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Review

Exploring the potential of genome editing CRISPR-Cas9 technology

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ABSTRACT

CRISPR-Cas9 is an RNA-mediated adaptive immune system that protects bacteria and archaea from viruses or plasmids. Herein we discuss the recent development of CRISPR-Cas9 into a key technology for genome editing, targeting, and regulation in a wide range of organisms and cell types. It requires a custom designed single guide-RNA (sgRNA), a Cas9 endonuclease, and PAM sequences in the target region. The sgRNA-Cas9 complex binds to its target and creates a double-strand break (DSB) that can be repaired by non-homologous end joining (NHEJ) or by the homology-directed repair (HDR) pathway, modifying or permanently replacing the genomic target sequence. Additionally, we highlight recent advances in the repurposing of CRISPR-Cas9 for repression, activation, and loci imaging. In this review, we underline the current progress and the future potential of the CRISPR-Cas9 system towards biomedical, therapeutic, industrial, and biotechnological applications.

Contents

Abbreviations: Cas, CRISPR-associated proteins; cccDNA, covalently closed circular DNA; CCR5, C-C chemokine receptor type 5; CHO, Chinese hamster ovary; CRISPRs, clustered regularly interspaced short palindromic repeats; CRISPRa, CRISPR activation; CRISPRi, CRISPR interference; crRNAs, CRISPR RNAs; dCas9, dead Cas9; DMD, Duchenne muscular dystrophy; DSB, double-strand break; eGFP, enhanced green fluorescent protein; HDR, homology-directed repair; HEK 293, human embryonic kidney 293; Indel, insertion and deletion; iPSCs, induced pluripotent stem cells; KRAB, Krüppel associated box; LTR, long terminal repeat; mRFP, monomeric red fluorescent protein; NHEJ, non-homologous end joining; PAM, protospacer adjacent motif; RBS, ribosome binding site; sfGFP, superfolder green fluorescent protein; sgRNA, single guide RNA; TSS, transcription start site; tracrRNA, trans-activating crRNA.

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1. Introduction

Synthetic biology is an emergent field that employs the application of engineering principles to biology. It has been used to design novel parts, devices, and systems for the better understanding of gene networks and for a variety of biotechnological applications (Endy, 2005; Purnick and Weiss, 2009; Khalil and Collins, 2010; Qi and Arkin, 2014; Singh, 2014a). In the past decades, a number of promoters (Alper et al., 2005), ribosome-binding sites (Salis et al., 2009), scaffolds (Dueber et al., 2009), RNAs and proteins (Pfleger et al., 2006; Win and Smolke, 2008) have been designed and characterized. There are currently a number of complex circuits including biologic gates (Tamsir et al., 2011; Moon et al., 2012; Shis and Bennett, 2013; Singh, 2014b), toggle switches (Gardner et al., 2000; Atkinson et al., 2003), genetic oscillators (Elowitz and Leibler, 2000; Stricker et al., 2008; Danino et al., 2010), riboregulators (Isaacs et al., 2004; Na et al., 2013), and riboswitches (Tucker and Breaker, 2005; Blount and Breaker, 2006), all of which have been deployed in many organisms.

Currently, the use of synthetic circuits and technologies are a high priority for the community. In recent years, synthetic circuits have been designed and implemented that have helped to control the morbidity and mortality of cancer development (Culler et al., 2010; Nissim and Bar-Ziv, 2010), acted as toggle switches for controlling of metabolic flux (Soma et al., 2014) and of T-cell population controllers (Chen et al., 2010), and have also assisted in artificial insemination (Kemmer et al., 2011). Other recently developed synthetic biology technologies include multiplex automated genome engineering (MAGE), which has been used to improve lycopene production (Wang et al., 2009), modify the genetic code (Isaacs et al., 2011), insert His-tags into the genome (Wang et al., 2012), and to incorporate non-standard amino acids into proteins (Lajoie et al., 2013; Rovner et al., 2015). Similarly, synthetic small RNAs have been used for improving tyrosine and cadaverine production (Na et al., 2013), and global transcription machine engineering (gTME) has been used to improve ethanol tolerance (Alper et al., 2006). These advances are expected to become extremely useful for accelerating invention and innovation in the biological sciences.

From the scientific and ethical point of view, the need to perform genome editing is well validated, due to a large number of genetic defects discovered from genomics data that have the potential and the capability of being treated using the novel technologies offered by genome editing. Even small mutation in the gene can lead to significant changes, highlighted strongly in the examples of sickle cell anemia (Rees et al., 2010), haemophilia (Manco-Johnson et al., 2007), and Duchenne muscular dystrophy (Long et al., 2014), among others. Synthetic biology has the potential to develop the tools and methods in order to correct such mutations. It has been also used for accelerating genomics research and the redesign of pathways and synthetic genomes (Gibson et al., 2009; Gibson et al., 2010; Kosuri et al., 2010; Kim et al., 2012; Hutchison et al., 2016) *via* genetic synthesis (Lartigue et al., 2009). Genome engineering is one of the most promising technologies in terms of medical and industrial interests due to the accelerating ease of genome editing towards fruitful applications. Recently, the synthetic biology toolbox has been expanded for targeted and precise genome engineering. For example, transcription activator-like effector nucleases (TALENs) and Zinc-finger nucleases (ZFNs) are synthetic endonucleases and designed to cleave specific sequences of DNA (Pabo et al., 2001; Szczepek et al., 2007; Boch et al., 2009; Moscou and Bogdanove, 2009; Boch, 2011; Carroll, 2011; Mashimo, 2014), although the design of sequence-specific DNA binding proteins remains laborious, time-consuming and expensive.

Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated proteins (Cas) are an RNA-mediated adaptive immune system of bacteria and archaea that protects from phages and plasmids. The CRISPR-Cas are categorized into three types referred to as Types I, II, and III (Barrangou et al., 2007; Marraffini and Sontheimer, 2008; Horvath and Barrangou, 2010). The Type II CRISPR-Cas system utilizes RNA-guided Cas9 endonuclease that has been used for the development of a genome editing technology. The CRISPR-Cas9 technology is simple, cost effective and efficient for targeted genome editing of bacteria, yeast and mammals (Cong et al., 2013; DiCarlo et al., 2013; Jiang et al., 2013; Mali et al., 2013; Bikard et al., 2014; Jakočiūnas et al., 2015). The technology has since been extended into the genome editing of zebrafish (Hwang et al., 2013; Hisano et al., 2015), *Drosophila* (Port et al., 2014; Ren et al., 2014a), the correction of defective genes in mammals (Long et al., 2014; Guan et al., 2016; Nelson et al., 2016), and the eradication of viruses such as HIV-1 (Ebina et al., 2013; Zhu et al., 2015), hepatitis B virus (Lin et al., 2014; Zhen et al., 2015), human papillomavirus (Kennedy et al., 2014), and the latent Epstein-Barr virus from human cells (Wang and Quake, 2014).

In addition, CRISPR-Cas9 can be repurposed through the use of a mutated Cas9 that targets specific genes, permitting control through the repression and activation of genes and the imaging of genomic loci in a wide variety of organisms (Bikard et al., 2013; Chen et al., 2013; Gilbert et al., 2013; Qi et al., 2013; Ma et al., 2015). The aim of this present review is to underline recent advances within the field, and to explore the potential and versatility of the CRISPR-Cas9 technology for targeted genome editing in medically and industrially important organisms.

2. Role and mechanism of the CRISPR-Cas system

Ishino et al. (1987) first discovered the CRISPRs in *Escherichia coli*. It was involved in a variety of functions such as replicon partitioning (Mojica et al., 1995), adaptation at high temperature (Riehle et al., 2001), chromosomal rearrangements (DeBoy et al., 2006), and repairing of DNA (Makarova et al., 2002). Subsequently, it was observed that bacteria and archaea have developed defense mechanisms *via* CRISPR and Cas proteins enabling acquired resistance against invading viruses (phages) and plasmids. This is underlined by the fact that approximately 40% of currently sequenced bacterial genomes and 90% of archaeal genomes have CRISPRs (Horvath and Barrangou, 2010; Al-Attar et al., 2011; Richter et al., 2012; Szczepankowska, 2012).

The functional CRISPR-Cas system requires a CRISPR locus/array that contains the hypervariable spacers that the defending host acquires from phages or plasmids, and is located in the host genome. In addition, it also requires the diverse group of Cas genes that are located in the nearby CRISPR locus and encodes the Cas proteins for the multistep defense against foreign DNA (Horvath et al., 2008; Bhaya et al., 2011; Sapranauskas et al., 2011). The CRISPR-Cas defense mechanism is a sequential three-step process beginning with the acquisition, followed by RNA processing, and finishing with interference (Fig. 1). During the acquisition phase, the foreign DNA is first recognized (as being foreign/invasive) and it is captured and subsequently integrated as spacers between the two contiguous repeat sequences located in the CRISPR locus. Spacers are derived from phage or plasmid, and are also known as protospacer (Deveau et al., 2008). Small nucleotides present near the protospacer are referred to as the protospacer adjacent motif (PAM), which is particularly important during the acquisition of DNA (Deveau et al., 2010).

The Cas1 and Cas2, and their encoding genes are universally present in the genome and they process foreign DNA to generate a functional CRISPR-Cas system (Bhaya et al., 2011). In the second phase of processing, the RNAP transcribed CRISPR locus and produces a preCRISPR RNA (pre-crRNA) and endonucleases cleave the pre-crRNAs into active CRISPR RNAs (crRNAs). Whereas, the third phase is interference (Cui et al., 2008) or immunity (Garneau et al., 2010) where crRNAs form a multiprotein complex that can recognize, through the base pairing and with great specificity, the regions of incoming foreign DNA (or RNA). This complex degrades the foreign DNA and maintains phage immunity (Brouns et al., 2008). On the other hand, if the base pairing of seen sequences is not homologous or in absence of the PAM

