 systems have full complements of tools necessary for the key defence mechanism. The in-depth sequence analysis recently permitted additional generalization of the CRISPR/Cas classification at least for the type I and IIIsystems [18].

3.1. CRISPR/Cas system type 1

CRISPR/Cas system 1 is categorized by effector components that have many further sub units. Almost 90% of CRISPR/Cas loci are recognized in archaea and bacteria and it can focus RNA as well as DNA [19]. CRISPR/Cas system type 1 consists of many big and small sub units and these sub units have the selection of target DNA and it contains many "Cascade interference complexes" [20].

All type-I CRISPR/Cas loci have a signature gene cas3. This gene codes for many proteins along with the helicase, possessing a single-stranded DNA stimulated activity of ATPase coupled to unwind the RNA-DNA and DNA-DNA duplexes. The helicase domain and the HD family domain are fused together and create the endonuclease activity that cleaves the targeted DNA [21].

3.2. CRISPR/Cas system type 2

Type 2 system is obtained from Cas9 and it is most widely used and developed system [9] and it encodes a protein of multi-domain which combines effector complexes and the target DNA cleavage function and very important for crRNA maturation [22]. Apart from Cas1 and Cas2 genes, CRISPR/Cas is categorized on the basis of Cas9 genes. They are divided into two more groups due to the occurrence of other genes (1) sub2-A that is CRISPR/Cas and CRISPR/Cas of *S. thermophiles* that consists of csn2 gene (2) sub type 2-B consist of Cas4 gene [19]. This system was originated from the *Streptococcus pyogenes* [23, 24]. Type 2 is very simple of all the CRISPR/Cas systems and for foreign DNA; the actions of endonucleases are necessary that is condensed in its own domain of protein called the cas9 endonuclease.

The progress of biotechnology of CRISPR/Cas

system type 2 has generated opportunities to unveil different functions making it easy to understand its Cas proteins and related RNAs [6]. CRISPR/Cas system type 2 is not present in Achaea and in bacterial genes they are only 5% but present in large amount in pathogens. Type 2 Cas genes are only four in number and it is good choice to use in genetic engineering [18]. For working of innate Cas9, the essential parts that it comprised are Cas9 nuclease enzyme crRNA and tracrRNA. It should be ensured that they all are shown in external sequence [25]. The proof of the presence of adaptive immune system of type 2 CRISPR/Cas was revealed by Danisco Company. Horvath and colleagues were working on *Streptococcus thermophiles* for the production of daily products where thy confirmed the presence of CRISPR nucleic-acid-based immune system. Also the role of Cas protein as a spacer regulator and to provide safety against various phages was revealed [26].

3.3. CRISPR/Cas system type 3

CRISPR/Cas system type 3 is present in bacteria as well as in archaea. It comprises of one fourth of the CRISPR systems and includes pathogenic bacteria like *Mycobacteria* and *Staphylococci* [27]. All type III systems have the cas10 signature gene. This gene encodes multidomain protein having a palm domain. This palm domain is similar to the polymerases and cyclases domains of the family PolB. That's why, this protein was considered as a polymerase. More recently, the structure of Cas10 protein has shown with four discrete domains [28].

The cyclase-like domains have N-terminal that assumes the same RRM fold as the palm domain does. On the other hand, this domain has no enzymatic activity. It is reported that, a helical domain with Zn-binding motif, the C-terminal alpha helical domain, the palm domain and the alpha helical protein are mostly present in few type III CRISPR/Cas systems.

The Cas10 protein is big subunit of effector complexes of type III systems. The locus for type III system also codes other effector complexes subunits i.e. one gene for Cas5 group RAMP protein, one for small subunit and many genes for RAMP proteins of Cas7. Often Cas10 fused to nuclease domain of HD family which is different from type I CRISPR/Cas the HD domains. But a circular permutation of the conserved motifs is present in the later one [28]

4. COMPARISON OF CRISPR/Cas WITH OTHER GENOME EDITING TOOLS

There are three main categories of genes manipulation tools named as ZFNs (Zinc-finger nucleases) TALENs (Transcription activator-like effector nucleases) and CRISPR/Cas as shown in Table 1. They are categorized as same type of nucleases called as "programmable nucleases" and the process of all these genome manipulation tools vary from each other [24]. These all are are revolutionizing systems and they have ability to

Table 1. Comparison between genome editing tools (ZFNs, TALENS & CRISPR/Cas-9)

Parameters	CRISPR/Cas 9	Zinc finger nuclease (ZFNs)	Talens (TALENs)	References
Construction	Simple	Complicated	Normal	[37]
Target region specifying unit	Base pair between the sgRNA and target DNA	DNA triplets	Single base pair	[37]
Enzyme type	RNA-guided DNA endonuclease	zinc-finger nucleases	Transcription activator-like effector nucleases	[34]
Restriction site	3 NTS base pair upstream of PAM	non-palindromic DNA sites	Fokl cleavage domain	[34]
Modifications	sgRNA sequence	Targeted DSBs	TALE proteins	[34]
Base pair length of target site	15 to 23 bases	9-bp target for 3 Zinc fingers	Single base pair	[38]
Off target activity	Fluctuate depends upon sequence	Extreme	Less	[39]

modernize scientific study on living things and are also helpful in the formation of tailor-made drugs [23]. In the last few years, specialized genetic manipulation by CRISPR/Cas9, ZFNs and TALENs had developed more progressively. In 1996, the processes of ZFNs and TALENs were improved. Many studies concerning the action and harmfulness of these processes have urged scientists to manipulate living organism's genome on trial basis and their subsequent utilization in gene therapy [29, 30].

Certainly, these developing tools have the capability to change living organism's genome and modifying the genes to cure various diseases. ZFNs and TALENs are potent systems for scientific study and these are chimeric nucleases, made up of DNA binding unit connected with the DNA domains. It could be possible to manipulate DSB (Double Strand Breaks) with ZFNs and TALENs. In contrast to ZFNs and TALENs, CRISPR-Cas system has many more benefits and uses.

ZFNs are a good program for genome editing and now days, it is used for the cure of HIV (Human Immunodeficiency Virus). Nevertheless, it is not easy to build a proficient and specific tool for clinical uses and extensive knowledge of protein engineering is required. On the other side, TALENs give extensive modesty and they are very easy to construct as compared to the ZFNs. However, TALENs also needs some complicated molecular cloning procedures [23].

