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Review

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Advancing CRISPR-Based Programmable Platforms beyond Genome Editing in Mammalian Cells

Yasutomi Higashikuni[1-5]*, Timothy K. Lu[1-4]*

[1]Synthetic Biology Center, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[2]Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[3]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[4]Department of Electrical Engineering & Computer Science, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[5]Present affiliation: Department of Cardiovascular Medicine, The University of Tokyo, Tokyo 113-8655, Japan

*Corresponding Authors:

Yasutomi Higashikuni (tommy-h@tc4.so-net.ne.jp)

Timothy K. Lu (timlu@mit.edu)

Lead contact: Timothy K. Lu (timlu@mit.edu)

Room NE47-221, 500 Technology Square, Cambridge, MA 02139, USA

Tel: +1-617-715-4808, Fax: +1-619-789-4808, Email: timlu@mit.edu

ABSTRACT

Human diseases are caused by dysregulation of cellular biological programs that are encoded in DNA. Unveiling the endogenous programs and encoding new programs into the genome are key to creating novel diagnostic and therapeutic strategies. CRISPR/Cas9, originally identified in bacteria, has revolutionized genome editing in mammalian cells. Recent advances in CRISPR technologies have provided new programmable platforms for modifying cell function and behavior. CRISPR-based transcriptional regulators and modified gRNAs have enabled multiplexed regulation and visualization of genome dynamics with spatiotemporal precision. Using these toolkits, genome-scale screening platforms can identify key genetic elements or combinations thereof that modulate phenotypes in mammalian cells. In addition, imaging platforms for multiplexed genomic labeling have been created to study the conformation and dynamics of chromatin in living cells, which are essential for genome function. Furthermore, CRISPR-based computation and memory platforms have been built in living mammalian cells by using DNA as a data processing and storage medium to regulate and monitor cellular behaviors. The conditional regulation of CRISPR-based parts has enabled the design of complex multilayered biological programs. CRISPR-based memory platforms can continuously record biological events as mutations in defined DNA loci. By making use of base editors, CRISPR-based computation and memory platforms have been interconnected to perform logic operations based on past events. These technologies open up new avenues for understanding biological phenomena and designing mammalian cells as living machines for biomedical applications.

KEY WORDS: CRISPR, screening, imaging, computation, memory, mammalian cells

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Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 was originally identified as a prokaryotic adaptive immune system that protects bacterial cells against foreign genetic elements such as viruses and plasmids.[1, 2] In this system, the Cas9 endonuclease degrades DNA fragments by introducing a double-strand break at a target location recognized by a guide RNA (gRNA).[3] Each gRNA is composed of a scaffold sequence for Cas9 binding and a specificity determining spacer sequence (SDS) for target recognition and directs Cas9 to those DNA fragments that contain a sequence complementary to the SDS and lie immediately adjacent to a protospacer adjacent motif (PAM) sequence. Double-strand breaks introduced by Cas9 into DNA can be repaired by intracellular DNA repair mechanisms, such as non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Figure 1A). These repair mechanisms modify the original DNA sequences by the insertion or deletion (indel) of nucleic acids, which can represent information encoded into DNA. The CRISPR/Cas9 system, which modifies DNA information with only 2 components (Cas9 and a gRNA), works not only in prokaryotic cells but also in mammalian cells and so has ushered in a new era of DNA editing across a wide array of cell types for many applications.[4, 5]

Novel therapeutic strategies can be created by discovering the endogenous programs whose dysregulation underlies human disease[6, 7] and then encoding new biological programs into the genome.[8] Recent advances in CRISPR technologies that involve modifying Cas9 and designing synthetic gRNAs have enabled researchers to harness the CRISPR/Cas9 system both for editing genetic information at desired loci and for encoding biological programs in DNA, which functions as

a data processing and storage medium in living cells.[9-12] Cas9 endonuclease mutants, such as nuclease dead Cas9 (dCas9) and nickase Cas9 (nCas9), retain the ability to bind to DNA sequences targeted by the gRNA, independent of endonuclease activity. By fusing various effectors such as transcriptional regulators or deaminases to these enzymes, researchers have gained the ability to regulate transcriptional activity or to introduce specific substitution mutations at target loci.[11, 13] New strategies to express functional gRNAs from RNA polymerase II promoters or under the control of small molecules or endogenous proteins have enabled the conditional regulation of gRNA expression and function.[14-16] These toolkits exhibit high modularity and scalability, which greatly facilitate the regulation of endogenous genome dynamics and the design of synthetic gene circuits to execute complex cellular computation and memory operations. In addition, integration of fluorescent proteins into these toolkits enables visualization of genome dynamics in mammalian cells.

With these toolkits, CRISPR technologies are being applied to genome-scale phenotypic screening and multiplexed genomic imaging, which are essential to clarifying the mechanisms of complex biological processes.[17, 18] Phenotypic screening can directly assess the causal relationship between the activity of specific genetic elements and cellular phenotypes in mammalian cells. Genomic imaging platforms for living cells can be used to study chromatin dynamics with spatiotemporal precision, which play critical roles in genome function.