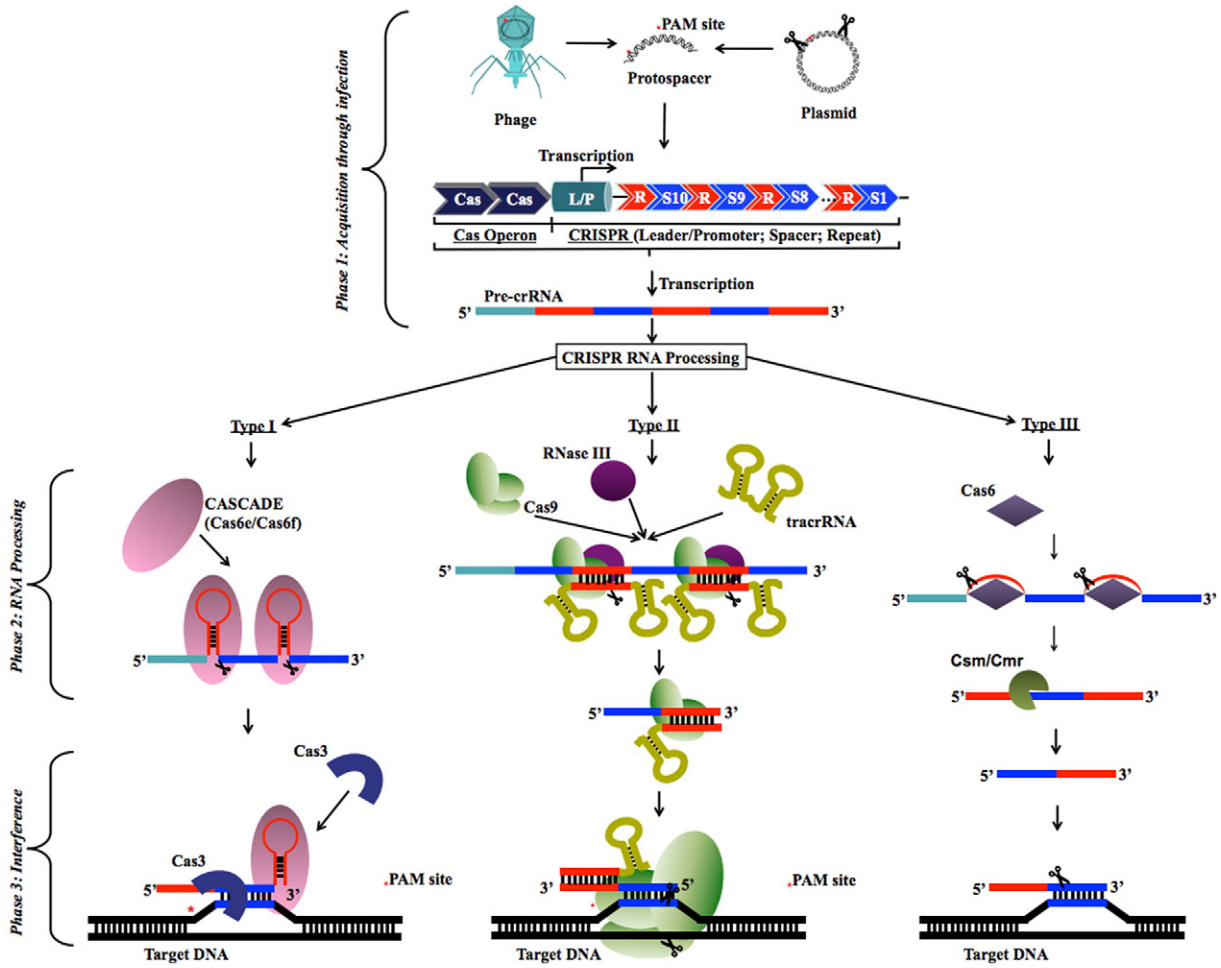

Fig. 1. CRISPR-Cas defense through acquisition, RNA processing and interference (Type I, Type II, and Type III). The CRISPR-Cas defense mechanism follows a three-step process (acquisition, RNA processing, and interference). In acquisition, foreign DNA possessing the protospacer adjacent motif (*PAM) known as protospacer (phage/plasmid origin) are captured and integrated as spacers (S) between adjacent repeats (R) in the CRISPR locus (Deveau et al., 2008). Following this, foreign DNA is degraded through three routes – Type I, Type II, and Type III. In Type I (L) the multisubunit (Cas6e/Cas6f) CASCADE binds, processing the crRNA, and creating a ribonucleoprotein complex. Following this, the helicase/nuclease Cas3 joins the complex that acts in the interference phase through sequence-driven degradation of the foreign DNA. Type II (center) utilizes the Cas9 protein to generate crRNA and to target/degrade foreign DNA (Garneau et al., 2010; Jinek et al., 2012). It is enabled through single guide RNA (tracrRNA-sgRNA). Type III (R) operates through Cas6, which is assisted by other proteins in a complex (Csm or Cmr). This activity as a whole does not require PAM sequences, and so it acts non-specifically, still degrading foreign DNA in the interference phase (Barrangou et al., 2007; Horvath and Barrangou, 2010; Bhaya et al., 2011).

sequence then the bacterial host does not have resistance against the phage, leading to infection and subsequent host cell lysis with the release of a new phage for the following infections (Bhaya et al., 2011).

CRISPR-Cas systems are categorized into three types, Type I, Type II and Type III (Fig. 1). Type I features the ubiquitous presence of the signature protein Cas3, a helicase/nuclease that is a large multidomain protein, but has distinct activity from DNA nuclease and helicase enzymes (Brouns et al., 2008; Sinkunas et al., 2011). Multi-subunit (Cas6e/Cas6f) CASCADE-like complexes are involved in the interference phase. It is associated with processed crRNA and forms a ribonucleoprotein complex through the seed sequence driven base pairing and degrades foreign/invasive DNA. The Type II CRISPR-Cas system features the essential Cas9 signature protein. The Cas9 protein contains the two active domains, the RuvC-like and the HNH domains that are essential for its endonuclease activity (Jinek et al., 2012; Chen et al., 2014). The Cas9 is a large multifunctional protein and it forms a complex with crRNA-tracrRNA that binds upon phage and plasmid DNA for further cleavage (Garneau et al., 2010). Specifically, the single guide RNA (sgRNA) is a hybrid of regions comprising of a 20–25 nucleotide long base pairing region (seed region) for target DNA binding (crRNA) and a 42 nucleotide long hairpin trans-activating crRNA (tracrRNA) for Cas9 binding (Deltcheva et al., 2011).

CRISPR-Cas9 requires an sgRNA, an endonuclease (Cas9), and the PAM sequences (5′-NGG-3′) present in target sites (Gasiunas et al., 2012; Jinek et al., 2012), although sometimes less frequently 5′-NAG-3′ (Hsu et al., 2013). In the last phase, the Type III CRISPR-Cas that requires a number of RAMP proteins, Cas6, and Cas10 which involve in the processing of crRNA and in targeted DNA cleavage (Anantharaman et al., 2010). Type III does not require PAM sequences for the recognition of foreign DNA, which makes it a non-specific system, although it is still capable of degrading foreign DNA (Bhaya et al., 2011). The CRISPR-Cas is regarded as one of the earliest defense systems that have been developed in prokaryotes, and it is also inheritable (Horvath and Barrangou, 2010; Richter et al., 2012; Szczepankowska, 2012). Recently, the Type II CRISPR-Cas system has become more widely used for genome editing using the well-characterized Cas9 endonuclease from *Streptococcus pyogenes* (Cong et al., 2013; Hwang et al., 2013; Jinek et al., 2013; Mali et al., 2013; Hisano et al., 2015). In addition, other Cas9s derived from different bacteria include those of *Streptococcus thermophilus*, *Staphylococcus aureus*, and *Neisseria meningitidis* (Table 1), and they show different cleavage efficiencies and PAM sequence requirements (Deveau et al., 2008; Mojica et al., 2009; Esvelt et al., 2013; Hou et al., 2013; Ran et al., 2015).

The expression of sgRNA and Cas9 in the cell forms the sgRNA-Cas9 complex, which binds to the targeted region in the presence of its PAM

Table 1
The Cas9 endonuclease derived from different bacteria and their PAM sequences.

Variant of Cas9	PAM sequences	References
Streptococcus pyogenes, SpCas9	5′-NGG-3′	Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013; Jiang et al., 2013
Staphylococcus aureus, SaCas9	5′-NNGRRT-3′ or 5′-NNGRR(N)- 3′	Friedland et al., 2015; Ran et al., 2015;
Neisseria meningitidis, NmCas9	5′-NNNNGATT-3′	Hou et al., 2013
Streptococcus thermophilus, StCas9	5′-NNAGAAW-3′	Cong et al., 2013; Deveau et al., 2008; Mojica et al., 2009;
Francisella novicida, FnCas9	5′-NGG-3′	Price et al., 2015

sequences. It creates a double-strand break (DSB) that can be repaired either by a homology-directed repair (HDR) pathway or by a non-homologous end-joining (NHEJ) pathway (Fig. 2). Gene disruption by small insertions/deletions (Fig. 2A) or gene correction/insertion by assisted recombination (Fig. 2B) can follow (Cong et al., 2013; Cristea et al., 2013; Mali et al., 2013; Maresca et al., 2013). Currently, the interest to edit the genomes of desired organisms is increasing, and accordingly to target these genes of interest there is a need to design a number of specific sgRNAs. These sgRNAs can be designed manually and also computationally. However, in order to computationally design sgRNA, a number of web-based tools (Table 2) have been developed that enable the easy design of sgRNA. These can be benefitted with the highest sequence specificity for the targeted gene by considering the PAM sequence (NGG), and can help avoid off-target effects (Bae et al., 2014; Liu et al., 2015; Naito et al., 2015; Park et al., 2015; Stemmer et al., 2015). Doench et al. (2016) have developed a portal for large-scale screening of sgRNA library for on target and off target effects in human and mouse. It can effective maximize the genome edit and genetic screens. These sgRNAs can be subsequently chemically synthesized (through oligos) and used for genomic changes towards the generation of desired phenotypes.

3. Exploring the potential of the CRISPR-Cas9 system

3.1. Genome editing of microorganisms

The genome editing of harmful microorganisms is essential for the better control of infection, virulence, and drug resistance. On the other hand, genome editing of beneficial microorganisms is also essential, as such organisms have a positive history of mankind, having been used to make foods and drinks such as cheese, sourdough, beer, wine, and vinegar for a long time, and a there is accordingly an arising desire to make these microorganisms faster and more robust for industrial benefit. In order to improve the quality and robustness of microorganisms,

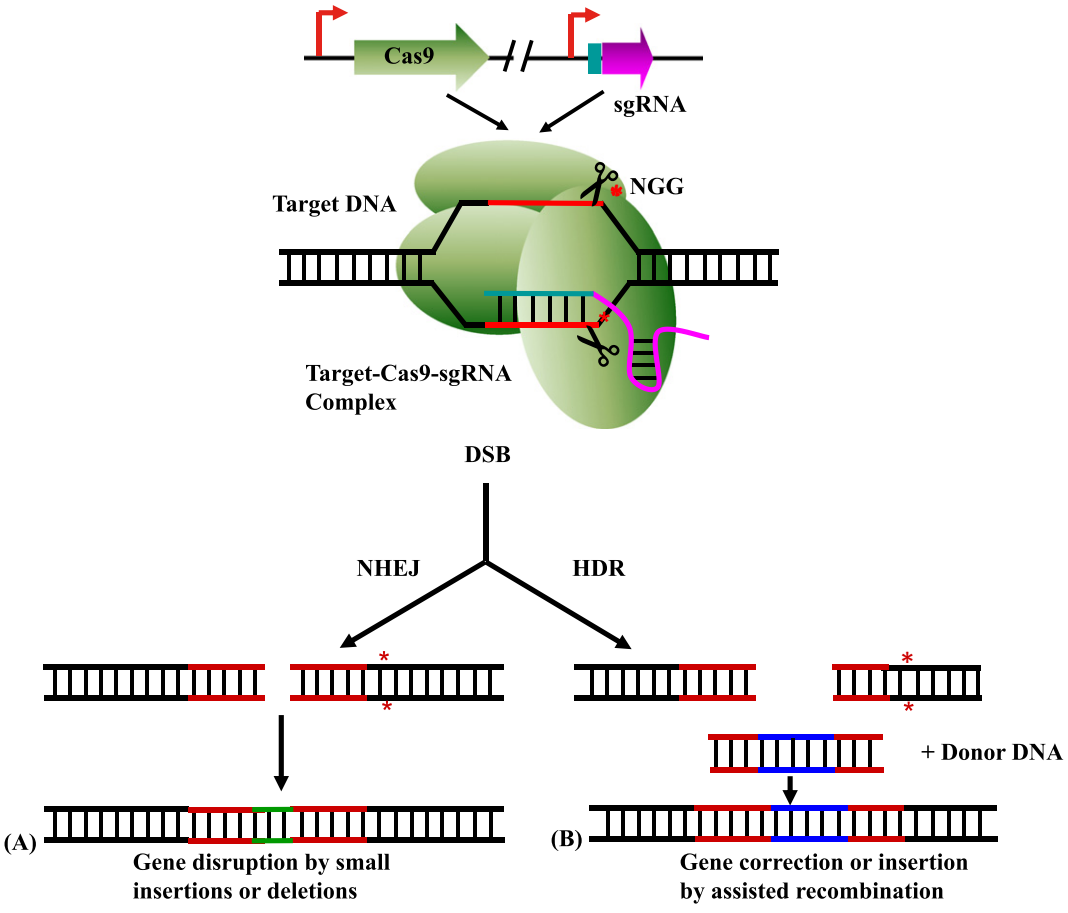

Fig. 2. Targeted genome editing *via* CRISPR-Cas9 system. The Cas9 and sgRNA are expressed and create a complex that binds on targeted DNA near the NGG (*PAM) site. A double stranded break (DSB) is generated at a targeted site that can be repaired either by non-homologous end joining (NHEJ) or homology directed repair (HDR). (A) Repair by NHEJ usually results in the insertion or deletion (indel) or frameshift mutation that causes gene knockout by disruption (green). (B) If a donor DNA is provided with end homology this can be inserted at the targeted site to modify a gene by introducing nucleotide changes or gene insertion (blue) (Jinek et al., 2012; Mali et al., 2013; Cong et al., 2013).