ZFNs and TALENs are able to cure the disease permanently with very accurate gene manipulation. The ordinary kind of DNA-binding motifs is Cys2–His2 zinc-finger domain which occurs in eukaryotes and it highly occurs in the human genome. Once zinc-finger has almost thirty amino acids and flexible shape of zinc-finger proteins make them background to adjust DNA binding proteins. The ZFNs and TALENs have enough flexibility which makes them able to fit and identify any sequence and DNA binding domains. Advancing the action of nucleases for gene study and other medical approaches they should be very specific to their targets but sometime the genome of higher organisms there are many copies that are very similar to each other and it can cause cell injury and other off-target activities. Apart from ZFNs and TALENs, CRISPR/Cas system is very good alternate because it is very proficient and easy to use tool for gene manipulation. The CRISPR/Cas system can be conveniently transformed to the human cells by help of plasmid with Cas9 endonuclease and some other important parts such as crRNA[31].

5. CRISPR/CasAPPLICATIONS IN PLANTS

5.1. IncRNA knockouts

In plants, the system is being used when it comes to discover the functional characterization of non-coding RNAs. The CRISPR/Cas9 system is now being used on large scale for their discovery because of its greater potential for generation of knock-in and knockout mutants in plants as demonstrated recently [32, 33] The CRISPR/Cas9 system ensures the functional characterization and provides a deeper understanding of ncRNAs involved biological systems and processes. This was demonstrated in a wide range of horticultural plant species including *Citrus sinensis,* [41] *Malus pumila*, [35] *Solanum lycopersicum* [36] and *Solanum tuberosum* [37]. With other low-target activities, it has the advantage of the complete gene knockout [38]. If compared with the RNA interference, the RNAi has a limitation of the incomplete gene knock down and extensive off-target activities. It is restricted in the cytoplasm where the RNA-induced silencing complexes are present [39]. This technology has been used to induce knockouts in plants such as soybean [40]. A dead variant of CRISPR/Cas9 like the dCas9 method deployed long noncoding lncRNAs into the DNA loci. It occurs by incorporating Specific chromatin domains (Cargo) into the sgRNA which provides initial insights into the function of ncRNAs [41].

5.2. Non-homologous end joining (NHEJ) gene knockouts

The NHEJ gene knockouts include enzyme genes or microRNAs (miRNAs) via gene mutation [42].

5.3. Enzyme genes

Jiang *et al*. [43] revealed the genome editing procedures in the Arabidopsis, tobacco, rice, and sorghum using CRISPR/Cas9 system. They

performed Agrobacterium or PEG-mediated transfer by constructinga binary vector carrying CRISPR and Cas9 recombination and expressing them into these four plants. For the sweet orange, targeting the Phytoene Desaturase Gene CsPDS, a new tool is developed for its expression which showed no off-target effects [44]. A VIGE (virus-based guide RNA delivery system) for CRISPR/Cas9 mediated plant genome editing was reported by Yin *et al*., [45] that it could be used for the transient expression targeting NbPDS3 and NbIspH, which cause a photo-bleaching phenotype when expressed in tobacco. Lawrenson [46] used the CRISPR/Cas9 to edit the HvPM19 gene in *Hordeum vulgare* and BolC. GA4 in *Brassica oleracea* via a transgenic system.

5.4. MircroRNAs

They are known as the stimulators or inhibitors of the gene expression in plants. Two miRNAs were used and the CRISPR/Cas9 system was applied in the soybean by Jacobs *et al*. [47]. Vectors having the sgRNA were bombarded for transient expression. Lei *et al*. [48] demonstrated the co-expression of the Cas9 and gRNA to target the AcrII site of NbPDS gene by a DNA template in the protoplasts of tobacco.Yang *et al.* [49] demonstrated the global diversification of the freshwater bloom-*Microcystis aeruginosa* forming cyanobacterium. They produce toxic and secondary metabolites such as the microcystins which are considered harmful to human health. They isolated the two stains, sequenced them, found a total number of 71 loci's of CRISPR. The CRISPR spacers provide resistance to bacteriophage and plasmids and also target any exogenous material in the natural environment [49].

Anderson *et al*. [50] utilized the spacers derived from the CRISPR putative system for the analysis of a viral metagenome in Pacific hydrothermal vent. The virome of the marine vent matched with the spacer which showed that those viruses came from a diverse taxonomy in the environment.

The acute form of pneumonia is the Legionnaires' disease which is caused by the *Legionella pneumophilia* [51]. The CRISPR loci which consisted of leader sequences [21-72 bp] and several spacers, encode sgRNA provide immunity and protection against the invading DNA and the viruses [52].

crRNA is a multi-spacer RNA, made by the small subunits and flanking in-between the two partial repeats by the CRISPR-Cas. This provides specific guidance to the machinery of Cas protein to avoid the nucleic acids with the matching sequences. They are evident in showing the past viral infections remnants for acquired immunity [53].

Sikiasa *et al*. [54] demonstrated the community viruses in the oral cavity through CRISPR loci, which were known to be involved in bacterial and archaeal resistance against the invading viruses by acquiring the short sequences of a viral genome. 2065 246 CRISPR spacers were reported from five separate and repeat motifs found in the oral bacterial species of *Gemella veillonella, Leptotrichia* and *Streptococcus* which determined the oral exposure in their cavity to the environment when exposed to similar viruses.

Studies of Andersson and Banfield [55], Tyson and Banfield, [56], Heidelberg *et al*. [57], Anderson *et al*. [50], Held *et al*. [58], Emerson *et al.* [59], Maier *et al*. [60] and Vestergaard *et al*. [61] demonstrated that CRISPR systems potentially provide *Haloarchaea* to respond and defend against *Haloarchaea* viruses.

Nigro *et al*. [62] demonstrated through CRISPR, the scaffolds in the metagenomes of the microbial life in the igneous crust of the seafloor with taxonomy of 163 out of 737, of which showed that archeal were 51 to 55% and bacterial were appeared to be 45% to 49%. The results implied that in the buried oceans with the highly-altered fluids were found several distinct novel viruses which may infect archaea. These viruses were found to be an active participant in the ecology of the basement of the microbiome.

6. CRISPR/Cas APPLICATIONS IN GENETIC DISORDERS

The CRISPR gene editing applications provides a great therapeutic benefit for the genetic disorders because of their ability to knock out the mutant genes [63]. In one study, Zhu *et al*. [64] demonstrated the genome editing of CRISPR/Cas9 to correct the

Dystrophin mutation in skeletal muscle cells by a mouse model which had muscle dystrophy. They used a fibrin gel culture system to expand muscle stem cells from the crude skeletal muscle cells of mdx mice. With the help of CRISPR/Cas9, they corrected the expanded Dystrophin mutation by the expression upon translation.

The CRISPR induced deletion or excision of mutated exon 23 of DMD (Duchenne Muscular Dystrophy) mouse resulted in the excision of the intervening DNA, restoring the expression of truncated version of the Dystrophin protein in myofibers, cardiomyocytes, and even in the muscles stem cells (MuSCs) [65-66-67]. The results improved the muscle biochemistry and significant enhancement of muscle force in mice. [64]

Wang *et al.* [68] reported the corrections in the genes of Amyotrophic Lateral Sclerosis (ALS)-a complex neurodegenerative disease which were SOD1 and FUS known to be associated with ALS. They reported SOD1 +/A272C and FUS +/G1566A mutations in the iPSCs from the patient's fibroblasts. They generated the corrected gene using the CRISPR/Cas9 system and analyzed it through genome wide RNA sequencing. This method provided novel therapeutic strategy for treating the ALS.

