

## **Characterization and applications of Type I CRISPR-Cas systems**

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## **Abstract**

CRISPR-Cas constitutes the adaptive immune system of bacteria and archaea. This RNA-mediated sequence-specific recognition and targeting machinery has been used broadly for diverse applications in a wide range of organisms across the tree of life. The compact class 2 systems, that hinge on a single Cas effector nuclease have been harnessed for genome editing, transcriptional regulation, detection, imaging and other applications, in different research areas. However, most of the CRISPR-Cas systems belong to class 1, and the molecular machinery of the most widespread and diverse Type I systems afford tremendous opportunities for a broad range of applications. These highly abundant systems rely on a multi-protein effector complex, the CRISPR associated complex for antiviral defense (Cascade), which drives DNA targeting and cleavage. The complexity of these systems has somewhat hindered their widespread usage, but the pool of thousands of diverse Type I CRISPR-Cas systems opens new avenues for CRISPR-based applications in bacteria, archaea and eukaryotes. Here, we describe the features and mechanism of action of Type I CRISPR-Cas systems, illustrate how endogenous systems can be reprogrammed to target the host genome and perform genome editing and transcriptional regulation by co-delivering a minimal CRISPR array together with a repair template. Moreover, we discuss how these systems can also be used in eukaryotes. This review provides a framework for expanding the CRISPR toolbox, and repurposing the most abundant CRISPR-Cas systems for a wide range of applications.

## Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated proteins (Cas) provide adaptive immunity in bacteria and archaea against invasive genetic elements, such as viruses (bacteriophages) and plasmids (1-3). CRISPR-Cas systems are DNA-encoded, and provide CRISPR RNA-mediated (crRNA) targeting of nucleic acids (4) (Figure 1). Two major classes, six types and forty-four subtypes CRISPR-Cas systems subtypes have been described (5, 6). The different CRISPR-Cas types and subtypes all carry out DNA-encoded, RNA-mediated nucleic acid targeting, but they vary in their biochemical mechanisms of actions, the nature of the nucleic acid target (DNA or RNA), crRNA sequences and structures, and protospacer adjacent motif (PAM) requirements (7, 8). Class 1 systems are defined by the presence of a multi- protein effector complex that constitute the CRISPR-associated complex for antiviral defense (Cascade). Class 1 systems encompass Types I, III and IV, with Cas3, Cas10 and DinG as signature nucleases, respectively. In contrast, Class 2 systems rely on a single effector nuclease, namely Cas9 (Type II), Cas12 and Cas14 (Type V) and Cas13 (Type VI). In nature, CRISPR-Cas systems are widely distributed, as they occur in approximately 46% of bacterial genomes and 90% of archaeal chromosomes, although their occurrence does not correlate within ecological niches nor phylogenetic clades (9). Interestingly, Cas3, Cas10 and Cas12 occur in both bacteria and archaea, whereas Cas9 and Cas13 occur uniquely in bacteria, and Cas14 is uniquely present in archaea. Moreover, Cas3, Cas9 and Cas10 are the most predominant in nature and can be easily identified in human commensal and bacterial pathogens (9). Also, Cas12 (*Lachnospiraceae*) and Cas13 (*Prevotella*, *Allistipes*, *Leptotrichia* or *Fusobacterium*) have been identified in human and animal microbiome data, whereas the archaeal Cas14 was identified in water samples from different origins (10). Altogether, various

CRISPR-Cas systems can be identified from numerous microbiomes with variability in terms of occurrence, distribution and complexity.

The most popular and studied CRISPR-Cas systems primarily consist of Cas9-related Type II systems, which have been heavily used for genome editing in a wide range of organisms relevant for medicine, agriculture and biotechnology (11, 12). More recently, Type V and Type VI systems have been also repurposed for genetic engineering applications also encompassing RNA editing and diagnostics (13). Although these systems are less abundant in nature, their simplicity and convenience due to their reliance on a single effector nuclease provide portability for heterologous applications in various organisms. However, Type I CRISPR-Cas systems are the most abundant in nature. Yet only a handful of model systems have been characterized, with most of the efforts focused on the model Type I-E from *Escherichia coli* (3, 14-17). Elucidating the commonalities and differences among Type I systems and understanding their native components will enable the reprogramming of these prokaryotic immune systems for a wide range of applications. Moreover, the endogenous Type I CRISPR-Cas systems that are naturally present in bacteria and archaeal chromosomes can theoretically be repurposed with the need to “only” deliver a synthetic minimal CRISPR array. This would enable the re-directing of the endogenous Cascade-Cas3 complex towards the genome, and repurposing for targeting killing, genome editing or transcriptional control. This would open new avenues to alter the genome, transcriptome or microbiome of industrial environments and workhorses (18). Here, we described the main features of Type I CRISPR-Cas systems, and provide a framework for the development of Cascade-based technologies and applications for genome editing and beyond, in bacteria, archaea and eukaryotes.