Table 2
Tools for design of sgRNA.

sgRNA design tool	Supports for organisms	Reference/developer Lab
Cas-OFFinder	Human, *Arabidopsis*, *C. elegans*, *Drosophila*, zebrafish, mouse, rat, cow, dog, pig, *Thale cress*, rice, tomato, corn and monkey	Bae et al., 2014
Cas-Designer	Human, *Arabidopsis*, *C. elegans*, *Drosophila*, zebrafish, mouse, rat, cow, dog, pig, *Thale cress*, rice, tomato, Corn and monkey	Park et al., 2015
CCTop	Human, *Arabidopsis*, *C. elegans*, zebrafish, sea squirt, cavefish, Chinese hamster, fruit fly, rice, fish, mouse, silk worm, stickleback, tobacco, tomato and frog	Stemmer et al., 2015
CRISPR MultiTargetor	Human, *Arabidopsis*, zebrafish, mouse, rat, chicken, frog, fly, worm, maize, and rice	Prykhozhij Lab (http://www.multicrispr.net/)
CRISPRdirect	Human, *Arabidopsis*, *C. elegans*, *Drosophila*, zebrafish, mouse, rat, marmoset, pig, chicken, frog, sea squirt, rice, sorghum, silkworm and yeast	Naito et al., 2015
Target Finder	Human, *Arabidopsis*, *C. elegans*, *Drosophila*, zebrafish, mouse, rat, rabbit, pig, possum, chicken, dog and mosquito	Zhang Lab (http://crispr.mit.edu/)
E-CRISPR	Human, *Arabidopsis*, *C. elegans*, *Drosophila*, zebrafish, mouse, rat, yeast, frog, *Brachypodium distachyon*, *Oryza sativa*, and *Oryzias latipes*	Boutron Lab (http://www.e-crisp.org/E-CRISP/)
CRISPR gRNA	Human, *Arabidopsis*, mouse, *E. coli*, and yeast	DNA2.0 (https://www.dna20.com/eCommerce/cas9/input)
CRISPOR	Human, *Arabidopsis*, *C. elegans*, *Drosophila*, zebrafish, mouse, rat, *Bombyx mori*, *Anopheles gambiae*, *Aedes aegypti*, *Oryza sativa*, *Zea mays*	http://crispor.tefor.net/
CRISPR-ERA	Human, *C. elegans*, *Drosophila*, zebrafish, mouse, rat, yeast, *E. coli*, *B. subtilis*, and fruit fly	Liu et al., 2015

genome editing plays a vital role in creating gene knock-outs, knock-ins, and replacements of sequences. Despite this, microbial genome editing remains an unexplored and underrepresented application of CRISPR-Cas systems (Selle and Barrangou, 2015). The possession of the CRISPR-Cas system in the microorganisms that have it has been considered to be part of a resistance mechanism. Young (2008) has suggested that the CRISPR-Cas complex is a secret weapon that is incorporated into the bacterial genome for the prevention of phage attack.

In addition, Agari et al. (2010) have published interesting data from *Thermus thermophilus* HB8, in which they identified 12 CRISPR loci. The genome-wide transcription profile was obtained by DNA microarray data where the strain was infected with the lytic phage PhiYS40. Following phage infection, they have observed that the two CRISPR-associated (Cas) operons were up-regulated. As an extension to this, they also looked at the catabolic repressor protein (CRP) regulated genes, and in a crp deficient strain these genes were less up-regulated as when compared to the wild-type strain. These results suggest that cAMP is a signaling molecule that transmits the information about phage infection, and is part of the pathway in the resistance mechanism of CRISPR-Cas.

The use of multiplex mutagenesis has always been of great interest for rapid genetic engineering. Recently, Jiang et al. (2013) have reprogrammed the dual-RNA:Cas9 specificity by changing the nucleotides of short crRNAs to build single and multinucleotide changes carried upon targets. They used simultaneously two crRNAs that permitted multiplex mutagenesis with CRISPR-Cas9, and in a test with *S. pneumoniae* they found cells that contained up to 100% of the desired mutations, and in *E. coli* about 65% of desired mutations, demonstrating potent *in vivo* rates with this technique.

A major issue to human health is that some bacteria can easily gain resistance against commonly used antibiotics. There are more than 200 conserved and essential proteins present in the bacteria, but only a relatively small number of these are currently exploited antibiotic targets (Bugg et al., 2011). However, the current antibiotics are not specific to selectively kill the desired strains from a complex community (Smith and Romesberg, 2007; Chen et al., 2012; Bikard et al., 2014). Another issue to consider is that most of the antibiotic resistance genes are derived from plasmids (and have multiple copies) that are capable of autonomous transfer into microbial populations (Nordmann et al., 2012). Potentially the build-up of resistance over time may have also occurred due to random mutations in genes and the evolutionary adaptation of strains, and the excess and inappropriate use of antibiotics. Current antibiotics have a tendency to be broad spectrum, and therefore cannot readily discriminate between the beneficial and harmful strains within a mixed community. In order to specifically target harmful strains, a programmable CRISPR-Cas9 system has been successfully developed for targeting even highly related strains in pure or mixed cultures. This could also offer the development of a new way to control multi-drug resistance (MDR) and to discriminate between the harmful and the beneficial microorganisms within a community (Gomaa et al., 2014).

Similarly, a sequence-specific programmable CRISPR-Cas9 system has been developed and deployed through bacteriophage. In this work, they have constructed a staphylococcal vector and inserted Cas9 and tracrRNAs for crRNA biogenesis that target the aph-3 kanamycin resistance gene. This system enables the killing of virulent *Staphylococcus aureus* in a sequence-specific manner, whilst leaving behind avirulent strains. This was shown to prevent the further spread and the transfer of antibiotic resistance plasmids into non-pathogenic staphylococci (Bikard et al., 2014). To test the multiplex ability of this CRISPR-Cas9 antimicrobial system, they have expanded the CRISPR array by phagemid to produce a second set of crRNAs that targeted either the super antigen enterotoxin *sek* gene or a region of the *mecA* gene. It was shown that targeted strains were killed with comparable efficiencies. The delivery of sequence-specific Cas9 dramatically decreases the plasmid content in a population without killing host cells, an exciting find as this could be used to immunize avirulent strains against the transfer of antibiotics resistance plasmid (Bikard et al., 2014). Studies of this kind provide evidence for the potential of a potent alternative to antibiotics, and thus a novel concept has been demonstrated; to reprogram CRISPR-Cas9 for the simultaneous targeting of multiple loci for the effective targeting of pathogens, and assist human healthcare against the significant and pressing threat of resistant microorganisms.

In related work, Citorik et al. (2014) have developed a CRISPR-Cas9 system for the targeting of a specific DNA sequence within a complex microbial community. It has been delivered through the bacteriophage and bacteria carrying conjugation plasmids. In order to establish a sequence-specific RNA-guided nuclease (RGN) system, they have designed RGNs to induce DSBs in blaSHV-18 and blaNDM-1 strains, which encode respectively extended-spectrum and pan-resistance against β-lactam antibiotics. The RGN system has been transformed into *E. coli* that contains a chromosomal copy of these target genes and they obtained a 1000-fold reduction in the efficiency of transformation as compared to wild-type strains (Citorik et al., 2014). Similarly to test the phage-based delivery of RGNs, they have engineered a phagemid for targeting blaNDM-1 and blaSHV-18. The phage packaged RGNNDM-1 was accomplished by transducing a population of *E. coli* EMG2 which then enabled adaptation of a complex community through the specific reduction in the targeted strains (Citorik et al., 2014). This could be expanded in other microorganisms for the precise and the effective control of targeted strains in future. Still there are issues remaining to be resolved for phage therapy, including the fact that the phages in use are often poorly characterized. There may be a synthetic phage that could be an alternative in future phage therapy for more precise and effective control of MDR bacteria. CRISPR-Cas9 could thus provide

a promising solution to the growing obstacle of antibiotic resistance bacteria (Beisel et al., 2014). A CRISPR-Cas9 system like this could be a key technology for further control of MDR bacteria, including those existing within a complex microbial community, in a sequence-specific manner, which is something that could be also expanded to the human gut system whereby we can still retain our important and beneficial microflora but selectively target the pathogenic bacterial infections.

One important application of CRISPR-Cas9 is in the dairy industry due to the huge losses of dairy products through the bacterial contamination. *Lactococcus lactis* is a biotechnological and industrially important microorganism within the dairy industry. It has dominant defense systems against horizontal gene transfer and mobile elements that also encode phage defense systems for protection from viruses. However, after the analysis of eight recently sequenced genomes of lactic acid bacteria (LAB), it was discovered that none of these bacteria possessed a CRISPR-Cas system (Millen et al., 2012). However, LAB strain has been modified by the insertion of spacer sequences from lactococcal phage, subsequently showing enhanced resistance against phage and therefore exhibiting this useful tool for the dairy industry (Millen et al., 2012). The introduction of a phage resistance mechanism like CRISPR-Cas9 could enhance a bacterial species overall defense mechanisms, improving their utility for industry. Another use of CRISPR-Cas9 could be within industrially important actinomycetales that are well-known and industrially established sources for the production of secondary metabolites and pharmaceutically active components for a long time. Recently, Tong et al. (2015) have developed a CRISPR-Cas9 system for the genome editing of actinomycetales and they have used it to delete genes and gene clusters, to implement a precise gene replacement, and to reversibly control the gene expression. Two genes (actIORF1 and actVB) have been targeted from the actinorhodin pathway of *Streptomyces coelicolor* A3(2) and both were successfully inactivated. They could inactive with 100% efficiency if templates for HDR were available (Tong et al., 2015). Similarly, the CRISPR-Cas9 and the λ-Red recombination system were combined with MAGE technology (CRMAGE) to create a high-efficiency method for genome engineering, finding high success rates with values between 96.5-99.7% as compared to success rates of traditional recombineering, typically between 0.68-5.4% (Ronda et al., 2016).

DiCarlo et al. (2013) have used the CRISPR-Cas9 system in *Saccharomyces cerevisiae* for targeting endogenous genomic loci. The Cas9 is constitutively expressed with the sgRNA cassette, showing that targeted DSBs can increase homologous recombination rates of single-stranded DNA (5-fold) and double-stranded DNA (130-fold) donors. This approach provides a key foundation for a simple and powerful genome-engineering tool for site-specific mutagenesis and allelic replacement. In addition, Jakočiūnas et al. (2015) have recently developed CRISPR-Cas9-based multiplex genome editing using sgRNAs for the knock-out of up to 5 different genomic loci (bts1, ypl062W, yjl064w, rox1, and erg9) in a single transformation step with 100% efficiency in *S. cerevisiae*. They have used all possible combinations to investigate high mevalonate producing strains, and have identified 20 strains with significantly higher mevalonate levels (between 0.34 and 10.38 μM) as compared to the wild-type strain (0.25 μM). In this approach, even without the overexpression of any genes in the mevalonate pathway, it was possible to identify strains producing 41-fold higher mevalonate than the wild-type strain. The technology of CRISPR-Cas9 shows great potential and versatility towards medical, therapeutics and industrial applications in these areas. More developments are certain to come in its future.

3.2. Genome editing of mammalian cells

The sequencing of the human genome holds a number of benefits such as early disease diagnosis, genetics, gene function, and gene therapy for improving and obtaining better health (Venter et al., 2001). Targeted genome editing has been previously performed using engineered ZFNs (Szczepek et al., 2007; Carroll, 2011) and TALENs (Moscou and Bogdanove, 2009; Boch, 2011). However, these methods remain difficult to design, time-consuming, and overall expensive. In 2013, a number of studies were launched to explore more to the potential of expanding genome engineering within the higher organisms. CRISPR systems of the Type II family from *Streptococcus thermophilus* (Cong et al., 2013) and *Streptococcus pyogenes* (Cong et al., 2013; Mali et al., 2013) have been engineered to achieve targeted genome editing in mammalian cells. Cong et al. (2013) have designed a CRISPR system (SpCas9, SpRNase III, tracrRNA, and pre-crRNA) for targeting the mammalian genome. They have used a different combination of CRISPR-Cas9 components and transfected these into 293FT cells, finding efficient cleavage. In addition, Mali et al. (2013) have made recent advances in human genome editing through the development of a CRISPR-Cas9 system, especially for the endogenous AAVS1 locus. They have designed human codon-optimized Cas9 and fused this at the C-terminus with the SV40 (Simian virus 40) nuclear localization signal. They have fused custom designed chimeric crRNA-tracrRNA (sgRNA). They found noticeably variable targeting rates in a variety of cells, including rates of 10–25% in 293T cells, 13–8% in K562 cells, and 2–4% in induced pluripotent stem cells (iPSCs). They show that this process is sequence-specific and relies upon CRISPR components, and upon the simultaneous introduction of multiple sgRNAs, can result in the multiplex editing of targeted loci. These studies provide a key foundational tool for mammalian genome editing.