Kolli *et al*. [69] demonstrated that the Huntington's disease can be treated by CRISPR-Cas9 genome editing method. Huntington'sis an autosomal dominant neurodegenerative genetic disease which causes cognitive deficits and abnormal development of the limbs along with the psychiatric symptoms [70]. The disease is caused by a mutation in the Huntington gene which codes for the Huntington protein due to which the translated protein undergoes inappropriate post-translational modification, results a toxic function. They constructed two CRISPR plasmids, among which one breaks the DNA at the upstream open reading frame (uORF) region [71] where no translation occurred, and the other plasmid at the exon-1 intron boundary. The study implied application of plasmid into mesenchymal stem cells (MSCs) which were extracted from the bone marrow of YAC128, because it carries transgene for the HD. Their results showed the disruption of uORF influencing negative translation of Huntington, disrupts the exon-1 intron boundary, which also affects the translation of Huntington protein [69].

Zhou *et al*. [72] confirmed that the non-coding regions of the genomic DNA use CRISPR/CAS9 gene editing method to prove a relationship between genetic defects and the pathophysiology of schizophrenia.

Lee *et al*. [73] validated the CRISPR/Cas9 which used the exon 1 of the hypoxanthine-guanine phopspho ribosyltransferase locus for genetic modification and restore the activity of the arginase and induced Pluripotent stem cells and hepatocyte-like derivatives in genetically distinct patient-specific humans.

Im *et al*. [74] reviewed the preclinical studies of CRISPR/Cas9 in hereditary movement disorders including Parkinson's disease**,** Huntington's disease, Dystonia and Ataxia.

Ou *et al*. [75] reported that the utilization of CRISPR/Cas9 system promote the hematopoietic differentiation and corrects the β-thalassemia mutations in their patient specific iPSCs. In their study, no tumor in the lungs, livers, kidneys or bone marrow for duration of 10 weeks was noticed when the CRISPR/Cas9 corrected stem cells were injected into the NSI mice. This suggested a safe strategy for the treatment of β-thalassemia.

To reengineer TSS site in K562 cells in genes of GATA1, CRISPR/Cas9 system can be used to check the arrival of myeloid leukemia [76]. With CRISPR/Cas9, they generated mammalian cells with shortened kind of the protein of GATA1 genes which were same as seen in the ML-DS.

Cystic fibrosis is a persistent and advanced disease of lungs and pancreatic ducts characterized by the dysfunctional secretory epithelial cells [77]. Changes in CFTR gene with is the major cause of the disease which are responsible for encoding apical membrane in the Cl-/HCO3- channel [76, 78, 79]. Sanz *et al*. [80] utilized CRISPR/Cas9 to find mutations in the genes of CFTR region with the characterization of 281 genes as disease causing. They designed a gRNAs of Cas9 to target the DSBs in the genes of CFTR region on each side of mutation which cause in high proficiency

excision of the targeted regions by the NHEJ repair. They also designed it for the correction of unusual splicing signals or removal of disturbing regulatory factors of transcription [81].

7. CRISPR/Cas9 APPLICATIONS BEYOND GENOME EDITING

It has been said that the libraries have frequently abundant undesired sequences. So, the undesired extra sequences having DSBs entirely engulfed by Cas9 with 99% effectiveness having no off-target effects as seen with restriction enzymes. While in RNA sequence libraries, it can expand sensitivity of pathogen and treating with cas9 can reduce abundant rRNA [82]. CRISPR/CAS9 system also has ability for treating many diseases like hepatitis and cancer [83]. Scientists also try to undertake research on CRISPR/Cas9 by editing reproductive cells. Nevertheless, some ethical obligations are concerned with it. Because any change in germ line will pass to the next generations as well and is banned in many countries including the United States of America and the United Kingdom [84].

8. CRISPR/Cas in CANCER

Various applications of CRISPR/Cas9 are used in oncology and cancer biology to perform robust site-specific gene editing, thereby becoming more useful for biological and clinical applications. Many variants and applications of CRISPR/Cas9 are being rapidly developed. The first ever medical test by CRISPR/Cas9 was initiated against cancer cell lines in 2016. By using CRISPR/Cas9, the PD-1 genes for the first time were scissor from the immune cells of a person having lung cancer. Moreover, in 2016, the United States Food and Drug Administration (FDA) have permitted a trial in which CRISPR system was used to modify T-cells taken from people suffering with various kinds of cancer [85].

The precision cancer medicine has provided clues for the development of a lot of targeted anticancer drugs. Targeted therapy has already proved a huge benefit, but there are still many challenges exist. The patients who only show a certain altered gene expression or mutation respond to the targeted drug treatment and drug resistance to the therapy still occurs. The functional genome-screening methods by using CRISPR system have the potential to unveil gene expression changes after treatment and pinpoint genes connected with resistance to targeted drugs. This identifies new biomarkers for cancer treatment and provides value able new insights for cancer development [86]. An example involved with the screening of cancer metastasis causing gene with a CRISPR/Cas9 mediated loss of function screen. In a study, a non-metastatic lung cancer cell was infected with mGeCKO (mouse genome scale CRISPR knockout) sgRNA library and transduced cells were intravenously transferred into immune compromised mice. After six weeks, the mice showed metastasis lung cancer and were selected for sgRNA sequencing [87]. On the other hand, a lot of candidate genes causing lung cancer metastasis have been identified and validated likes *miR-152*, and *miR-345*, *Pten*, *Nf2, a Trim72,* and Fga [86, 87].

Other screens for loss-of-function were also functional to scrutinize suppressor tumor genes in liver. Here, the mouse embryonic liver progenitor cells like $p53^{-/-}$/Mycwere infected by mGeCKO library. The infected cells were then transplanted into mice. sgRNAs that were increased 8-fold were chosen as candidates and *Nf1*, *Plxnb1*, *Flrt2*, and *B9d1* were identified as new tumor suppressors involved in liver cancer formation. In one more study CRISPR interference (CRISPRi) to screen for functional lncRNA loci, which could modify cell growth.

9. BIOMEDICINE

Sequences of interest including antibiotic resistance genes, virulence factors and others can also be targeted by RNA guided nucleases. This technique is used to manipulate bacterial inhabitants and it signifies antimicrobial therapy [88].