## **Biology of CRISPR-Cas systems**

CRISPR-Cas systems represent a sequence-specific adaptive immune system in bacteria and archaea, which provides resistance against invasive nucleic acids (1). The CRISPR-Cas locus encompasses a set of *cas* genes and the CRISPR array, which encompasses conserved repeat sequences separated by spacers. CRISPR spacers are hypervariable sequences that constitute the immunization record of the bacteria, when DNA sequences from invasive genetic elements such as phages and plasmids are copied and pasted iteratively into the CRISPR array. Among the Cas proteins, two main modules can be distinguished: a set of genes involved in adaptation and another responsible for targeting. The adaptation module, primarily constituted by Cas1 and Cas2 is highly conserved across CRISPR-Cas systems (Figure 1), regardless of the Class, type and subtype, and is responsible for the acquisition of new spacer during the infection event and the immunization process. On the other hand, the effector module is highly variable between CRISPR-Cas types and subtypes, and encompasses the signature and effector Cas protein(s). This effector module is involved in nucleic acid targeting and cleavage that is carried out during the interference stage, after RNA-guided recognition and targeting of nucleic acids complementary to the crRNA sequence (16) (Figure 1). Different CRISPR-Cas systems present various effector module and signature nucleases. Accordingly, the mechanism of DNA (or RNA) cleavage varies, with differences encompassing the targeted DNA strand, and the nature of the cleavage, both in terms of process (endo- vs. exo-nucleolytic) and outcome (ss vs. ds processing). Moreover, the effector module is involved in the maturation of the CRISPR RNA (crRNA), which requires processing of the precursor CRISPR RNA (pre-crRNA) into a mature crRNA which guides the effector complex.

During the acquisition step, a DNA sequence is copied from the invasive nucleic acid and incorporated as a new spacer into the CRISPR array, providing vaccination against the matching predator (Figure 1). Expression of the CRISPR array, the second step, leads to the genesis of small crRNAs that contain a portion of the repeat-spacer pair, and acts as sequence-specific guide RNA for the Cas effector machinery to specifically detect and target complementary invasive nucleic acids. Finally, during the interference step, the homology between the spacer portion of the crRNA and the complementary protospacer located within the invasive nucleic acid (16), drives the Cas effector machinery to specifically recognize, bind, and cleave complementary sequences (16, 17), if they are flanked by a protospacer adjacent motif (PAM) (19-21). Then, the interference hinges on sequence-specific recognition and cleavage of the target nucleic acid leading to the invader's DNA degradation and subsequently genome eradication.

### **Type I CRISPR-Cas systems peculiarities**

Among CRISPR-Cas systems, the Type I systems are defined by the presence of the signature nuclease Cas3, and are the most abundant and diverse CRISPR-Cas systems in nature (9). Type I systems display complex architectures that include the conserved adaptation module (Cas1 and Cas2), and the effector module encompassing the signature nuclease Cas3 and a multi-protein effector complex that constitute the Cascade. The Cascade proteins of Type I systems typically encompass Cas5, Cas6, Cas7, and Cas8 (Cse1), with some subtypes also presenting Cas4 and / or Cas11(Cse2) (5). The different genetic organization of the *cas* genes within Type I systems is related with the CRISPR subtype, which is also correlated with the repeat sequence and length (28 – 30 nt), leading to seven defined distinct subtypes: I-A, I-B, I-C, I-U, I-D, I-E,

and I-F (5, 9). Noteworthy, subtype I-F are the most distinct ones with Cas2 and Cas3 fused into one subunit that is involved in spacer acquisition and also in DNA degradation (22). Subtype I-F represents the minimal Type I system with significantly smaller size and Cascade architecture (23, 24).