Ranganathan et al. (2014) have developed the CRISPR-Cas9 technology towards the modification of sgRNA expression that would normally use the U6 promoter. This promoter needs a guanosine nucleotide to initiate transcription and is thus constrained in its genomic targeting sites to GN19NGG. To perform this, they modified endogenous genes using the H1 promoter, which expresses sgRNAs and targets both AN19NGG and GN19NGG sites (striking the former ~15% more frequently). Accordingly it has been found that the versatility of CRISPR-Cas9 could be enhanced through the doubling of the number of targeting sites within the genome. Fu et al. (2014) have used truncated gRNAs with less than 20 bp complimentary region of target that could be reduced the undesired mutations at some off-target site by 5000-fold without scarifying the on-target editing efficiency. Recently, X. Liang et al. (2015) have developed an efficient method based on liposome-mediated transfection/electroporation for the delivery of Cas9/sgRNA ribonucleoprotein (RNP) complexes into different types of mammalian cells. When they targeted only a single locus and found indel (deletion) rates were 94% in Jurkat T cells and 87% in iPSCs. The indel rates were also obtained by 93% and 65% through the targeting of 2- and 3-loci in Jurkat cells, respectively. Additionally, they have also found that off-target effects, occurring when the sgRNA binds to another region in the genome, were reduced using this system as compared to the plasmid-based transfection. This method can be useful towards the technique of high-throughput and efficient multiplex genome editing.

In the past decade, mammalian cells have posed an increasingly more attractive proposition for the production of recombinant proteins and therapeutics because of their superior capabilities for protein folding, assembly, and post-translational modification (Wurm, 2004). Plasmid-based expression of recombinant proteins and the insertion of foreign genes into the genome have both been major challenges. To tackle these issues, CRISPR-Cas9 has been recently used for insertion of up to 5 kb of plasmid into the genome *via* NHEJ, finding an efficiency of up to 0.17% in human embryonic kidney 293 (HEK293) cells and 0.45% in Chinese hamster ovary (CHO) cells (Bachu et al., 2015). Similarly, Sakuma et al. (2015) have integrated 9.6-kb plasmid and 7.6-kb DNA into a targeted region in genomic of CHO could be able to produce recombinant scFv-Fc protein of the knock-in cells. However, this demonstrates that CRISPR-Cas9 can be a quick and efficient tool for the insertion of large foreign DNA into targeted genomic sites in mammalian cells, and can thus be one of the more stable and efficient platforms for the production of desired recombinant proteins. Whilst the CRISPR-Cas9 system is limited by the low efficiency of HDR as compared

to NHEJ pathway, and in a related study, the team of Chu et al. (2015) tested the efficiency of insertion of foreign DNA by both pathways, which were studied and improved. To enhance HDR, NHEJ inhibiting molecules KU70 and DNA ligase IV (through inhibition by SCR7) have been used to induce a 4–5 fold increased efficiency in HDR. The insertion efficiency has been further increased by co-expression of E1B55K and E4orf6, rising up to 8-fold increases, leading to a complete alteration of the NHEJ pathway (Chu et al., 2015). In addition, they have found up to a 19-fold increase in the efficiency of HDR-mediated genome editing by CRISPR-Cas9 in mammalian cells, including those of mice. Here, they observed an improvement in the insertion efficiency of small and long lengths of DNA at the TSG101 and the Tap1 target loci (Maruyama et al., 2015). The earlier study demonstrated that HDR is less efficient than NHEJ. Therefore; He et al. (2016) used NHEJ pathway for integration of a 4.6 kb promoterless IRES-eGFP fragment into the GAPDH locus. They found that up to 20% GFP + cells in somatic LO2 cells and 1.70% GFP + ESCs. This study also proved that NHEJ-based knock-in is more efficient than HDR-mediated gene targeting in all human cell types.

CHO cells are attractive tools and represent the most widely used cells for the production of therapeutic proteins. The CRISPR-Cas9 system has been used for the integration of a 3.7 kb gene expression cassette into targeted loci of the genome for stable transgene expression, selecting on drug marker expression (Lee et al., 2015). The regulation of gene expression could be assisted by the versatility of CRISPR-Cas9. DNA methylation is a heritable epigenetic marker. DNA methyltransferase (DNMT) enzymes help to transfer of methyl group to the C-5 position of cytosine ring (Robertson, 2005). It is also involved in regulating gene expression and maintaining genomic integrity. Studies have used CRISPR-Cas9 for the inactivation of all three catalytically active DNMTs enzymes in human embryonic stem cells and found that deletion of DNMT1 resulted in rapid cell death (Liao et al., 2015).

Recently, Price et al. (2015) have identified another type of Cas9 derived from *Francisella novicida* (FnCas9) that has the ability to target endogenous bacterial RNA. They have expanded the use of FnCas9, which is also mediated by sgRNA, to target and inhibit the human + ssRNA virus, as well as the hepatitis C virus (targeted 5′ UTR and 3′ UTR) within eukaryotic cells. This work demonstrates the effectiveness of this tool as a versatile and portable RNA-targeting system that can operate in mammalian cells for the control of viral infections. In addition, another version of Cas9 derived from *Neisseria meningitidis* has been used for the genome editing of human pluripotent stem cells (hPSCs), something that holds a great promise towards biomedical research and regenerative medicine (Hou et al., 2013). They have demonstrated within three different hPSC lines the efficient targeting of an endogenous gene using HDR. The Cas9 RNA-guided endonuclease from *N. meningitidis* (NmCas9) recognizes a 5′-NNNNGATT-3′ PAM different from those recognized by Cas9 from *S. pyogenes* (SpCas9) and *S. thermophilus* (StCas9). Similarly to SpCas9, NmCas9 is also able to use an sgRNA to direct its activity. Because of its distinct PAM, the *N. meningitidis* CRISPR-Cas9 machinery increases the sequence contexts amenable to RNA-mediated genome editing (Hou et al., 2013).

A genetic disorder is a serious genetic problem that is caused by one or more abnormalities in the genome due to mutations or changes in the DNA sequence. Disorders can manifest through possibly damaged gene function. Recently, CRISPR-Cas9 has been used for the correction of mutation in *Fah* (fumarylacetoacetate hydrolase) gene in the hepatocytes in a mouse model of the hereditary human disease tyrosinemia. The correction of the mutation in *Fah* by CRISPR-Cas9, and the finding of *Fah*-positive hepatocytes clones that could rescue the loss of body weight phenotype show the potential of the technique to be a solution in addressing and correcting human genetic diseases as well (Yin et al., 2014a). Indeed, this approach can be useful for replacing a defective gene with its correct sequence. Another study looked into the mutations of the gene encoding dystrophin that results in a serious X-linked inherited disease called Duchenne muscular dystrophy (DMD). DMD is characterized by progressive muscle weakness and shortened life span. Currently, there are no effective treatments available. CRISPR-Cas9 has the potential to restore expression of the modified dystrophin gene. It has been used to correct the dystrophin gene (*dmd*) mutation in an mdx mouse germline and the correction rates between 2-100% were found (Long et al., 2014; Nelson et al., 2016). The variation in corrective efficiency can be further optimized and expanded for a better cure of human patients with DMD. Very recently, in the same issue of Science have published three major articles on DMD for correction and kept muscle function. Long et al. (2016) used *in vivo* approach using adeno-associated virus-9 (AAV9) to deliver CRISPR-Cas9 in gene postnatal mdx mice, a model of DMD. It restored dystrophin expression in cardiac and the skeletal muscle with different level and expression was more increased from 3 to 12 weeks after the injection and enhanced the skeletal muscle function. Similarly, CRSIPSR-Cas9 approach to modify exon 23 deletion and modified dystrophin gene showed recovery of functional dystrophin protein in skeletal myofibers and cardiac muscle. It could also be enhanced the muscle function (Nelson et al., 2016; Tabebordbar et al., 2016).

Haemophilia B is a blood clotting X-linked genetic disorder that is caused by a mutation in the factor IX gene, F9, leading to a deficiency of the factor IX protein. This may also be a target for gene therapy. Most recently, Guan et al. (2016) have identified a family with haemophilia B who carry a mutation (specifically Y371D) in the F9 gene. To test the effectiveness of the CRISPR-Cas9 system, they used a mouse model to generate the mutation Y371D in F9 and found the development of the haemophilia B phenotype. Subsequently, they used a naked DNA construct and adenoviral vectors to deliver Cas9 for the correction of the F9 Y371D mutation, resulting in a correction rate of 0.56% of F9 alleles within hepatocytes that was sufficient to restore haemostasis. This study again demonstrates that inherited diseases of humans could be addressed and hopefully cured by CRISPR-Cas9 systems.

The specificity of Cas9 in mammalian cells remains an important issue for the use of the technology towards clinical practice. When compared to the bacterial genome, the several 100-fold larger sizes of mammalian genomes may present more off-target effects that must be extensively examined. The versatility of Cas9 system has been successfully used for studying human disease for the correction of equivalent gene mutations in animal models (Platt et al., 2014; Xue et al., 2014; Yin et al., 2014a). A number of other challenges remain ahead towards the effective treatments of serious human diseases. These may be addressed by the use of genome editing, regulation, and targeting. Furthermore, this could be benefitted by the use of iPSCs that could be applied for these purposes, although there needs to be a consideration of the ethical issues therein (discussed below).

3.3. Genome editing of Drosophila

The fruit fly *Drosophila* is widely used as a model organism, most especially the species *D. melanogaster*, and it is used for studies of genetics, physiology, microbial pathogenesis, and evolution. Functional genomics studies of *D. melanogaster* were made with a recently developed CRISPR-Cas9 system (Ren et al., 2014a). In this study, they found that the specificity and the efficiency of sgRNAs plays a crucial role, and interestingly observed that off-target effects did not occur in regions of genomic DNA that presented three or more nucleotide mismatches to sgRNAs. The efficiency of mutation through the NHEJ pathway for DSBs with different sgRNAs targeting the same gene can vary around 10-fold. In contrast, the efficiency of mutation through HDR was 5% and created null alleles of HP1a (Ren et al., 2014a). In general, the CRISPR-Cas9 system requires either injection of *in vitro* transcribed RNAs or the injection into transgenic Cas9-expressing embryos. Therefore, for efficient gene targeting rates, Gokcezade et al. (2014) have developed a simple and versatile genome-editing tool for *Drosophila via* bicistronic-Cas9/sgRNA expression vectors that provide an alternative

method for CRISPR-Cas9 use. It was used for knock-out and knock-in of alleles, and mutants could be quickly screened, demonstrating this as a simple and efficient tool for genome editing.

Similarly, Xue et al. (2014) have developed a CRISPR-Cas9 mediated conditional mutagenesis system. They have combined tissue-specific expression of Cas9 that is driven by a Gal4/upstream activating site (UAS) with various ubiquitously expressed sgRNAs to effectively inactivate gene expression in a controlled manner. This study shows the use of a number of sgRNAs to target a single gene and the subsequent creation of a high degree of mutations in specific tissues. To overcome the issue of off-target effects when using the CRISPR-Cas9 system, Ren et al. (2014b) have mutated the Cas9(D10A) nickase, affording a greater capability to reduce the off-target effects *in vitro*. They have injected sgRNA and the Cas9(D10A) nickase plasmids into *Drosophila* and efficiently generated indel mutants and also reduced the off-target effects. In addition, Kondo and Ueda (2013) have used two transgenic strains, one of which expresses Cas9 from a germline-specific nanos promoter and the other one that expresses an sgRNA that targets a unique site in the genome. The genetic cross between both strains forms an active Cas9-sgRNA complex specifically in germ cells, capable of generating mutations at this target site. They have also found that simultaneous cleavage of two sites by co-expression of two sgRNAs efficiently could induce internal deletion to a frequency between of 4.3 and 23%, creating a simple and highly efficient CRISPR-Cas9 method that can be used for generating and detecting mutations of any gene in *D. melanogaster*. Bassett et al. (2013) have modified the system by injecting sgRNA into the embryos of *Drosophila* and finding mutagenesis of targeted genes by up to 88%.

A new type of CRISPR-Cas9 system for the high-efficiency genome editing of *D. melanogaster* has been established that containing Cas9 lines and versatile sgRNA expression plasmids (Port et al., 2014). With this system, they have demonstrated the differential activity of the same sgRNA expressed from different U6 snRNA promoters. Therein they found that appropriate combinations of Cas9 and sgRNA allowed for targeting of essential and non-essential genes with a transmission rate of between 25 and 100%. The versatility of this system shows that this form of mutation operates in a precise and efficient way *via* HDR (Port et al., 2014). In addition, an effective and economical CRISPR-Cas9 method for genome editing of this organism exists whereby the sgRNAs are expressed under the control of U6b promoter that is then injected into the transgenic flies where Cas9 is expressed in the germline under the control of nanos promoter. Here, a mutagenesis rate of 74.2% was achieved (Ren et al., 2013). The delivery system of Cas9 and sgRNA remains an important issue that needs to be improved to uncover the full potency of the CRISPR-Cas9 system.