CRISPR technologies have also enabled us to encode logic and memory operations into the genome of mammalian cells; such operations are key to programming cells for a broad range of

biomedical applications. Complex logic circuits can be used to recapitulate or reprogram multiplexed gene networks in mammalian cells. A better grasp of these networks is likely to reveal mechanisms underlying natural biological processes, while engineered gene networks can outfit cells with new functions for diagnostic or therapeutic applications.[8] *In vivo* memory systems can capture longitudinal biological information rather than a snapshot. This is useful because mapping the relationship and timing between individual events can yield a chronicle of complex biological processes, which would be otherwise hard to study. In addition, by connecting memory systems to logic circuits, we can regulate cellular behavior based not only on the current condition or environment but also on past cellular events. This review discusses recent advances in CRISPR-based programmable platforms beyond genome editing in mammalian cells, as well as current limitations and future directions.

CRISPR-BASED TRANSCRIPTIONAL REGULATORS and BASE EDITORS

Using dCas9 as a sequence-specific DNA binding platform, researchers have invented various CRISPR-based transcriptional regulators that inhibit or activate gene expression, whether endogenous or exogenous (Figure 1B).[19-25] A fusion protein consisting of a KRAB transcriptional repressor domain and dCas9 (dCas9-KRAB) can efficiently suppress endogenous gene expression when introduced with a targeting gRNA in mammalian cells.[19] Yeo *et al*. created a more potent CRISPR-based repressor by fusing MeCP2 to dCas9-KRAB.[25] Fusion of a multiple number of VP16

transcriptional activation domains, or of 4xVP16, p65, and Rta activation domains (VPR) as a single polypeptide, to dCas9 allows transcriptional activation of reporters and endogenous genes with gRNAs targeting their promoter elements.[19-22] Konermann *et al*. developed the CRISPR Synergistic Activation Mediator (SAM) system by modifying gRNAs so that they have minimal hairpin aptamers that selectively bind dimerized MS2 bacteriophage coat proteins.[23] The modified gRNAs recruit dCas9-VP64 and MS2-p65-HSF1 fusion proteins to targeted promoter elements, resulting in potent synergistic transcriptional activation. Tanenbaum *et al*. created the SunTag system, in which a fusion protein made of repetitive peptide epitopes fused to dCas9 recruits multiple copies of antibody-fused VP64 and induces strong transcriptional activation of the genes targeted by the gRNAs.[24]

CRISPR-based epigenetic regulators, which target histones or CpG motifs, have also been developed.[26-33] A fusion protein consisting of dCas9 and the histone demethylase LSD1 removes H3K4me2 marks in a targeted active enhancer and induces gene silencing,[26] whereas the fusion of dCas9 with the histone methyltransferase PRDM9 induces H2K4me3 marks and reactivates silenced target genes.[32] Similarly, dCas9 fused with the catalytic histone acetyltransferase core domain of p300 can add H3K27ac marks in targeted regulatory regions and thereby upregulates gene expression,[27] whereas dCas9-LSD1 or a fusion protein of dCas9 and histone deacetylase 3 (HDAC3) reduces H3K27ac marks and the gene expression of the target loci.[26, 33] Fusing dCas9 with the methyltransferase Dnmt3a produces a protein that methylates CpG motifs in a target window of several hundred bases and silences endogenous genes efficiently.[28, 29, 31] Fusing dCas9 with the

catalytic domain of the 5-methylcytosine dioxygenase Tet1 or a full-length Tet1 leads to targeted reduction in the methylation of CpG motifs and the upregulation of targeted genes.[28, 30]

These highly efficient CRISPR-based transcriptional regulators have enabled simultaneous multiplexed gene perturbation in mammalian cells; thus, they have been used to design logic computing in mammalian cells. In combination with synthetic gRNA libraries, these transcriptional regulators have been used to create genome-scale pooled screening platforms that can identify key genes that modulate phenotypes in mammalian cells.[34-36]

CRISPR-based base editors have been created by fusing the cytidine deaminases APOBEC1 or PmCDA1 to dCas9 or nCas9 so that substitution mutations can be made at target sites with single nucleotide resolution (Figure 1C).[37, 38] These enzymes deaminate cytosine (C) to uracil (U) at target loci in single-strand DNA unwound by Cas9 helicase activity; DNA repair and replication mechanisms then replace uracil (U) with thymidine (T) without introducing a double-strand break. The efficiency of the C-to-T substitution mutation was improved by 1) using nCas9 to enhance the DNA mismatch repair of the non-deaminated strand in response to the single-strand break caused by the nickase, and 2) inhibiting the repair of the deaminated strand by fusing a uracil DNA glycosylase inhibitor to nCas9.[37, 38] Recently, various base editors have been created by fusing Cas9 and deaminase variants.[13] Adenosine base editors, which convert an A/T base pair to a G/C base pair, have been developed by fusing TadA, an *Escherichia coli* tRNA adenosine deaminase enzyme, and a mutant version of TadA, to nCas9.[39] In theory, adenosine base editors can reverse a C-to-T mutation

introduced by a cytidine base editor, thus having the potential to erase or rewrite DNA data. Collectively, the advent of base editors has yielded DNA writing platforms having precise single-nucleotide resolution, which can be used not only for genome editing but also for computation and memory systems in living cells.