In type I CRISPR-Cas systems, after the CRISPR array is expressed as a premature CRISPR RNA (pre-crRNA), the 3'-end of one repeat is processed by Cas6 (Cas5 in subtype I-C) to generate a mature crRNA with a 7-8 nucleotides handle at the 5'-end of the flanking CRISPR repeat (25-27). This handle is followed by the CRISPR spacer sequence that defines the target of CRISPR-Cas immunity, and a processed repeat that generates a hairpin structure, representing the guide RNA of Type I systems. Often times, the palindromic nature of CRISPR repeats leads to the formation of secondary structures, and the crRNA is processed at the 3' base of the hairpin. The crRNA is loaded into the Cascade where Cas5 interacts with the 5' handle of the crRNA and the crRNA hairpin backbone interacts with various Cas7 subunits throughout the length of the guide (Figure 1B). Noteworthy, the number of Cas7 subunits is correlated with the spacer length to properly accommodate the crRNA (28, 29).

The Cascade complex, crRNA and Cas proteins, altogether constitute a double-stranded DNA recognition and targeting machinery that drives the sequence-specific cleavage of complementary DNA, based on nucleotide base-pairing between the spacer sequence of the crRNA and the complementary strand of the target DNA. The interaction between the crRNA: Cascade and the targeted DNA region loops out the non-targeted strand by generating an R-loop structure (30-33). Cascade binding to DNA is a prerequisite for subsequent Cas3 recruitment. Then, the signature Cas3 unwind and degrades the non-target DNA strand in a 3' to 5' direction through ATP-dependent helicase and nuclease activities, after PAM recognition (34-

36) (Figure 1C). This processive single stranded DNA degradation typically causes cell death, especially in the absence of proper DNA repair mechanisms that help to overcome Cas3 damage (37). Indeed, there is a relative paucity of DNA repair pathways in bacteria and archaea compared to complex eukaryotes, which render DNA targeting more lethal, and thus useful when used for genome editing, screening, or as antimicrobials.

The PAM sequence represents a second step of specificity that reinforces the sequence-based recognition of the target DNA encoded within the crRNA portion derived from the CRISPR spacer (19-21). Different Cas nucleases recognize different PAM sequences, even within CRISPR subtypes. Noteworthy, the same CRISPR subtype in different organisms display preferences for different PAM sequences. For instance, the Type I-E Cascade-Cas3 from *E. coli* recognizes a 5'-ATG-3' PAM sequence (36, 37), whereas the *Lactobacillus crispatus* Cascade-Cas3\_I-E recognizes 5'-AAA-3' (38) (as displayed in Figure 1) and the *Bifidobacterium longum* Cascade-Cas3\_I-E PAM was predicted to be 5'-AAG-3' (39). Thus, the different PAM sequences that are recognized by orthologous CRISPR-Cas systems provide the opportunity to select a particular system depending on the specific nucleotide sequence to be targeted, with various G-C content, or specific nucleotide enrichment and relative density in target regions of interest. Noteworthy, Type I systems have shown promiscuous PAM recognition, meaning that a particular Cas3 from a given organism is able to recognize different PAM sequences with similar efficiencies in DNA cleavage, as shown for the different validated PAM sequences of EcoCas3 (5'- ATG, AAG, AGG, GAG-3') (37, 40). Moreover, the nucleotide base pairing between the seed sequence, the first eight nucleotides of the spacer contained in the crRNA, and the protospacer region are essential for target recognition, with mismatches allowed at position 6, and incrementally throughout the rest of the spacer sequence (41).

## **Genome targeting applications**

The current CRISPR-Cas tool box is mainly based on the use of single effector nuclease like Cas9 or Cas12 for DNA targeting, and Cas13 for RNA applications. Portable heterologous tools have been developed to use CRISPR-Cas technologies in a wide range of organisms, from bacteria to mammalian cells (42, 43). With thousands of naturally occurring CRISPR-Cas systems, there are virtually endless opportunities to repurpose a wide variety of other systems, with Type I being the most abundant in nature. However, Type I CRISPR-Cas systems have been used sparsely to perform genome editing or transcriptional regulation applications, despite their relative abundance.