In order to reduce the growth of herpes virus, CRISPR is a very efficient method and in the Epstein-Barr virus (EBV) it can remove viral DNA. In anti-herpesvirus, CRISPR can rejuvenate transplantation of organs of animals but transplanted recipients can be damaged by retroviruses present in their genes. A studyon retrovirus in 2015 with CRISPR showed the removal of 62 mutant disease causing copies [89]. It has been reported that the

expression of MHC class 2 proteins causes rejection of the transplant. CRISPR may be crucial in tissue engineering and may eliminate or inhibits the genes that cause expression of MHC class 2 proteins [90].

10. GENE DRIVE

The CRISPR/Cas9 utilization in mosquitoes follows from an idea, which was perceived in 2003, that naturally occurring genes producing endonuclease enzymes could be used to generate gene drive. Theoretically, constructs integrating the CRISPR/Cas9 system and natural endonuclease genes spread in same manner, nonetheless the stress-free manipulation of the ribonucleic acid (RNA) permits the targeting of wide range of gene sequences.

Computational modelling on the basis of other gene drive systems recommends that the type of drive obtained with CRISPR/Cas9 could be very effective that discharge of low numbers of modified mosquitoes into the environment could result in establishment of the genetic modification in the natural interbreeding population [91]. That's how Gene drive technology provides the promise for a cost-effective, high-impact, and durable method to control malaria transmission that causing a significant influence to removal. Gene drive systems, such as those based on CRISPR associated protein, have the potential to spread beneficial traits through interbreeding populations of malaria mosquitoes [92].

11. CRISPR/CAS9 IN NEURO-DEGENERATIVE DISEASES (NDs)

Neurodegenerative diseases (NDs) like Huntington's, Alzheimer's, and Parkinson's, are very lethal due to the lack of precise diagnostic tools or treatments for these devastating diseases [93]. Under these circumstances, it is necessary to create more powerful drugs for them and to speed up the process of drug discovery. An important neurological disorder, where CRISPR/Cas9 tool has proved promising results is the Huntington disease (HD). In the exon-1 of the HTT gene, the expansion of CAG repeats ciphers for mutant hunting in (mHTT), a huge protein in the N-terminal domain of mHTT containing of many polyglutamine repeats causes a toxic-gain of function ensuing in HD. In order to treat this disease, one prominent technique is by using CRISPR/Cas9 to specifically suppress the mHTT expression of [93, 94].

Despite of having few off target properties with Cas9, newly a one investigation revealed an innovative variant of the gene editing system. It emerges to be clear-cut and prudent by using a nickase version of Cas9 [93, 94, 95]. This Cas9 edition is favorable because it only cuts downsingle strand of DNA instead of two that proves the trust worthiness with which Cas9 can edit or remove genomic regions.

Double stranded breaks are being introduced by CRISPR/Cas9 system which can be used to create knock-in in spite of knock out of target gene. Coding region of gene can be changed by exact incorporation of a donor template to repair a mutation then a protein tag has been placed there. Instead of double strand cut a single stranded cut is used to make exact insertions so in this way Cas9 action has been changed. Homology-directed repair can be achieved by double stranded DNA and it can also be manipulated with crRNA, tracrRNA and sgRNA on other strand [27-95]. Genome level monitoring of cancer cell feasibility and resistance of drug can be performed by pooled lent viral libraries at low MOI [96-97].

In quintessence, an innovation like this makes it convenient to use CRISPR/Cas9 to trigger the expression of mutant protein in specific regions of the brain. It could open up the doors of a novel treatment plans not only for Huntington disease but other neurodegenerative ailments including other diseases like Alzheimer's, dementia and Parkinson's could be treated.

12. CRISPR/Cas9 ENGINEERING

There are numerous options regarding the delivery of Cas9-gRNA:

- ✓ In insects, the major engineering pattern is through microinjection. Early stage embryos are frequently used because at this stage, there are very few cells. Reagents are injected in these embryos so that they can contact nuclei of both germinaland somatic cells.
- ✓ In insects, cells transfection is another important engineering process. To send cas9, microfluidic

membrane deformation method is used.

- ✓ While electroporation is not been found in insects, yet but it is mainly used in mammalian ovary.
- ✓ Cas9-gRNA can also be transferred through virus.
- ✓ In addition, virus-mediated Cas9-gRNA delivery is also an option. In mammals and zebrafish, virus mediated Cas9-gRNA method is proved to be good [91].

In bacteria and archaea, the CRISPR/Cas9 system is used for genome editing in many models like mice. To make a mutant mice having edited genome CRISPR/Cas9 reagents are delivered directly into the mouse zygote [98]. In knock out system, which is based on NHEJ, one sgRNA for frame shift mutation or two sgRNAs by deleting a gene sequence are used. Main concerns of this process include:

- ✓ Do not target ATG region as it can be used for production of truncated proteins.
- ✓ When splicing is done care should be done as wrong splicing can cause abnormal product especially when targeting exon and also save the donor and acceptor region.
- ✓ Usually mutations created by CRISPR/Cas9 are very little, not more than 20 nucleotides, so such type of exon should be selected that is big to adjust the mutation leaving donor and acceptor sites unaltered.
- ✓ A double strand break is kept nearby the mutation region for creating a knock in a model, so that target site will be restricted. Before doing the process of CRISPR, a proper region must be measured for it [99].

13. SPECIFICITY OF CRISPR/Cas

Guide RNA mostly has some specific sequences of 20bp. These sequences are corresponding to target sequence of gene to be edited. But not all of these 20bp are required to counterpart for guide RNA so that it can bind to gene to be edited. There is probability for guide RNA to bind somewhere else as an alternative. For instance, 19 of the 20bp may subsist somewhere that is completely different than that of genome which leads to binding of guide RNA there as an alternative [100]. In this case, cas9