The system can be optimized for *Drosophila* genome editing and regulation to resolve many health related issues. Advancement of genomics and proteomics research suggest that about 75% of known human disease-causing genes and about 50% of protein sequences resemble sequences found within the genome of *Drosophila* (Reiter et al., 2001). Due to this, and as expected, *Drosophila* is being used as a genetic model organism for a number of human diseases that include spinocerebellar ataxia, Alzheimer's disease, Parkinson's disease, and Huntington's disease. It is also being used to study the mechanisms underlying stress, immunity, diabetes and cancer. Thus, CRISPR-Cas9 represents a sensitive, efficient and powerful tool for *Drosophila* genome editing that can be readily expanded towards creating models for therapeutic and biomedical applications.

3.4. Genome editing of zebrafish

The zebrafish (*Danio rerio*) is a tropical freshwater and popular aquarium fish. The zebrafish is an important and widely used vertebrate model organism for a variety of applications such as in the study of cancer (Ceol et al., 2011) and cardiovascular disease (Drummond, 2005), in drug discovery, and in the study of its regenerative abilities (Goldshmit et al., 2012). The sensitive and efficient genome editing of zebrafish was recently achieved using the CRISPR-Cas9 system. Jao et al. (2013) developed a simple CRISPR-Cas9 based method for mutagenesis. To make it more robust and efficient, they used sgRNAs and a zebrafish codon-optimized Cas9 that can efficiently target a reporter transgene Tg(−5.1mnx1:egfp) and the four endogenous loci of *tyr*, *golden*, *mitfa*, and *ddx19*. A mutagenesis rate was achieved at frequencies between 75 and 99%. They have also simultaneously targeted five genomic loci and successfully found loss-of-function phenotypes. Furthermore, Hwang et al. (2013) have studied the *in vivo* induction of targeted genome editing in zebrafish embryos using the CRISPR-Cas9 system. They have microinjected different concentrations of fh-targeted sgRNA and the Cas9 encoding mRNAs into one-cell stage zebrafish embryos. They obtained the induction of targeted indels mutations at frequencies ranging from 10.0 to 52.7% with different concentrations of sgRNAs. Similarly, Hruscha et al. (2013) have demonstrated that mutagenesis in zebrafish using CRISPR-Cas9 can be a highly efficient tool reaching a success rate of up to 86.0%. This work also used targeted knock-in of a protein tag provided by a donor oligonucleotide and found knock-in efficiencies of 3.5–15.6%, contrasting with mutation rates at potential off-target sites at only 1.1–2.5%, demonstrating the specificity of the CRISPR-Cas9 system in this application.

In a recent study, Ota et al. (2014) have verified the editing of multiple genes by CRISPR-Cas9 in this species. The selected genes included those for the golden (*gol*) and tyrosinase (*tyr*) phenotypes, associated with pigment formation, and *s1pr2* and *spns2*, associated with cardiac development. All of these genes were disrupted with insertion or indel mutations that were introduced by co-injection of multiple sgRNAs and the Cas9 mRNA. They observed the hypopigmentation of skin melanophores and the two different heart phenotypes in the injected F0 embryos. It was also shown that CRISPR-Cas9 induced indel mutations and a locus-specific deletion was heritable in F1 embryos. Furthermore, they have improved the heteroduplex mobility assay (HMA) for the simultaneous detection of indel mutations at different target loci. In addition, it has been shown that more than 35% of site-specific somatic mutations using the Cas9/sgRNA system for *in vivo* targeted the genes in either *etsrp* and *gata4*, or in *gata5*, for the zebrafish embryos (Chang et al., 2013). The CRISPR-Cas9 system is a simple, efficient and target specific genome editing technology that shows potential use in zebrafish, demonstrating its versatility to be further expanded for biomedical and biotechnology applications in this important model species.

3.5. Genome editing of human viruses

A virus is a small infectious particle that replicates inside the cells. They can infect all types of life forms including animals, plants, fungi, bacteria and archaea (Koonin et al., 2006). Viruses are a therapeutic challenge because its life cycle occurs within the host cells, and they use the host machinery for replication and propagation (White et al., 2015). When a virus infects an animal it can cause serious diseases resulting in grave morbidity, mortality, and further infectious transmission. Infection by a virus may also reduce the animal immune system, although this can be improved and assisted by vaccination. Many of the infections such as those including HIV, HBV, and HPV, and as well as others, are important human viruses that result in a major and global health problem that remains difficult to control. There still exists a need to develop a highly effective vaccine or antiviral drug, a challenge limited so far due to therapies tending to be time-consuming, expensive, and fundamentally inefficient for the control of viruses. Thus, a pressing need arises to control and cure the viral infection in humans. Recently, the potential of the CRISPR-Cas9 system has been demonstrated as a simple and effective tool for genome editing to eradicate some human viruses (Table 3). The C-C chemokine receptor type 5 (CCR5) in humans is a protein found on the surface of white blood cells (WBCs). CCR5 serves as an essential co-receptor for HIV-1 entry and it has been

Table 3
The use of the CRISPR-Cas9 system for eradicating of human viruses.

Organisms	Targeted genes	Cells	Use	References
HIV-1	LTR	T-cells	Loss of HIV-1	Ebina et al., 2013
	LTR U3	T-cells	Loss of HIV-1	Hu et al., 2014
	CCR5	$CD4^+$ cells	Resistance to HIV-1	Wang et al., 2014; Ye et al., 2014
	LTR	human-iPSCs	Reduced HIV-I	H.K. Liao et al., 2015
	LTR	T-cells	Decrease HIV-1	Kaminski et al., 2016a
HBV	*P1* and *XCp*	Huh-7	Reduced HbsAg level	Lin et al., 2014
	HbsAg, Core, and TR	HepAD38	Reduced viral DNA and cccDNA	Kennedy et al., 2015
	HBsAg	HepG2.2.15	Reduced HbsAg level	Zhen et al., 2015
HPV18	E6 and E7	HeLa	Inactivated	Kennedy et al., 2014
HPV16	E6 and E7	SiLa	Inactivated	Kennedy et al., 2014
HCV	UTR	Huh-7.5	Decreased viral load	Price et al., 2015

observed that individuals with the CCR5Δ32 variant appear to be healthy and also resistant against HIV-1, therefore highlighting CCR5 as an attractive target for the control of HIV-1 infection (Li et al., 2015).

AIDS, which can result from HIV infection, remains a major global public health concern. In one study, the design of sgRNAs and the transfer of CRISPR-Cas9 were made into CD4 + T-cells. This targeted the CCR5 gene, resulting in the disruption of CCR5 expression. This subsequently drove the knock-down of CCR5 expression on the cell surface, conferring HIV-1 resistance (Li et al., 2015). This represents a similarity with the people who carry the mutation (CCR5Δ32) that is protective against HIV infection. In addition, Ye et al. (2014) have used CRISPR-Cas9 for the editing of the CCR5 gene with 32-bp deletions (CCR5Δ32). Therein, they found that the CCR5Δ32 mutation occurs naturally, and they also modified the iPSCs into monocytes/macrophages and this demonstrated their capability to gain resistance to the HIV-1 challenge. Wang et al. (2014) targeted the CCR5 gene by sgRNAs and Cas9 into HIV-1 susceptible human CD4 + cells and found a high frequency of disruption of CCR5 and these cells also became resistant to HIV-I infection. Recently, Saayman et al. (2015) have modified a nuclease-deficient Cas9 fused to transcription activation domains that can result in targeted activation of proviral gene expression. In addition, Saayman et al. (2016) have used CRISPR-dCas9 fused with VP16 domain for activating the latently integrated HIV-1. They targeted the 23 sites of the long terminal repeat promoter of HIV-1 that could be reactivated towards functional cure of HIV/AIDS. The purpose of their approach is to reactivate latent HIV-1 genomes in order to eliminate the viral reservoir.

Ebina et al. (2013) used CRISPR-Cas9 for editing of the HIV-1 genome to inhibit gene expression. The long terminal repeat (LTR) targeting CRISPR-Cas9 was transfected into HIV-1 LTR expression-dormant and inducible T-cells. A major loss of LTR-driven expression was found after induction and it was also observed that CRISPR-Cas9 efficiently cleaved and mutated LTR target sites. It was also able to eliminate the internal viral genes from the genome, showing the potential of the technology towards curing of HIV-1 infection (Table 3). In addition, Hu et al. (2014) have reported the use of both singleplex and multiplex CRISPR-Cas9 systems to eliminate the HIV-1 genome. They have identified highly specific targets in the HIV-1 LTR U3 region that were efficiently edited, inactivating gene expression and subsequent replication in the latently infected different cell types (promonocytic, microglial, and T-cells). In this study, it was demonstrated that the presence of multiplex sgRNAs within Cas9-expressing cells strongly prevented HIV-1 infection.

Recently, Zhu et al. (2015) have designed sgRNAs and tested them upon 10 sites of the HIV-1 genome that were targeted by a CRISPR-Cas9 system. This was introduced into JLat10.6 cells that were latently infected by HIV-1. Here, each target site in the HIV-1 genome was mutated by CRISPR-Cas9, especially in the second exon of Rev (T10), revealing a high rate of mutation that led a 20-fold reduction in virus replication. In addition, H.K. Liao et al. (2015) used HIV-1 as a model organism and used a CRISPR-Cas9 system to eradicate the latently integrated viral genome by targeting the LTR and establishing the provision of long-term protection against subsequent viral infection. The human-iPSCs were engineered to stably express HIV-targeted CRISPR-Cas9 that efficiently discriminates into HIV reservoir cell types and therefore maintains their resistance to HIV-1 challenge. These in particular, as well as the others, underscore the potential utility of CRISPR-Cas9 as a novel antiviral therapeutic strategy. Very recently, Kaminski et al. (2016a) have used CRISPR-Cas9 for the precise elimination of the complete genome of HIV-1 from latently infected human CD4 + T-cells. They have expressed Cas9 and specific targeting sgRNAs in HIV-1 eradicated T-cells that can be then gained protection against subsequent HIV-1 infection. Through targeting the LTR, they found significant reductions in HIV-1 replication in infected primary CD4 + T-cell cultures, and decreased viral loads in *ex vivo* cultures of CD4 + T-cells from HIV-1 infected patients. This study demonstrates that CRISPR-Cas9 could further become an effective and precise platform for addressing AIDS through the elimination of HIV-1.

Very recently, Yin et al. (2016) used CRISPR-Cas9 system that was co-transformed into HEK293T cells and lentiviral vector for delivery of gRNA. They have selected two sites and found combinations the gRNAs that targeted structural gene and LTR could effectively eliminate genome of HIV-1. In addition, Wang et al. (2016) demonstrated that somehow indel in HIV-1 genome is deleterious (some indel is not deleterious that leads to refractory) but on the other hand, it leads to the development of replication competent viruses and are resistant to Cas9/sgRNA. This is one of limiting factor to use Cas9/sgRNA for viral therapy. Choi et al. (2016) developed a lentiviral particles based CRISPR/Cas9 system to edit genome and minimizing off target effect. They have edited CCR5 in TZM-bl cells and also LTR of HIV provirus in the J-LAT model. This study reveals that it is a safer and effective approach for human gene therapy. Similarly, Kaminski et al. (2016b) have developed short version of Cas9 and used multiplex of sgRNAs for targeting the HIV LTR and *Gag* gene from in transgenic mice and rats. They injected in transgenic mice with a recombinant Adeno-associated virus 9 (rAAV9) vector, expressing saCas9 and gRNAs. They found that the cleavage of integrated HIV-1 and excision of a 978 bp DNA segment between LTR and Gag gene in spleen, lung, heart, liver, kidney and lymphocytes. This study can be further used for *in vivo* delivery and elimination of HIV and other human viruses in future.