The endogenous CRISPR-Cas system present in the chromosome of archaea and bacteria can be harnessed to perform chromosomal targeting by just delivering a crRNA, without the need of complex plasmids constructs that include the Cas protein(s). Delivering a crRNA inside of the cell reprograms the endogenous system to conveniently and programmably target the desired chromosomal or plasmid sequence of interest. This enables the targeting of chromosomal sequences for three distinct outcomes and applications (Figure 2): the lethal destruction of chromosomal DNA to generate sequence-specific antimicrobials; the co-delivery of targeting RNA with repair templates to generate genome editing; the blocking of RNA polymerase by the Cascade complex to drive transcriptional repression.

Chromosomal targeting by CRISPR-Cas3 complexes typically generates extensive DNA damage and leads to cell death (Figure 2A). This enables scientists to use Type-I CRISPR-Cas systems as antimicrobials for selective eradication of pathogenic bacteria from different microbiomes. Indeed, the ability to repurpose the endogenous Type I-E CRISPR-Cas system to

induce cell suicide has been used as strategy to develop CRISPR-based next generation antimicrobials with outstanding efficiency and specificity in *E. coli*, *Salmonella* and *Streptococcus* (44, 45) showing great potential to mimic this in other human pathogenic bacteria such as *Clostridium difficile*, *Pseudomonas aeruginosa* responsible for infection disease. that also carry endogenous CRISPR-Cas system and are responsible for the rise and spread of the multidrug resistant infections that represent a threat to human health.

In order to escape lethal DNA degradation, a designed donor DNA template can be provided and co-delivered with the targeting machinery to escape cleavage and replace the wild type genotypes with the designed template, in which mutations, insertions or deletions can be engineered. Indeed, providing a repair template enables the cell to overcome Cas3 lethality, as the DNA repair mechanisms are able to use the provided donor DNA as a template for homologous directed repair (HDR) of the degraded strand. Alternatively, the template could provide a basis for homologous recombination in a small proportion of the microbial population, enabling the screening of recombinant bacteria when Casade-Cas3 targeting is used to eliminate the wild type genotype. Either way, this strategy can be used to perform a wide range of genome editing applications (Figure 2B), based on the nature of the engineered template. The endogenous Type I systems have been previously repurposed for genome editing applications in archaea (46, 47) and in industrially-relevant bacteria such as *Clostridium tyrobutyricum* and *Clostridium pasteurianum* (48, 49). Moreover, the endogenous Type I-E of *E. coli* was used to perform phage engineering of the well-known T7 phage, during the infection stage on the host (50). Recently, our group has shown that plasmid-based co-delivery of a crRNA together with a design repair template enables genetic engineering in the genetically recalcitrant probiotic *L. crispatus*, for various editing outcomes, like insertion, deletion and single base substitution for

precise mutations (38). This approach opens new avenues for the harnessing of endogenous CRISPR-Cas systems to engineer next generation probiotics and industrial workhorses that were genetically inaccessible before CRISPR (12).

Type I systems have also been used also for transcriptional regulation, using the Cascade complex DNA binding capability, without Cas3 cleavage. This enables the targeting crRNA: Cascade complex to bind to DNA sequences of interest, such as promoter regions, and sterically block and prevent the progress of RNA polymerase, reducing or completely blocking transcription (Figure 2C). In order to achieve this, *cas3* needs to be deleted or inactivated first, and then, a crRNA can be delivered to direct the endogenous Cascade machinery towards the region of interest. Downregulation of gene expression using the endogenous Cascade has been shown with the type I-E system of *E. coli*, with various efficiencies targeting the promoter region, the start codon or other positions within the gene (51, 52). A similar approach has also been shown using type I-B CRISPR-Cas systems in archaea (53). CRISPR-based transcriptional inhibition or interference, CRISPRi, provides the ability to alter gene function and gene regulatory networks and thus investigate metabolic pathways without altering DNA (54). This application allows to unravel gene functionality generating fundamental knowledge and also provides potential therapeutic applications changing gene expression, without altering the cell genomic content.