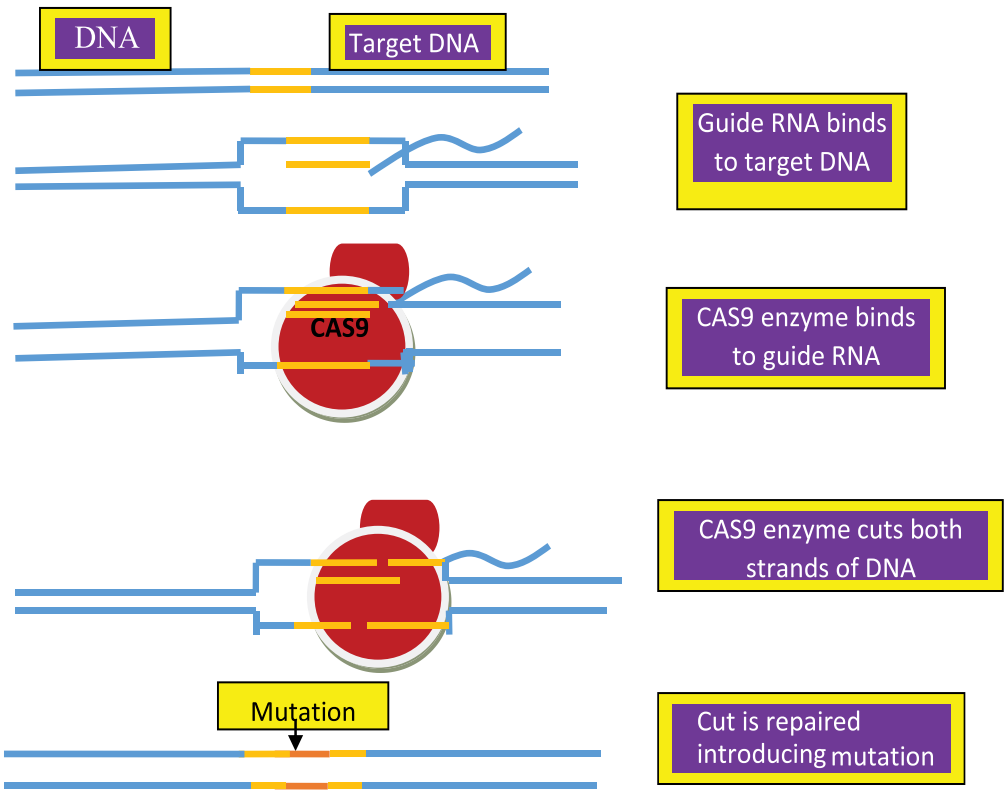

Fig. 4. Binding of RNA to the sequence (targeted) and the activity of Cas9 enzyme.

enzyme will bind at wrong side and thus can induce mutation at an incorrect location. This kind of mutation may or may not affect individual (it could either be a positive or negative mutation).

However, some mutations can add additional traits whereas, some mutations can knock out genes/sequences that are crucial for an individual to survive. Specificity of Cas9 can be achieved by using Cas9 system that can cut at single strand of DNA rather than double strand. This can somehow reduce the chances for Cas9 enzyme to cut at wrong position and thus diminishes chances of mutations. Another possible solution to minimize error rate is to design more specific RNA by using our knowledge about sequence of genome. Online tools are developed to design guide RNA with a future insight of higher precision and accuracy for instance CRISPR fly design and fly CRISPR. Categorization of novel CRISPR system and enlargement of Cas9 variants of Cas9 enzymes can assure to make genome progressively and gladly handy to our on-demand double stranded DNA breaks.

14. FUTURE PROSPECTS

For efficient and precise gene modification, it is highly likely that the future solutions will be found in as of yet unexplored corners of the rich biological diversity of nature. For effector domain localization, off target activity of Cas9 may be quite broad than Cas9 mediated genome editing. Cas9 based chromatin immune precipitation sequencing analysis (ChIP-seq analysis) at multiple target sites could be a high-throughput solution for understanding binding degeneracy [100] whereas techniques used for detecting and labeling double-strand breaks will help to accomplish a comprehensive map of Cas9-induced off-target idles [101]. All these data together will help in creating some highly productive models that would be able to maximize the on target activity of Cas9 in genetic therapies or few other applications of high levels of precisions and accuracy. Understanding Cas9 activity of binding and cleavage, in the context epigenetic, states of chromatin accessibility and will also inform better computational evaluation to specificity of guide RNA.

Another important future advancement includes development of expression systems and flexible delivery for applications of Cas9. Adeno-associated virus (AAV) or lenti-virus and some other viral vectors are commonly used for delivery of gene of interest into cell types resistant to common transfection methods, such as immune cells. Because of their less immunogenic threat and reduced oncogenic risk due to host genome integration these vectors have also been used for efficient *invivo* gene delivery. However, the mostly used Cas9 nuclease-encoding gene from *Streptococcus pyogenes* is >4 kb in length, which is difficult to transduce using AAV due to its 4.7 kb packaging capacity. For introducing CRISPR reagents in non-viral approaches *invivo* present a productive ground for developing fresh and new delivery strategies, from liposomes and aptamers to the molecular Trojan horse and cell-penetrating peptides [102].

15. CONCLUSION

It is concluded that the CRISPR/Cas9 system holds phenomenal capabilities in order to revolutionize research in all biological fields and the ability of expansion of genetic manipulation could be ensured with the help of this tool. It is evident from recent research that using this system, any gene could be target with the aim of removing or modifying it in a better way. The CRISPR/Cas9 system have instigated over and done with a same procedure, Once the mechanism essential for how the CRISPR/Cas9 system actually works was known, then it can be easily connected with other applications in genetics and molecular biology which were not before hand anticipated.

16. REFRENCES

1. Cui, J., S.J.L. Chew., Y. Shi., Z. Gong., & H.M. Shen. CRISPR system for genome engineering: the application for autophagy study. *BMB reports* 50(5): 247.(2017).
2. Cao, H. X., W. Wang., H.T. Le., & G.T. Vu. The Power of CRISPR-Cas9-Induced Genome Editing to Speed Up Plant Breeding. *International journal of genomics* (2016).
3. Chylinski, K., K.S. Makarova., E. Charpentier., & E.V. Koonin. Classification and evolution of type II CRISPR-Cas systems. *Nucleic acids research* 42(10): 6091-6105 (2014).
4. Singh, A., D, Chakraborty., & S, Maiti. CRISPR/Cas9: a historical and chemical biology perspective

of targeted genome engineering. *Chemical Society Reviews*45(24): 6666-6684 (2016)