The hepatitis viruses are distinct and highly unusual human pathogens. They have emerged as a major global public health issue. Hepatitis B virus causes hepatitis B, which is one of the major causes of liver infection (Zeng, 2014; Zhang et al., 2014). Lin et al. (2014) designed eight sgRNAs for targeting *P1* and *XCp* of HBV. In this study, they found a significant reduction in the production of core and HBsAg proteins in Huh-7 hepatocytes derived from cellular carcinoma cells. In a mouse model it was found that there had been a clearance of the HBV genome and reduction in the serum levels of HBsAg (Lin et al., 2014). Additionally, CRISPR-Cas9 has been used to target the HBsAg of HBV in cell culture and within *in vivo* systems that were confirmed by quantitative enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR). The total amount of HBsAg secreted into

the cell culture and in the mouse serum was reduced after the treatment with CRISPR-Cas9. In the same study, they have also found that no HBsAg-positive cells persisted in the liver tissue of CRISPR-Cas9-S1 + X3 treated mice, and this was confirmed by immunohistochemistry. Likewise, the CRISPR-Cas9 system efficiently generates mutations in HBV DNA and drives downstream inhibition of replication (Zhen et al., 2015). It was used to target the HBV cccDNA (covalently closed circular DNA), which is highly stable, and a prime target for the inhibition of HBV infection. This work also showed the reduction in the generation of the virus in Huh7 cells and in HepG2.2.15 HBV-replication cells (Dong et al., 2015). Similarly, Kennedy et al. (2015) have used Cas9 and HBV-specific sgRNAs. The total viral DNA load was reduced by up to 1000-fold and cccDNA was reduced by up to 10-fold, and noticeably remaining viral DNA was mutated. This study can be further expanded and explored, hopefully towards deployment as a novel therapeutic strategy, not only against chronic HBV infection, but also against the wider family of other hepatitis viruses in the future.

The CRISPR-Cas9 system has been used to eradicate another virus. It has been used for targeting the genomes of the Epstein–Barr virus (EBV) in the human cell line Raji. The Cas9-sgRNA complex binds on the genome that dramatically arrests proliferation and also decreases in viral load (Wang and Quake, 2014). Recently, Yuen et al. (2015) designed two sgRNAs and used CRISPR-Cas9 for editing of the EBV genome in human cells. The sgRNAs targeted for deleting a 558 bp region in the promoter region of BART (*Bam*HI A rightward transcript), which encodes the viral microRNAs (miRNAs). It was achieved in a number of human epithelial cell lines that were latently infected with EBV, including nasopharyngeal carcinoma C666-1 cells. This work has shown the loss of BART miRNA expression, indicating potential in the treatment of the infection by this virus (Yuen et al., 2015).

Another virus, the human papillomavirus (HPV), is a highly important and most widely prevalent sexually transmitted infection globally and one of the major risk factors for certain subtypes of cancer including especially cervical cancer (Schiffman et al., 2007). HPV is a DNA virus belonging to the papillomavirus family and is capable of causing infection in humans, whereby its viral E6 and E7 oncoproteins are necessary for malignant conversion. There is medical interest in the high-risk HPV E6 and E7 proteins that are associated with the tumor suppressor p53 and pRB (Yim and Park, 2005). The persistent infections of low-risk types of HPV (type 6 or type 11) are the major causes of genital warts. Kennedy et al. (2014) have employed CRISPR-Cas9 for the targeting of the E6 and E7 genes in both HPV18 and HPV16. They have used HeLa and SiHa cervical carcinoma cell lines and inactivated the E6 and E7 genes by indels. Recently, CRISPR-Cas9 has been also used to target and inactivate the E7 gene in both HPV type 6 and type 11 in keratinocytes. It was shown that the silencing of E7 led to the inhibition of cell proliferation and the downstream trigger of apoptosis induction in E7-transformed keratinocytes. It has the potential to lead into the development of an adjuvant therapy for the control of genital warts (Liu et al., 2016). Flaviviruses are a large group of human viruses and infect millions of people annually. There is yet no effective antiviral therapy available. More recently Zhang et al. (2016) used CRISPR/Cas9 system to perform genome wide screen to identify host genes that are associated in infection when it was edited that reduced the flavivirus infection. They have identified nine genes associated with viral infectivity. Those were associated with function of endoplasmic reticulum such as protein degradation, translocation and N-linked glycosylation. A subset of endoplasmic reticulum-associated signal peptidase complex (SPCS) proteins are essential for cleavage of the flavivirus structural proteins (prM and E) and secretion of viral particles that was tested in Japanese encephalitis, Dengue, West Nile, yellow fever, Zika and hepatitis C viruses.

To date, there are viral vectors such as lentivirus vectors (LVs), adenovirus vectors (AdVs), adeno-associated virus vectors (AAVs) and herpes simplex-1 virus vectors (HSV-1s) used for this purpose. A number of non-viral vectors including lipid nanoparticles (LNPs), liposome, polymers, conjugates, and cell-derived membrane vesicles (CMVs) have been used (Thomas et al., 2003; Yin et al., 2014b). The large size of the CRISPR/Cas system presents an obstacle for its delivery *in vivo*. It also hampers the delivery of the CRISPR/Cas system to targeted tissues in mammalian cells for genome editing therapy *in vivo*. In closing this section of successful and striking viral studies, it must be recognized that CRISPR-Cas9 can be further expanded towards the control of other important and highly pathogenic viruses. Potentially these viruses may belong to the family of those that infect humans, inflicting morbidity and mortality, and further may extend into the category of those viruses that affect other animals, and may then related towards the benefits of economic and welfare aims in a wider context including and extending beyond human health.

4. Expanding the potential of repurposing the CRISPR-Cas9 system

The complexity and dynamic transcriptional regulation of a number of genes and their pathways play a key role in a wide range of cellular activities such as genome replication, repair, cell division, and inheritance. A precise manipulation and perturbation of the expression of the desired gene by repression or activation is important to understand gene function (Dominguez et al., 2016). In order to repurpose the CRISPR-Cas9 system, two mutations were made in the RuvC (D10A) and HNH (H840A) active sites of the Cas9 endonuclease. The mutated Cas9 is also known as “dead” Cas9 or dCas9 that is catalytically inactive but retains its binding ability (Bikard et al., 2013; Qi et al., 2013). The repurposed CRISPR-Cas9 system is commonly called CRISPR interference (CRISPRi) and it was established for transcriptional interference through gene silencing, and it has since expanded further towards the activation of transcription, epigenetic modifications, and the imaging of genomic loci (Bikard et al., 2013; Chen et al., 2013; Gilbert et al., 2013; Qi et al., 2013; Anton et al., 2014; Ma et al., 2015).

4.1. Transcriptional repression via CRISPRi

The regulation of any endogenous gene within the cells remains a challenging issue and bacteria lack the machinery for RNA interference (RNAi). However, this may be alleviated by the recent development of CRISPRi that can sterically repress transcription either by blocking of transcription initiation (Fig. 3A) or blocking of transcription elongation (Fig. 3B). This is achieved by the design of sgRNA that is complementary to the promoter or exonic sequences. The sgRNA complementary to the non-template strand more strongly represses the transcription as compared to the template strand. In prokaryotes, the target gene repression was achieved up to 1000-fold, without any off-target effects. Work in this field has also generated a dual color fluorescent reporter with mRFP (monomeric red fluorescent protein) and sfGFP (superfolder green fluorescent protein) that can report and show knock-down of gene function (Qi et al., 2013). Similarly, they have also developed the CRISPRi system in eukaryotic HEK293 cells and used SV40 promoter for eGFP (enhanced green fluorescent protein) expression. They observed that eGFP knock-down was up to 46%, and this value may be further improved by the design of sgRNA for multiple locations of the gene (Qi et al., 2013).

In addition, Bikard et al. (2013) have also constructed a repression system using dCas9, and they have tested this in *E. coli*. They have found that a programmable transcription repressor could be created through the blocking of the binding of RNA polymerase (RNAP) to the promoter sequences or, in contrast, blocking the running RNAP could create a transcription termination. In this study, they observed the more than 100-fold repression of GFP-mut2 when targeting using crRNA near to the promoter elements, especially within the −35 and −10 regions, and the RBS region (Table 4). Similarly, they tested dCas9 for the repression of transcription elongation using crRNA by targeting the coding and non-coding strand of GFP-mut2. They have found a 20 to 40-fold repression with the non-coding strand whilst a 6 to 35-fold repression was observed with the coding strand.

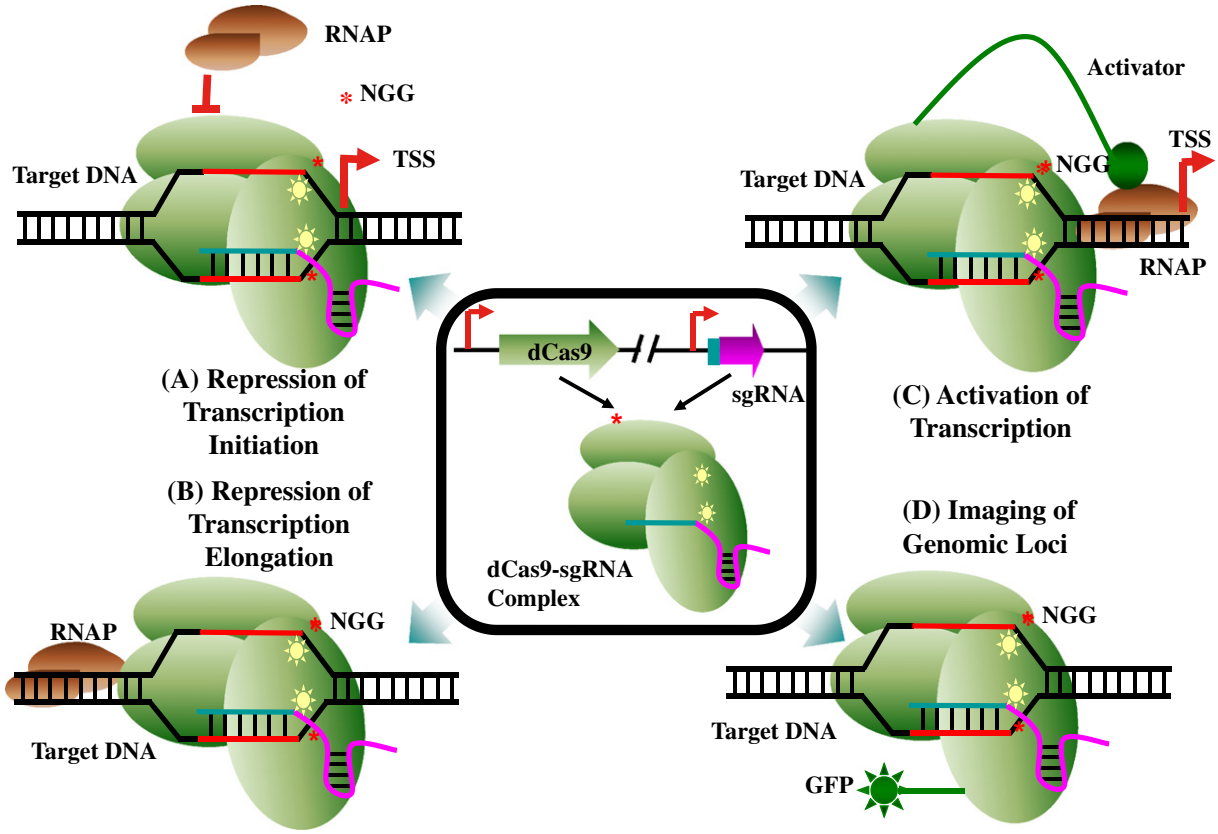

Fig. 3. Repurposing CRISPR-Cas9 system for gene repression, activation and loci imaging. The dCas9 is mutated (✲) form of Cas9 and the sgRNA are expressed and forms a dCas9-sgRNA complex. (A) The dCas9-sgRNA complex binds upon the target region and blocks RNA polymerase (RNAP) function by repressing the transcription initiation (Qi et al., 2013; Bikard et al., 2013). (B) The dCas9-sgRNA complex binds upon the target region and terminates the RNAP that represses the transcription elongation (Qi et al., 2013; Bikard et al., 2013). (C) The dCas9 is fused with an activator and forms a complex with sgRNA that binds upon the target region and activates gene expression (Bikard et al., 2013; Gilbert et al., 2013). (D) The dCas9 fused with GFP and forms a complex with sgRNA that binds on a targeted region for the imaging of genomic loci (Chen et al., 2013; Ma et al., 2015).