The relatively low usage of endogenous CRISPR-Cas systems in general, and perhaps Cascade-Cas3 in particular, is presumably due to the lack of fundamental understanding and characterization of diverse CRISPR-Cas systems. A detailed approach on how to perform this characterization was previously described by our group (18). Repurposing endogenous systems open new avenues for functional enhancement of bacterial strains and communities with

potential applications in human health and also for industrial workhorses. Moreover, it provides an opportunity to perform large genome-wide screening for bacterial genetic content interrogation to understand metabolic pathways and essential genes, as well as other genetic features of functional interest.

Plasmid-based heterologous expression of Type I systems has recently been used in eukaryotic cells, mainly for transcriptional regulation purposes. First, the various Cas proteins constituting Cascade need to be expressed from a plasmid vector, together with the designed crRNA or guide RNA to target and bind DNA in the host cell. This approach is challenging as the genes encoding Cascade typically represent 4.8 - 5 kb, and codon optimization and promoter adaptation needs to be performed for each specific host. Moreover, promoter optimization represents a challenging step in order to express each Cascade protein in the corresponding relative amounts and maintain the stoichiometry and architecture of Cascade, presenting at the same time an opportunity for enhancement. The Type I-E Cascade of *E. coli* and Type I-B Cascade of *Listeria monocytogenes* have been repurposed for transcriptional regulation in human cells (55). Moreover, the authors were not only able to downregulate gene expression, but also to perform transcriptional activation when an activator was fused to Cascade. This approach was also recently used to repurpose the Type I CRISPR-Cas system from *Streptococcus thermophilus* in plants, showing how broadly applicable this approach is (56). Presumably, the modular and complex nature of Cascade also affords more opportunities to append effector proteins to a larger complex, such as transcriptional activators, transcriptional repressors, base editors, acetylases and methylases and various DNA binding and altering motifs, active sites and proteins. Moreover, heterologous expression of Type I-E systems has recently shown the ability to perform long-range chromosomal deletions in human cells when Cas3 is also co-delivered with

Cascade (57). Using this approach, the authors were able to generate ribonucleoproteins (RNPs) to directly deliver the Cas3 and the Cascade proteins of *Thermobifida fusca*, instead of using a plasmid-based expression system.

Altogether, these proof of concept studies illustrate how Type I CRISPR-Cas systems can provide a suitable path to expand genome engineering tools that can be applied to a wide range of organisms, encompassing bacteria, archaea and eukaryotes. Moreover, the expansion of the CRISPR toolbox increases our ability to flexibly target different sequences with various PAMs, and generate combinations of different effector Cas proteins and complexes for concurrent alteration of the genome, epigenome and transcriptome. This approach opens new avenues to perform genome editing and transcriptional regulation beyond the use of Class 2 systems.

## Perspectives

- **Importance of the field:** CRISPR-Cas technologies have revolutionized biology with limitless opportunities to alter the genome and transcriptome in virtually any organism of interest to medicine, biotechnology and agriculture.
- **Current thinking:** Relatively few CRISPR-Cas systems have been repurposed to date, and various types and subtypes of Cas effectors can be harnessed to expand the CRISPR genome editing toolbox.
- **Future directions:** Developing next-generation CRISPR-Cas tools creates opportunities to enhance organisms of interest with unprecedented flexibility and convenience.

## Figure legends

**Figure 1.** CRISPR-Cas Type I-E system mechanism of action. **(A)** The acquisition of a new spacer represents the vaccination of the bacteria against invasive nucleic acids. **(B)** The CRISPR array is expressed as a premature CRISPR RNA (pre-crRNA) that then is processed by Cas6 cutting the last 7 nucleotides (*L. crispatus* model) of the 3'-end that will constitute the 5'-handle of the next crRNA. **(C)** The crRNA interacts with the Cascade multi-effector protein to bind double stranded invasive DNA, based on crRNA-mediated recognition, and Cas3 is recruited to degrade one strand on 3'to 5' direction, after PAM recognition.

**Figure 2.** Repurposing endogenous CRISPR-Cas system for various applications. The CRISPR-Cas systems present in the bacterial (or archaea) chromosome can be harnessed by delivering a minimum crRNA. **(A)** Harnessing the endogenous system against a particular genetic feature in the genome lead to cell death due to Cas3 cleavage. **(B)** Co-delivering a designed repair template assists the cell to repair the DNA damage based on the provided donor DNA, introducing the desire genome editing outcome. **(C)** Harnessing the endogenous system in the absence of Cas3 (previously deleted) allows to perform DNA binding (but not cleavage) leading to downregulate gene expression.