5. Lau, V., & J.R. Davie. The discovery and development of the CRISPR system in applications in genome manipulation. *Biochemistry and Cell Biology* 95(2): 203-210. (2016).
6. Mei, Y., Y. Wang., H. Chen., Z.S. Sun., & X.D. Ju. Recent progress in CRISPR/Cas9 technology. *Journal of Genetics and Genomics*43(2): 63-75 (2016).
7. Makarova, K.S., Y.I. Wolf., O.S. Alkhnbashi., F. Costa., S.A. Shah., S.J. Saunders., & P. Horvath. An updated evolutionary classification of CRISPR-Cas systems. *Nature Reviews Microbiology* 13(11): 722-736 (2015).
8. Doetschman, T., & T. Georgieva. Gene editing with CRISPR/Cas9 RNA-directed nuclease. *Circulation research*, *120*(5):876-894. (2017).
9. Zhang, J. H., P. Adikaram., M. Pandey., A. Genis., & W.F. Simonds. Optimization of genome editing through CRISPR-Cas9 engineering. *Bioengineered*, *7*(3), 166-174. (2016).
10. Hsu, P. D., E.S. Lander., & F. Zhang. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157(6):1262-1278 (2014).
11. Zhou, Y., Y. Zong., & X. Kong. Research progress in the third-generation genomic editing technology-CRISPR/Cas9. *Zhonghua yi xue yi chuan xue za zhi= Zhonghua yixue yichuanxue zazhi= Chinese journal of medical genetics* 33(5): 713-716 (2016).
12. Dominguez, A.A., W.A. Lim., & L.S. Qi. Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nature Reviews Molecular Cell Biology*, *17*(1), 5-15. (2016).
13. Amitai, G., & R. Sorek. CRISPR–Cas adaptation: insights into the mechanism of action. *Nature Reviews Microbiology* 14(2): 67. (2016).
14. Anders, C., O. Niewoehner., A. Duerst., & M. Jinek. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* 513:(7519) 569 (2014).
15. Wang, H., Russa., M. La., & L. S. Qi. CRISPR/Cas9 in genome editing and beyond. *Annual review of biochemistry* 85: 227-264 (2016).
16. Bhaya, D., M. Davison., & R. Barrangou. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annual review of genetics*45:273-297 (2011).
17. Jiang, F., & J.A. Doudna. CRISPR–Cas9 structures and mechanisms. *Annual review of biophysics*46:505-529 (2017).
18. Zhang, Y., N. Heidrich., B.J. Ampattu., C.W. Gunderson., H.S. Seifert., C. Schoen., J. Vogel., & E.J. Sontheimer. Processing-independent CRISPR RNAs limit natural transformation in *Neisseria meningitidis*. *Molecular Cell*50:488–503 (2013)
19. Makarova, K.S. F. Zhang., & E.V. Koonin. Snapshot: class 1 CRISPR-Cas systems. *Cell* 168(5): 946-946 (2017).
20. Richter, H., J. Rompf., J. Wiegel., K. Rau., & L. Randau. Fragmentation of the CRISPR-Cas Type IB signature protein Cas8b. *Biochimica et Biophysica Acta (BBA)-General Subjects* (2017).
21. Sinkunas, T., G. Gasiunas., C. Fremaux., R. Barrangou., P. Horvath., & V. Siksnys. Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system. EMBO Journal 30: 1335–1342 (2011).
22. Cong, L., F.A. Ran., D. Cox., S. Lin., R. Barretto., N. Habib.,& F. Zhang. Multiplex genome engineering using CRISPR/Cas systems. *Science*339(6121) : 819-823 (2013).
23. Lockyer, E. J. The potential of CRISPR-Cas9 for treating genetic disorders. *Bioscience Horizons: The International Journal of Student Research* 9: 012 (2016).
24. Chylinski, K., K.S. Makarova., E. Charpentier., & E.V. Koonin. Classification and evolution of type II CRISPR-Cas systems. *Nucleic acids research* 42(10): 6091-6105 (2014).
25. Hsu, P.D., E.S. Lander., & F. Zhang. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157(6):1262-1278 (2014).
26. Hatoum-Aslan, A., K.L. Palmer., M.S. Gilmore., & L.A. Marraffini. Type III CRISPR-Cas Systems and the Roles of CRISPR-Cas in Bacterial Virulence. In *CRISPR-Cas Systems*(pp. 201-219). Springer Berlin Heidelberg (2013).
27. Toro, N., F. Martínez-Abarca., & A. González-Delgado. The Reverse Transcriptases Associated with CRISPR-Cas Systems. *Scientific reports7*: 7089 (2017).
28. Makarova, K.S., L. Aravind., N.V. Grishin., I.B. Rogozin., & E.V. Koonin. A DNA repair system specific for thermophilic Archaea and bacteria predicted by genomic context analysis. *Nucleic Acids Research* 30:482–496 (2002).
29. Gaj, T., C.A. Gersbach., & C.F. Barbas. ZFN, TALEN, and CRISPR/Cas-based methods for

genome engineering. *Trends in biotechnology* 31(7): 397-405 (2013).\

30. Kim, H., & J.S. Kim. A guide to genome engineering with programmable nucleases. *Nature Reviews Genetics* 15(5): 321-334 (2014).
31. Wu, X., A.J. Kriz., & P.A. Sharp. Target specificity of the CRISPR-Cas9 system. *Quantitative biology* 2(2): 59-70 (2014).
32. Pattanayak, V., C.L. Ramirez., J.K. Joung., & D.R. Liu. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nature methods* 8(9): 765 (2011).
33. Liu, D., R.Mewalal., Hu., R.G.A.Tuskan.,&X.Yang. New technologies accelerate the exploration of non-coding RNAs in horticultural plants. *Horticulture research* 4: 17031(2017).
34. Jia, H.,& N. Wang. Targeted genome editing of sweet orange using Cas9/sgRNA. *PloS one*9(4): e93806(2014)..
35. Nishitani, C., N. Hirai. S. Komori., K. M. Wada., Okada., K. Osakabe., & Y. Osakabe. Efficient genome editing in apple using a CRISPR/Cas9 system. *Scientific reports* 6: 31481(2016).
36. Brooks, C., V. Nekrasov., Z.B. Lippman., & J. Van Eck. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant physiology* 166(3): 1292-1297 (2014)
37. Butler, N.M., P.A. Atkins., D.F. Voytas., & D.S. Douches. Generation and inheritance of targeted mutations in potato (Solanum tuberosum L.) using the CRISPR/Cas system. *PloS one* 10(12): e0144591 (2015).
38. Shalem, O., N.E. Sanjana., & F. Zhang. High-throughput functional genomics using CRISPR–Cas9. *Nature Reviews Genetics*16(5): 299 (2015).
39. Zamore, P. D., T., Tuschl, P. A., Sharp, & D. Bartel, PRNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101(1):25-33 (2000).
40. Jacobs, T.B., P.R. LaFayette., R.J. Schmitz., & W.A. Parrott. Targeted genome modifications in soybean with CRISPR/Cas9. *BMC biotechnology*15(1): 16 (2015).
41. Shechner, D.M., E. Hacisuleyman., S.T. Younger., & J.L Rinn. Multiplexable, locus-specific targeting of long RNAs with CRISPR-Display. *Nature methods* 12(7):664 (2015).
42. Liu, X., S.W., J.Xu.,C.Sui.,& J. Wei. Application of CRISPR/Cas9 in plant biology. *Acta pharmaceutica sinica B* 7(3):292-302 (2017).
43. Jiang, W., H.Bi. Zhou., H.M. Fromm., B. Yang., & D.P. Weeks. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic acids research* 41(20): e188-e188 (2013).
44. Jia, H., & N. Wang. Targeted genome editing of sweet orange using Cas9/sgRNA. *PloS one*9(4): e93806 (2014).
45. Yin, K., T. Han., G. Liu., T. ChenWang., Y.A.Y.L. Yu., & Y.A. Liu. geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. *Scientific reports*5:14926 (2015).
46. Lawrenson, T., O. Shorinola., N. Li., C. Stacey., L. Østergaard., N. Patron.,& W. Harwood. Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. *Genome biology* 16(1): 258 (2015).
47. Jacobs, T.B., P.R. LaFayette., R.J. Schmitz., & W.A Parrott. Targeted genome modifications in soybean with CRISPR/Cas9. *BMC biotechnology* 15(1): 16 (2015).
48. Li, M., X. Li,. Z. Zhou., P. Wu., M. Fang., X. Pan., & H. Li. Reassessment of the four yield-related genes Gn1a, DEP1, GS3, and IPA1 in rice using a CRISPR/Cas9 system. *Frontiers in plant science* 7: 377 (2016).
49. Yang, C., F. Lin., Q. Li., T. Li., & J. Zhao. Comparative genomics reveals diversified CRISPR-Cas systems of globally distributed Microcystis aeruginosa, a freshwater bloom-forming cyanobacterium. *Frontiers in microbiology* 6:394 (2015).
50. Anderson, R.E., W.J. Brazelton., & J.A. Baross. Using CRISPRs as a metagenomic tool to identify microbial hosts of a diffuse flow hydrothermal vent viral assemblage. *FEMS microbiology ecology* 77(1):120-133 (2011).
51. Fraser, D.W., T.R.Tsai.,W.Orenstein., W.E. Parkin., H.J.Beecham., R.G. Sharra.,&C.C. Shepard. Legionnaires disease: description of an epidemic of pneumonia. *New England Journal of Medicine* 297(22):1189-1197 (1977).
52. Horvath, P., & R. Barrangou. CRISPR/Cas, the immune system of bacteria and archaea. *Science* 327(5962):167-170 (2010).
53. Faucher, S. P., & H. A. Shuman, Small regulatory RNA and Legionella pneumophila. *Frontiers in microbiology* 2: 98 (2011).
54. Robles-Sikisaka, R., M. Ly., T. Boehm., M. Naidu., J. Salzman., & D.T. Pride. Association between living environment and human oral viral ecology. *The*