In order to improve the repression efficiency of CRISPRi in mammalian cells, Gilbert et al. (2013) have developed a dCas9 system by fusing with repressor domains such as the KRAB (Krüppel associated box) domain of Kox1, CS (Chromo Shadow) domain of HP1α, and the WRPW domain of Hes1 (Margolin et al., 1994; Fisher et al., 1996; Hathaway et al., 2012). They used HEK293 cells that express GFP chromosomally under the control of the SV40 promoter, and sgRNA that targets GFP. The cells express the dCas9-KRAB fusion protein and this generate 5-fold repression, higher than the values previously reported for 2-fold repression of GFP (Qi et al., 2013) whereas within the cells expressing the single dCas9, dCas9-CS or dCas9-WRPW all showed only a 2-fold repression of GFP (Table 4) (Gilbert et al., 2013). They have further improved repression, using the dCas9-KRAB system by the design of different sgRNAs. Six novel sgRNAs out of eight sgRNAs could knock-down GFP expression by up to 75% with 15-fold repression (Gilbert et al., 2013). In addition, they have used both dCas9 and dCas9-KRAB for the knock-down of the expression of human endogenous CD71 and CXCR4, finding 60–80% repression. To test the versatility of CRISPRi, dCas9 and dCas9-Mxi1 were both cloned and separately transformed them into yeast (*Saccharomyces cerevisiae*) that endogenously expresses the TEF1-GFP fusion. The sgRNA expresses and targets the endogenous TEF1 locus that is expressed under the control of the Pol III SNR52 promoter, and with dCas9 alone they have found up to 18-fold repression which was further increased to up to 53-fold with dCas9-Mxi1 (Gilbert et al., 2013). Recently, Mandegar et al. (2016) have developed a tunable dCas9 system which is fused with the KRAB for reversibly inhibiting gene function in iPSCs and iPSC-derived cardiac progenitors, T lymphocytes and cardiomyocytes. It has the potential to silence the single alleles.

Table 4
The use of the repurposing CRISPR-Cas9 (CRISPRi) system for gene repression.

CRISPRi	Targeted region/genes	Organisms/cell types	Repression	References
dCas9	Promoter or exonic sequences	*E. coli*	1000-fold	Qi et al., 2013
	eGFP	HEK293 cells	2-fold	Qi et al., 2013
	GFP-mut2 targeting near promoter & RBS	*E. coli*	100-fold	Bikard et al., 2013
	GFP-mut2, non-coding strand	*E. coli*	20 to 40-fold	Bikard et al., 2013
	GFP-mut2, coding strand	*E. coli*	6 to 35-fold	Bikard et al., 2013
	RFP, targeting σ70 promoters	*E. coli*	56 to 440-fold	Nielsen and Voigt, 2014
	TEF1-GFP	*S. cerevisiae*	18-fold	Gilbert et al., 2013
	GFP	HEK293 cells	2-fold	Gilbert et al., 2013
dCas9-Mxi1	TEF1-GFP	*S. cerevisiae*	53-fold	Gilbert et al., 2013
dCas9-CS	GFP	HEK293 cells	2-fold	Gilbert et al., 2013
dCas9-WRPW	GFP	HEK293 cells	2-fold	Gilbert et al., 2013
dCas9-KRAB	GFP	HEK293 cells	15-fold	Gilbert et al., 2013

In recent developments, epigenomic editing with CRISPR-dCas9 has become a promising technology for modulating and regulating gene expression. In the following study, the dCas9-KRAB repressor was constructed through fusion and was capable of silencing the HS2 enhancer. That could induce H3K9 trimethylation (H3K9me3) specifically at the enhancer, which leads to a downstream decrease in the chromatin accessibility of enhancer and its promoter targets (Thakore et al., 2015). A light-inducible CRISPRi has been developed whereby the dCas9 constitutively expresses and sgRNA transcription is regulated by YF1-FixJ-PFixK2 in the presence of a blue light. This enables the sensor-CRISPRi to accurately disrupt the cellular activities (Wu et al., 2014). Lawhorn et al. (2014) used only dCas9 and sgRNA to target the endogenous human *TP53* gene, which included targeting of the promoter, transcript sequence, and its flanking regions. It was observed that repression of up to 86% could be achieved, even without a repressor domain; through the targeting of the complex to sites near the *TP53* transcription start site (TSS). In this work, they further confirmed that the repression efficiency depends on the choice of sgRNA target sites.

There are a limited number of known transcription factors available and it remains difficult to build a complex circuit that takes multiple inputs to control a phenotype. To address this, a number of orthogonal sgRNAs have been designed and its cognate site was inserted into promoter sequences for repressing gene function (Nielsen and Voigt, 2014; Didovyk et al., 2016). A set of NOT gates was made by the design of five synthetic *E. coli* σ70 promoters that were repressed by the corresponding sgRNAs. These sgRNAs exhibited 56–440 fold on-target repressions. This was further connected to build NOR gate and a 3-gate circuit consisting of four layered sgRNAs. This team has further verified the utility of the circuits by connecting them with the native *E. coli* regulatory network by designing output sgRNAs to target *malT*. It could convert the output of the circuit to a switch in phenotype including those concerning sugar utilization, chemotaxis and phage resistance (Nielsen and Voigt, 2014). This approach can be expanded for the building of even more complex circuits for the better control of phenotypes. From an industrial perspective, *Corynebacterium glutamicum* is a well-established bacterium that is widely used for the production of amino acids at industrial scales. CRISPRi has been used to repress the genes using sgRNA for both *pgi* and *pck* to up to 98%, and of *pyk* to up to 97%, enhancing L-lysine and L-glutamine production. This demonstrates the use of CRISPRi as a quick and effective tool for metabolic engineering, capable of enhancing the production of desired molecules (Cleto et al., 2016).

Cyanobacteria have been widely used for a long time for the production of top value-added products. These bacteria have the potential to trap sunlight, atmospheric carbon dioxide, and utilize water and trace elements for production of a high amount of desired molecules. Recently, they have gained more attention because of the development of synthetic biology toolboxes for gene and genome modifications (Huang et al., 2010; Lindblad et al., 2012; Singh et al., 2016). Very recently, CRISPRi has been used in the cyanobacterium *Synechocystis* sp. PCC 6803 for gene repression. It has used to knock-down genes associated with the creation of the molecules polyhydroxybutyrate (PHB) and glycogen. Here they have a simultaneous knock-down of four putative aldehyde reductases and dehydrogenases with up to 50–95% repression. This work has further demonstrated that tightly repressed promoters permit for inducible and reversible CRISPRi in cyanobacteria (Yao et al., 2016).

Now considering a medical perspective, *Mycobacterium tuberculosis* (Mtb) is the causal agent of tuberculosis (TB) that has latently infected ~2 billion people worldwide (Smith, 2003). One of the approaches to control TB could arise from the identification of conserved proteins in *M. tuberculosis* and their subsequent targeting by drugs (Singh and Somvanshi, 2009; Singh and Somvanshi, 2010; Singh et al., 2011). However, MDR still remains one of the major barriers to the control of TB. Recently, Choudhary et al. (2015) have used CRISPRi for the endogenous repression of mycobacterial genes. Specifically, it was used for repressing the function of two important genes, *yidCMS*, and *mmpL3MS*, finding that after targeting of these two genes by CRISPRi, there was a reduction in expression levels by the inducer in a concentration-dependant fashion. CRISPRi can be useful for controlling of Mtb by repressing the genes that are involved in host-pathogen interactions. In summary, CRISPRi has developed a powerful technology that has the potential to strongly repress genes, and it is a technology that could be further extended into many other organisms, enabling them to become more useful for acceleration in basic and applied biological science research.

4.2. Transcriptional activation via CRISPRa

The activation of a gene by CRISPR, termed CRISPRa, uses dCas9 fused with transcriptional activators (Bikard et al., 2013; Dominguez et al., 2016). A fusion of the ω-subunit of RNA polymerase (RNAP) with dCas9 at both its C-terminal and N-terminal regions (separately) allows the assembly of the holoenzyme at a target promoter for activation of the gene of interest in *E. coli* (Fig. 3C). In this work observed 2.8-fold activation of the LacZ reporter when dCas9-ω was at the C-terminal fusion (Bikard et al., 2013). They have also designed a GFP-mut2 reporter plasmid and used the dCas9-ω fusion for testing further activational capacity. Here, they tested 10 binding sites and found 2 sites, one at 43 bases and the other at 59 bases away from the −35 region of the promoter, showing 7.2-fold and 23-fold activation (Table 5) of GFP-mut2 respectively (Bikard et al., 2013). This is still a relatively low increase in activation when compared to the levels of activation that have been previously achieved (70-fold) by a cI-ω fusion (Dove and Hochschild, 1998). The activation could be further improved by changing size of linker between fusion protein by reducing the steric hindrance or by the use of different activation domains (Bikard et al., 2013). A pressing need arises to develop more CRISPRa system for bacteria to achieve robustly and consistent gene activation.

In mammalian cells, the fusion of VP16 or of the p65 activation domain (p65AD) to dCas9 has been made in order to achieve high levels of gene activation, capable of activating both reporter genes and endogenous genes (Gilbert et al., 2013; Maeder et al., 2013; Perez-Pinera et al., 2013). The dCas9 has been fused with four copies of the activation domain of VP16 (VP64) or a single copy of p65AD. Here, the co-transfection of dCas9-VP64 or dCas9-p65AD and the sgRNA (on plasmids) that targets the Gal4 UAS was made into HEK293 cells, which expressed the Gal4 UAS-GFP reporter. Gilbert et al. (2013) observed 25-fold activation by using dCas9-VP64 and 12-fold activation by using dCas9-p65D fusion (Table 5). In addition, dCas9 was fused with the transcriptional activation domain (VP64) at the C-terminal region for activation of the human *VEGFA* endogenous gene using sgRNAs. The 16 sgRNAs were designed for targeting DNase I hypersensitive sites (HSSs) located upstream, downstream, or at the *VEGFA* gene TSS. From these sgRNAs, 15 of them were shown to have activated VEGFA protein expression when co-expressed with dCas9-VP64 in HEK293 cells (Maeder et al., 2013). In addition, they have tested for the activation of the human NTF3 gene. In order to activate gene expression, they have designed six sgRNAs that target the human NTF3 promoter and used a codon-optimized dCas9-VP64. All of the six sgRNAs were showed to significantly induce NTF3 transcript levels, with a maximum of up to 4-fold increased induction. In this study, they noticed that through decreasing the amounts of both sgRNA and dCas9-VP64 expression plasmids in transfected cells, a reduced activation of NTF3 gene occurred, demonstrating a dose-dependent effect (Maeder et al., 2013). Chavez et al. (2015) have reprogrammed Cas9 with activation domains. They used a tripartite activator VP64-p65-Rta (VPR) that was fused with the nuclease-null Cas9. It could be used for activating the endogenous coding and noncoding genes. It has also used for targeting the number of genes simultaneously and stimulating the neuronal differentiation of iPSCs.

Polstein and Gersbach (2015) have developed a light-driven system called the light activated CRISPR-Cas9 effector (LACE) system, which is capable of transcriptional induction in the presence of blue light. LACE is the product of the fusion of the light-inducible heterodimerizing

Table 5
The use of the repurposing CRISPR-Cas9 (CRISPRa) system for gene activation.

CRISPRa	Targeted region/genes	Organisms/cell types	Activation	References
dCas9-ω	GFP-mut2, target sites 43 bases near promoter	*E. coli*	7.2-fold	Bikard et al., 2013
	GFP-mut2, target sites 59 bases near promoter	*E. coli*	23-fold	Bikard et al., 2013
dCas9-VP64	GFP	HEK293 cells	25-fold	Gilbert et al., 2013
	NTF3	HEK293 cells	4-fold	Maeder et al., 2013
dCas9-p65AD	GFP	HEK293 cells	12-fold	Gilbert et al., 2013

proteins CRY2 and CIB1 with a transactivation domain with dCas9. In this form, tools like LACE have the potential to be expanded towards the dynamic regulation of endogenous genes, capitalizing on their advantages that primarily include their relative cost effectiveness and ease of use. Recently, the fusion of a nuclease-null dCas9 together with the catalytic core of human acetyltransferase p300 has been made to create a programmable CRISPR-Cas9-based acetyltransferase system. This fusion catalyzes the acetylation of histone *H3* lysine 27 at its target sites that lead to the robust transcriptional activation of target genes from promoters, as well as both the proximal and the distal enhancers (Hilton et al., 2015). In summary, CRISPR-dCas9 can be employed as a simple and versatile approach for RNA-guided gene repression and activation in a wide range of organisms and cell types. These approaches could be further expanded into more organisms for the improved robustness, physiology, and the greater understanding of gene function within a complex gene networks.