## Conflict of interest

Rodolphe Barrangou and Claudio Hidalgo-Cantabrana are co-inventors on several patents related to CRISPR-Cas systems and their uses. RB is a shareholder of Caribou Biosciences, Intellia Therapeutics, Locus Biosciences and Inari Ag, and a co-founder of Intellia Therapeutics,

Locus Biosciences and TreeCo. Claudio Hidalgo Cantabrana is an advisor and co-founder of Microviable Therapeutics.

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## Author contribution

CHC and RB participated in the manuscript writing. Both authors approved the final manuscript.

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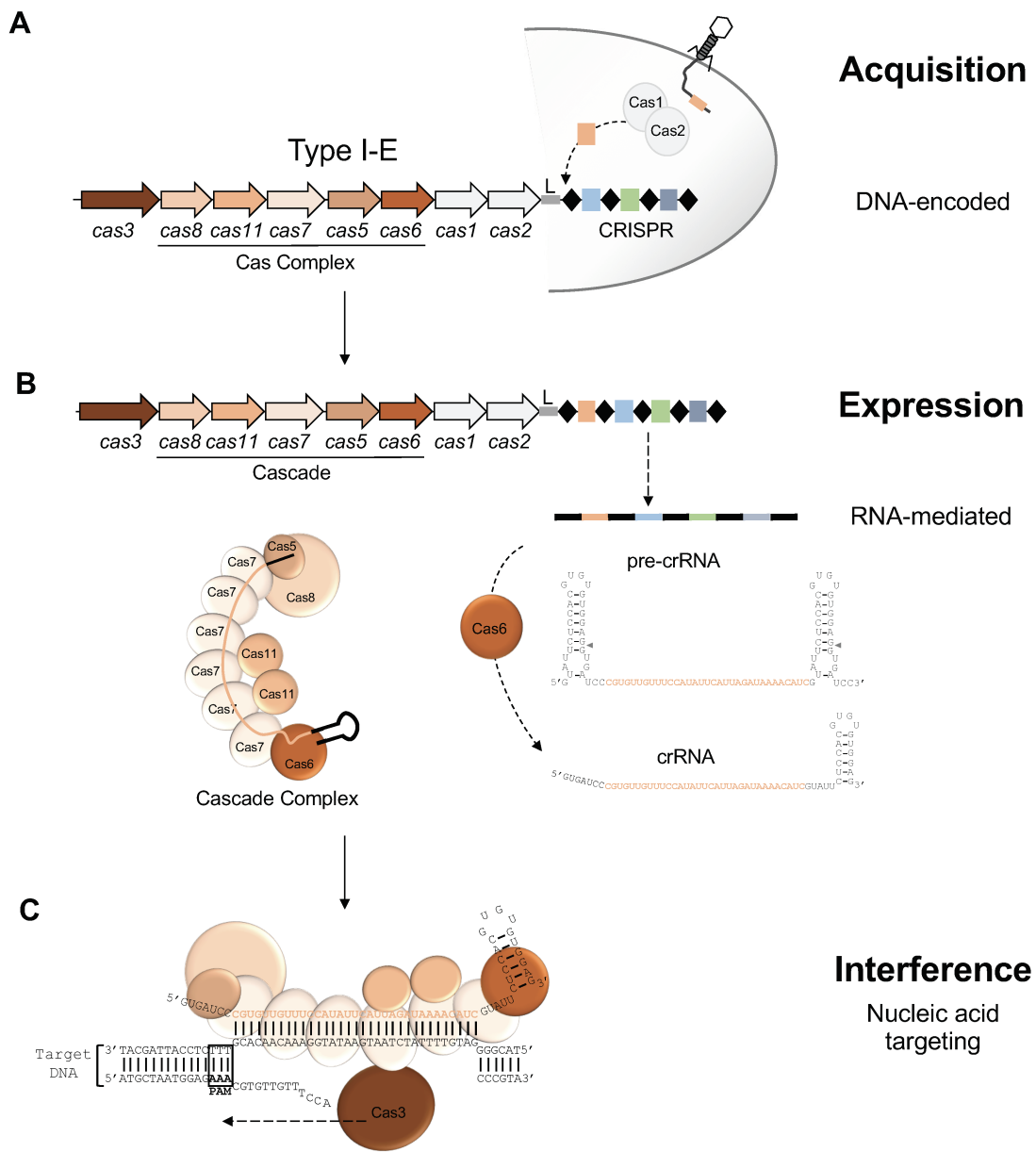