ISME journal, *7*(9):1710. (2013).

55. Andersson, A.F. & J.F. Banfield, Virus population dynamics and acquired virus resistance in natural microbial communities. *Science* 320(5879): 1047-1050 (2008).
56. Tyson, G.W., & J.F. Banfield. Rapidly evolving CRISPRs implicated in acquired resistance of microorganisms to viruses. *Environmental microbiology* 10(1):200-207 (2008).
57. Heidelberg, J.F., W.C. Nelson., T. Schoenfeld., & D. Bhaya. Germ warfare in a microbial mat community: CRISPRs provide insights into the co-evolution of host and viral genomes. *PLoS One* 4(1): e4169 (2009).
58. Held, N.L., A. Herrera., H. Cadillo-Quiroz., & R.J. Whitaker. CRISPR associated diversity within a population of Sulfolobus islandicus. *PLoS One* 5(9) e:12988 (2010).
59. Emerson, J.B., K. Andrade., B.C. Thomas., A. Norman., E.E. Allen., K.B. Heidelberg., & J.F. Banfield. Virus-host and CRISPR dynamics in Archaea-dominated hypersaline Lake Tyrrell, Victoria, Australia. *Archaea*(2013).
60. Maier, L.K., S.J.Lange., B.Stoll.,K.A Haas., S.M Fischer., E. Fischer&A. Marchfelder. Essential requirements for the detection and degradation of invaders by the Haloferax volcanii CRISPR/Cas system IB. *RNA biology*10(5):865-874 (2013).
61. Vestergaard, G., R.A. Garrett.,& S.A. Shah. CRISPR adaptive immune systems of Archaea. *RNA biology* 11(2):156-167 (2014).
62. Nigro, O. D., S. P Jungbluth,., H. T., Lin, C. C Hsieh,., J. A Miranda,., C. R Schvarcz,., ... & G. F. Steward, Viruses in the Oceanic Basement. *mBio* 8(2):e02129-16 (2017).
63. Sander, J.D., & J.K. Joung. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature biotechnology* 32(4):347 (2014).
64. Zhu, P., F. Wu., J. Mosenson., H. Zhang., T.C He., & W.S. Wu. CRISPR/Cas9-mediated genome editing corrects dystrophin mutation in skeletal muscle stem cells in a mouse model of muscle dystrophy. *Molecular Therapy-Nucleic Acids* 7:31-41 (2017).
65. Long, C., L. Amoasii., A.A. Mireault., J.R.McAnally., H. Li., E. Sanchez-Ortiz., & E.N. Olson. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science* 351(6271): 400-403 (2016).
66. Nelson, C.E., C.H. Hakim., D.G. Ousterout., P.I. Thakore, E.A. Moreb., R.M.C. Rivera., & A. Asokan., In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science* 351(6271):403-407 (2016).
67. Tabebordbar, M., K. Zhu., J.K. Cheng., W.L. Chew., J.J. Widrick., W.X. Yan., & L. Cong. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science* 351(6271): 407-411 (2016).
68. Wang, L., F. Yi., L. Fu., J. Yang., S. Wang. Z. Wang., & J. Qiao. CRISPR/Cas9-mediated targeted gene correction in amyotrophic lateral sclerosis patient iPSCs. *Protein & cell*8(5): 365-378(2017)
69. Kolli, N., M. Lu., P. Maiti., J. Rossignol., & G.L. Dunbar. CRISPR-Cas9 mediated gene-silencing of the mutant huntingtin gene in an in vitro model of Huntington's disease. *International journal of molecular sciences* 18(4): 754 (2017).
70. Paulsen, J.S., C.Nehl., K.F.Hoth.,J.E.Kanz., M. Benjamin., R. Conybeare., &B.Turner. Depression and stages of Huntington's disease. *The Journal of neuropsychiatry and clinical neurosciences* 17(4):496-502 (2005).
71. Lee, J., E.H. Park., Couture. G. Harvey.,I.P. Garneau., & J. Pelletier. An upstream open reading frame impedes translation of the huntingtin gene. *Nucleic acids research*, *30*(23): 5110-5119. (2002).
72. Zhuo, C., W. Hou., L. Hu., C. Lin., C. Chen., & X. Lin. Genomic editing of non-coding RNA genes with CRISPR/Cas9 ushers in a potential novel approach to study and treat schizophrenia. *Frontiers in molecular neuroscience* 10: 28. (2017).
73. Lee, P.C., B. Truong., A. Vega-Crespo., W.B. Gilmore., K. Hermann., S.A. Angarita., & B.E. Schoenberg. Restoring ureagenesis in hepatocytes by CRISPR/Cas9-mediated genomic addition to arginase-deficient induced pluripotent stem cells. *Molecular Therapy-Nucleic Acids*, *5*. (2016).
74. Im, W., J., Moon, & M. Kim, Applications of CRISPR/Cas9 for Gene Editing in Hereditary Movement Disorders. *Journal of movement disorders* 9(3):136 (2016).
75. Ou, Z., X. Niu., W. He., Y. Chen., B. Song., Y. Xian., & X. Sun. The Combination of CRISPR/Cas9 and iPSC Technologies in the Gene Therapy of Human β-thalassemia in Mice. *Scientific reports*6: 32463 (2016).
76. Bloh, K.M., P.A. Bialk., A. Gopalakrishnapillai., E.A. Kolb., & E.B. Kmiec. CRISPR/Cas9-Directed Reassignment of the GATA1 Initiation Codon in K562 Cells to Recapitulate AML in Down Syndrome. *Molecular Therapy-Nucleic Acids* 7: 288-298 (2017).