4.3. Imaging of genomic loci

In the early 1980s, fluorescence *in situ* hybridization (FISH) was developed and used for a wide range of cell biology applications. The technique employs fluorescently labeled probes for the localization of desired sequences on the chromosome (Langer-Safer et al., 1982), and has been further used in the identification of genetic disorders, genes, species identification, and for the study of cancer. FISH has been also used for studying the spatial-temporal patterns of gene expression within cells and tissues (Amann and Fuchs, 2008). However, in the field of genetics, there still remains one of its major challenges, which is for the labeling and tracing of desired sequences. To address this challenge, Chen et al. (2013) have fused dCas9 with eGFP and expressed the sgRNA for targeting of the repetitive elements in telomeres and for various coding genes to manage the live imaging of genomic loci (Fig. 3D). This technique could be useful for the study of telomere dynamics during the elongation and disruption phases. Additionally, this team also studied the subnuclear localization of the MUC4 loci and the cohesion of replicated MUC4 loci on sister chromatids, and their associated dynamics. This study provides a new insight for the study of chromosomes conformational changes and dynamics in living cells. Similarly, Anton et al. (2014) have also fused dCas9 with eGFP (dCas9-eGFP) and co-expressed sgRNAs to target the pericentric, centric, and telomeric repeats in the genome. They found a chromocenter (CC) pattern using major satellite specific sgRNAs. When sgRNAs targeted the minor satellites and telomeres they obtained smaller foci coinciding with the centromere protein B and the telomeric repeat-binding factor 2. The target specific labeling by gRNA/dCas9-eGFP complexes has since been directly visualized with 3D-FISH. Tanenbaum et al. (2014) have developed a protein scaffold to amplify the signal for imaging which is repeating peptide array termed as SunTag that recruits a multiple copy of antibody fusion protein. It has used for recruiting up to 24 copies of GFP for live imaging (termed as SunTag). It was also used as synthetic transcription factor by recruiting multiple copies of a transcriptional activation domain to a nuclease-deficient CRISPR/Cas9. It has been used for robust activation of endogenous gene expression and reengineered cell behavior. SunTag is a versatile platform for multimerization of protein on target protein scaffold and also shows a number of applications in imaging.

A programmable and labeled dCas9 can be further used for the study of functional nuclear architecture. Recently, Ma et al. (2015) have designed a multicolor version of CRISPR using dCas9 from three bacterial orthologs, with separate pairs of dCas9-fluorescent protein fusions and cognate sgRNAs for the efficient labeling of several target loci in live human cells. The colored dCas9-sgRNAs were used for determining the intranuclear distance between the loci. They have additionally determined the fluorescence spatial resolution in the same chromosome between the two genetic loci. This could be related to the linear distance between the loci on a physical map of the chromosome that permits the measurement of DNA compaction in regions of interest within living cells. These studies could be further expanded towards the design of different sgRNAs that can target the conserved and other significant regions of the genome. This could be useful for improving the spatiotemporal dynamics understanding, a question of growing importance and interest in the biological sciences.

5. Ethical concerns and safety issues

Following their launch into the public sphere, all transformative biological/genetic technologies have tended to capture a wide attention, some of which may be optimistic and positive, and some more cautious and even concerned. In many decades, we are benefitted by molecular technologies; it is now true that the use and refinement of CRISPR-Cas9 as a technique has opened doors that we previously have not had. The ever more rapid development and easy-of-use of technologies such as CRISPR-Cas9 have left us with an equally rapid development of ethical concerns and the moral questions that we and the field of science must consider. In general, one must consider that the transparency and the open nature of the public science world is one that is thankfully rare to transgress ethical and moral borders. This can be assessed at least partially by the success of experiments and their subsequent publication, and in this regards it is often recalled that the focus of ethics upon CRISPR-Cas9 was in fact highlighted largely by provocative publications.

For instance, genetically modified organisms (GMOs) have raised issues where genes from other organisms are inserted into the desired organism. A number of organisms have been genetically modified such as microbes, plants, grasshoppers, and mosquitoes, as well as others. There are a number of growing challenges that can be passed on ethical and regulatory issues. A primary example has advanced in the most ethically concerning areas of genetic engineering, that of the human germline (Lanphier et al., 2015). This lies in the use of CRISPR-Cas9 for the manipulation of the human genome, specifically in the germline towards generating heritable modifications. The use of the CRISPR-Cas9 technology for human embryo editing remains a risky proposition. It is currently in a premature stage of development where researchers are not fully aware to control the risk and health issues. It has been shown that, at this time, it may be impossible to know the genetic effect upon an embryo with precision until after the birth (Lanphier et al., 2015).

P. Liang et al. (2015) have tried to cure sickle cell anemia by correcting gene mutation of endogenous β-globin gene (HBB) at zygote (3PZ) level and human cells. It was also suggested that the efficiency and specificity of CRISPR-Cas9 should be enhanced for clinical applications. Recently, a group from China had used CRISPR-Cas9 for the eradication of the human β-globulin (HBB) gene from the germline of

human embryos. There are many issues arising that may prevent research at even premature stages (Cyranoski and Reardon, 2015). Dr. Francis Collins, the director of the US National Institute of Health (NIH, USA), has recently issued a statement that banned NIH-funded research into the genomic editing of human embryos (Collins, 2015). The government of China has not banned any particular type of research; whereas, governments of Italy and Germany have completely banned research on human embryos of this nature. Due to ethical and regulatory issues raised by the CRISPR-Cas9 genome editing system, there is a pressing need to encourage the scientific community, NGOs, and other stakeholders to debate further and drive discussion; and to encourage improvement into these systems for the future use of this technology (Otieno, 2015). With some reflection, it does not take a particularly adventurous nor scientific imagination to see the enormous potential of these experiments and research, as well as the great risks inherent.

To progress with confidence into the future there will be a level of public engagement, reflected in national and international policy. This will benefit from objective information that all levels of scientific knowledge can understand, and a forum-like structure will exist to allow input and the feedback from all relevant parties. To this end, recently a series of coordinated meetings titled the 'International Summit on Human Gene Editing' were held. Resulting from this summit has been drawn a number of conclusions, ones that form a solid ethical basis for the following steps into the future of genome editing with CRISPR-Cas9. There are at least three levels of concern for ethical responsibility from the use of CRISPR-Cas9 in human cells, specifically somatic cell modification, basic and preclinical use, and the heritable germline cell modification. Firstly, somatic cells (those which do not transmit hereditary information into the next generation) could be modified for the potentially great benefit. The ethical concerns are much lower, because therapies based upon somatic modification will only affect the direct patient, and thus ethical concerns are then limited simply to the safe and targeted deployment of such therapies. Secondly, with the preclinical (and basic) work, it is clear to see where a value may be obtained, but all the work must abide by the ethical guidelines and laws, and modified cells must never be capable of generating an embryo. The third of ethical responsibility concern the directed modification of the human germline, which as a scientific field is considered irresponsible and generally contravenes agreed and accepted legal as well as ethical rules. This remains true unless the related efficacy and safety concerns have been justified and resolved, that no better alternative exists, and it remains the appropriate course of action. Given the need for a protective and careful attitude to maintain our ethical standard, this is a field that needs constant update and review, to ensure it maintains a reasonable course of progression that is mindful and respectful of ethical concerns.

6. Conclusions and perspectives

The original functionality of CRISPR-Cas9 has risen from a bacterial defense mechanism against phage and plasmids to a powerful technique in the genome editing toolkit, capable of sequence-specific precise modification. Since its induction as a genome editing technique in the biological research field, it has been successfully applied to numerous model organisms, as well as organisms of medical, agricultural and industrial importance. This review has discussed applications specifically in human and mammalian cells, those of *Drosophila* and zebrafish, a range of bacterial microbes, and towards the control of viral infections such as HIV, HBV and HCV. Recently, an interesting and timely development in CRISPR-Cas9 technology has emerged that concerns the pressing topic of antibiotic resistance, leading from the use of a programmable CRISPR-Cas9 system that may specifically target pathogenic strains in among a wider flora. A technology like this could benefit enormously the ever-growing and prominent issue of MDR pathogens.

With many published successes, the technology behind CRISPR-Cas9 shows a great potential for genome editing. However, there is a pressing need to improve its efficiency and assist its expansion into more organisms. Cas9 should be studied efficiently where it could be possibly coupled with the λ-Red system to induce increased recombination efficiency (Pyne et al., 2015; Ronda et al., 2016). Toxicity is also a major concern that may be addressed by lowering the expression of Cas9 (Jiang et al., 2014; X. Liang et al., 2015) and of dCas9 (Nielsen and Voigt, 2014; Hara et al., 2015) although it is unclear whether it can still provide full potency towards genome editing and regulation at lower expression levels. CRISPR-Cas9 requires a 2–5 nt PAM sequence which is located downstream of the target sequence (Jinek et al., 2012). Hsu et al. (2013) reported upon an NAG PAM, which had only 20% efficiency of the NGG PAM for guiding DNA cleavage, demonstrating the importance of this specificity, also something that could be taken into consideration when developing the technology further.

Orthogonal Cas9 could be used independently with native Cas9 towards increasing the application of multiplex genome editing simultaneously (Briner et al., 2014; Zalatan et al., 2015). Esvelt et al. (2013) have characterized a set of fully orthogonal Cas9 that could edit and regulate gene function bacteria human cells. It was derived from *N. meningitidis* therefore we can use it for simultaneous genome editing and regulation. Horlbeck et al. (2016) have identified the issue of selecting sgRNA that can efficiently mediate the cas9 activity. In order to fill this gap, they analyzed the large scale genetic screens in human cells line using either cas9 or dCas9 nuclease. They found that highly active sgRNAs for Cas9 and dCas9 which is almost exclusively in the location of low nucleosome occupancy. Recently, a smaller Cas9 orthologue (25% small size) from *S. aureus* has been shown to have similar editing capabilities to the *S. pyogenes* Cas9 (Ran et al., 2015). The delivery of CRISPR-Cas9 system by microinjection into zygotes was earlier demonstrated however, in this study, they found that Cas9 was more efficient at homology-directed repair as compared to mRNA and TALENs (Ménoret et al., 2015). From the metabolic engineering point of view, CRISPR-Cas9 could be directly used in the native host to modify the genome and to regulate competitive pathways that can improve the flux towards increasing the production of desired chemicals and building blocks. It can be also used for the exchange of native promoters, RBSs, and TFs by well-characterized synthetic forms of them towards the improvement of the yield of targeted molecules.

A future innovation that would benefit the CRISPR-Cas9 technique is the perfection of editing efficiency through decreasing off-target effects. This is one topic of the technique that requires more research, and another topic would be about the innate differences found for nuclease cleavage between different sequences. These issues are a primary source of unpredictability and disadvantage for the technique, which otherwise by all other accounts remains an excellent tool and one of the best that we currently have for genome engineering of this kind. The related side-technology, CRISPRi, has intriguing potential in the context of fine-tuning repression and activation. It acts without altering the sequence of DNA, giving stable or transient control over gene expression. The primary disadvantage of this system is that it may repress downstream genes within an operon instead of an isolated targeted gene (Dominguez et al., 2016). Furthermore, dCas9 can be employed to enhance our understanding of genetic regulation, being capable of loci imaging which can help interpret chromosomal dynamics and organization, both of which greatly impacts upon the functioning of the genome.

The power of CRISPR-Cas9 technology to permanently correct genetic mutations leads to an enormous potential in the therapeutic development for humans. As it remains our duty as responsible scientists, we must consider too the principal ethical concerns facing the technology, and we have summarized what we have considered the best routes by which the scientific community may navigate forwards, striving for the best in innovation whilst being mindful of our responsibility towards ethical and safety concerns. Currently, bone marrow transplants

are the most widely used stem-cell therapy, but some therapies derived from umbilical cord blood are also in use. Successful clinical translations depend on upon suitable and efficacious delivery systems to target specific disease. To undertake this issue, to produce a high level of therapeutic efficacy, and to improve the homologous recombination efficiency will all be among the future challenges that should be addressed and significantly improved. Research is underway to develop various sources for stem cells that can apply further for treatment of a wide range of diseases including Parkinson's disease and Alzheimer's disease, diabetes, heart diseases, cancers, rheumatoid arthritis, osteoarthritis, stroke, traumatic brain injury, congenital learning disability, and the repair of hearing and of corneal damage.

The current acceptance of CRISPR-Cas9 technology, in general, has lead to a wide number of research facilities using it, employing it directly as well as adapting it to their needs and developing it beyond its current scope. It remains too then an interesting proposition that development momentum may well increase as a next-generation genome editing toolkit comes to fruition. Its potential for the creation of novel therapeutic approaches for human diseases offers an inspired future for genome engineering, one that will likely need to balance its curiosity and creativity with respect and care. In summary, CRISPR-Cas9 is a key technology for targeted genome editing in a wide range of organisms and cell types. It is a simple, cost-effective and efficient, and has the potential to be further expanded towards even greater biomedical, therapeutic, industrial and biotechnological applications.

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