Figure 1

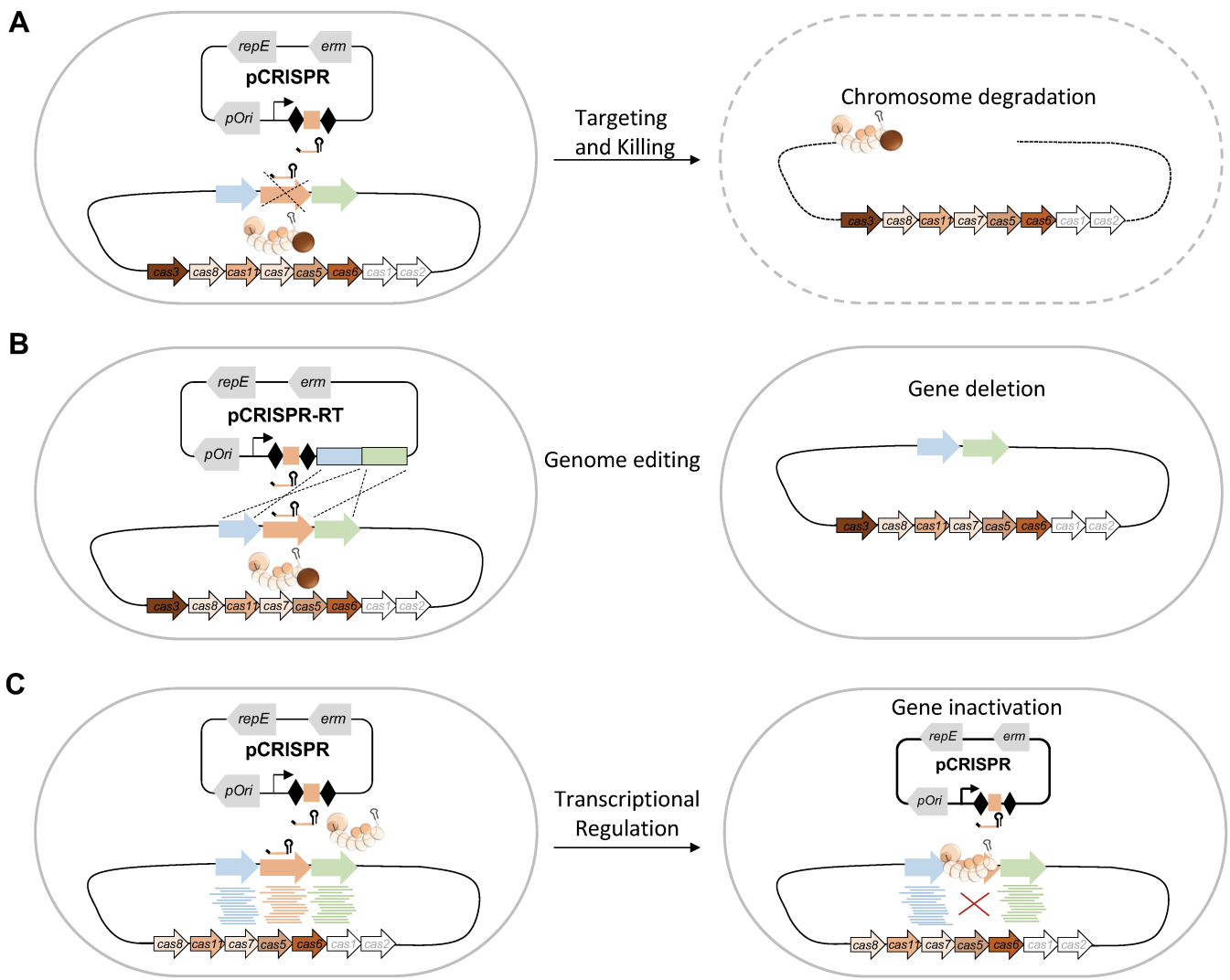


Figure 2