77. Cutting, G. R. Cystic fibrosis genetics: from molecular understanding to clinical application. *Nature Reviews Genetics*, *16*(1): 45 (2015).
78. Riordan, J.R., J.M. Rommens., B.S. Kerem., N. Alon., R. Rozmahel., Z. Grzelczak., & J.L. Chou.Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245(4922): 1066-1073 (1989).
79. Kerem, B.S., J.M. Rommens., J.A. Buchanan., D. Markiewicz., T.K. Cox.,A. Chakravarti., & L.C. Tsui. Identification of the cystic fibrosis gene: genetic analysis. *Science*245(4922):1073-1080. (1989).
80. Sanz, D.J., A. Hollywood., J.M.F. Scallan., & P.T. Harrison. Cas9/gRNA targeted excision of cystic fibrosis-causing deep-intronic splicing mutations restores normal splicing of CFTR mRNA. *PloS one* 12(9): e0184009 (2017).
81. Zhu, P., F. Wu., J. Mosenson., H. Zhang., T.C. He., & W.S. Wu. CRISPR/Cas9-mediated genome editing corrects dystrophin mutation in skeletal muscle stem cells in a mouse model of muscle dystrophy. *Molecular Therapy-Nucleic Acids* 7:31-41 (2017).
82. Lei, Y., X. Guo., Y.Liu., Y.Cao., Y.Deng., X.Chen., & H. Zhao. Efficient targeted gene disruption in Xenopus embryos using engineered transcription activator-like effector nucleases (TALENs). *Proceedings of the National Academy of Sciences* 109(43):17484-17489 (2012).
83. Taning, C. N. T., B. Van Eynde., N. Yu., S. Ma.,& G. Smagghe. CRISPR/Cas9 in insects: Applications, best practices and biosafety concerns. *Journal of insect physiology* 98: 245-257 (2017).
84. Ormond, K.E., D.P. Mortlock., D.T. Scholes., Y. Bombard., L.C. Brody., W.A. Faucett.,& K. Musunuru Human germline genome editing. *The American Journal of Human Genetics* 101(2):167-176 (2017).
85. Khan, F.A., N.S.Pandupuspitasari., H. Chun-Jie., Z. Ao., M. Jamal., A. Zohaib., & Z. ShuJun. CRISPR/Cas9 therapeutics: a cure for cancer and other genetic diseases. *Oncotarget* 7(32):52541 (2016).
86. Bester, A.C., J.D. Lee., A. Chavez., Y.R. Lee., D. Nachmani., S. Vora., J. Victor., M. Sauvageau., E. Monteleone., J.L. Rinn., P. Provero., G.M. &Church., J.G. Clohessy., P.P. Pandolfi. An integrated genome-wide CRISPRa approach to functionalize lncRNAs in drug resistance. *Cell* 173: 649–664 e620 (2018).
87. Chen, S., N.E. Sanjana., K. Zheng., O. Shalem., K. Lee., X. Shi., D.A. Scott., J. Song., J.Q. Pan, R., Weissleder., H. Lee., F. Zhang., & P.A. Sharp. Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell* 160: 1246–1260 (2015).
88. van Erp, P.B., G.Bloomer.,R.Wilkinson., &B. Wiedenheft. The history and market impact of CRISPR RNA-guided nucleases. *Current opinion in virology*12:85-90 (2015).
89. Maggio, I., &M.A.Goncalves. Genome editing at the crossroads of delivery, specificity, and fidelity. *Trends in biotechnology* 33(5):280-291 (2015).
90. Rath, D., L.Amlinger., A.Rath., &M. Lundgren.The CRISPR-Cas immune system: biology, mechanisms and applications. *Biochimie*117:119-128 (2015).
91. Burt, A. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proceedings of the Biological Sciences 270: 921–928 (2003).
92. Champer, J., A.Buchman., &O.S. Akbari. Cheating evolution: engineering gene drives to manipulate the fate of wild populations. *Nature Reviews Genetics* 17(3): 146-159 (2016).
93. Dabrowska, M., W. Juzwa., W.J. Krzyzosia., M. Olejniczak. Precise excision of the CAG tract from the huntingtin gene by cas9 nickases. *Frontiers in Neuroscience* 12: 75 (2018).
94. Saudou, F., & S. Humbert. The biology of huntingtin. *Neuron* 89: 910–926 (2016)
95. Ran, F. A., P.D.Hsu., C.Y Lin.,J.S.Gootenberg.,S. Konermann., A.E.Trevino.,&F. Zhang. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154(6): 1380-1389 (2013).
96. Wang, T., J.J.Wei., D.M.Sabatini., &E.S.Lander. Genetic screens in human cells using the CRISPR-Cas9 system. *Science* 343(6166):80-84 (2014).
97. Shalem, O., N.E.Sanjana., E.Hartenian., S.X. hiD. A.Scott., T.S. Mikkelsen., &F. Zhang. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343(6166): 84-87 (2014)
98. Riordan, S. M.,D.P.Heruth., L.Q.Zhang., &S.Q. Ye. Application of CRISPR/Cas9 for biomedical discoveries. *Cell & bioscience* 5(1): 33 (2015).
99. Qin, W., P. M.Kutny., R. S.Maser., S.L.Dion., J.D.Lamont., Y.Zhang., &H. Wang. Generating Mouse Models Using CRISPR/Cas9 Mediated Genome Editing. *Current protocols in mouse biology* 39-66 (2016).
100. Wu, X., D. A.Scott., A. J.Kriz., A. C.Chiu., P. D.Hsu.,

D. B. Dadon.,& R.Jaenisch. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nature biotechnology* 32(7): 670-676 (2014).

101. Gratz, S. J., A. M. Cummings., J. N. Nguyen., D. C. Hamm., L. K. Donohue., M. M. & O. Harrison, K. M.'Connor-Giles. Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. *Genetics*194(4):1029-1035 (2013).
102. Niewoehner, J., B.Bohrmann., L.Collin., E.UrichH., Sade., P. Maier., &H. Loetscher.