CRISPR-BASED PHENOTYPIC SCREENING PLATFORMS

Forward genetic screening is a powerful tool for understanding complex biological processes because it can be used to discover specific genes or epigenetic regulatory elements associated with a phenotype of interest in an unbiased and genome-wide manner. Recently, CRISPR-based phenotypic screening has become a popular method for systematic and high-throughput genetic screening in mammalian cells because of its high modularity and scalability for various experimental designs (Figure 2).[17] Pooled screening can be performed by designing gRNA libraries for the simultaneous testing of individual gene perturbations in a single batch, which can be performed at a reasonable cost compared with arrayed screening.

CRISPR-based pooled screening begins with building gRNA libraries that target the genes of interest (Figure 2A).[17] Then, these libraries are delivered into the genome of the target cell population using lentiviruses or transposons. After exposing the cell population to selective pressure, the genomic DNAs are isolated from surviving cells or from cells that have been sorted by flow cytometry based

on fluorescent signals as the output. The sequences of interest are amplified by PCR, followed by massively parallel next-generation sequencing (NGS). Potential hits determined by statistical analysis are then tested by validation experiments for confirming technical reproducibility and on-target effects. Beyond technical validation, these hits may also be examined to determine whether they can be generalized to other biological contexts.

Currently available CRISPR-based genetic perturbations include knockout, knockdown, activation, and epigenetic manipulation.[17, 34-36, 40-42] Pioneering work in CRISPR-based screening emerged with CRISPR knockout (KO) screening.[40-42] This approach can create null alleles and has been reported to provide experimental consistency with low noise and minimal off-target effects compared to RNA interference (RNAi) knockdown.[43] However, the generation of in-frame mutations and non-specific cell death caused by double-strand breaks can lead to phenotypic variations in screens that affect their reliability. Furthermore, the functions of genes essential for cellular survival might be difficult to test in this context because gene disruption leads to cell killing. The CRISPRi approach can overcome these limitations. In addition, CRISPRi may be useful to study non-coding regions; for example, Gilbert *et al*. demonstrated more than 80% knockdown for all except one of the long non-coding RNA genes tested by CRISPRi.[35] CRISPR activation (CRISPRa) approaches, using CRISPR-based transcriptional activators, can test the therapeutic potential of upregulated genes.[34, 35] In addition, CRISPRa may have advantages compared to simple overexpression of open reading frames by upregulating splicing variants of target genes without losing their diversity. CRISPR-based

epigenetic repressors or activators can be used for high-throughput functional annotation and discovery of regulatory elements for genes of interest,[36] as well as studying the relationships between cellular phenotypes and epigenetic modifications.

Complex biological systems are often regulated not by a single factor but by multiple factors. To identify combinations of factors that combinatorially affect a phenotype of interest, CRISPR-based combinatorial screening platforms have been developed (Figure 2B).[44-48] Wong *et al*. developed a novel cloning technology, termed Combinatorial Genetics *En Masse* (CombiGEM), to easily build (*n*)-wise combinatorial gRNA libraries only by repeating pooled digestion and ligation of plasmid libraries.[44] Boettcher *et al*. created a dual gene activation and knockout screening platform by expressing the *Streptocuccus pyogenes*-based SunTag system and Cas9 nuclease from *Staphylococcus aureus* (SaCas9) with corresponding gRNA libraries.[48] Although the number of target genes is limited by the exponential increase of library sizes with higher-order combinations, combinatorial screening platforms still enables insights into the interactions of multiple factors that contribute to complex biological networks.

CRISPR-based screening platforms combined with single-cell RNA sequencing have also been developed, such as Perturb-Seq,[49, 50] CROP-Seq,[51] CRISP-Seq,[52] and Mosaic-Seq (Figure 2C).[53] In these platforms, transcriptome data can be linked to each gRNA at a single cell level; thus, detailed information on the transcriptome-wide effects of genetic perturbations caused by individual gRNAs can be analyzed simultaneously. Furthermore, transcriptional phenotypes can be used as the readout

rather than a distinct cellular phenotype such as cellular survival, which facilitates the functional dissection of complex biological programs and heterogeneous cell populations. Currently, data analysis and interpretation for these approaches remain challenging due to the technical and biological noise in single-cell data. In addition, library size is limited in scale because of the technical difficulty and high cost of single-cell RNA sequencing. Nevertheless, we expect that these platforms will become increasingly powerful tools to identify key regulators of biological programs.

Microenvironments and interactions among multiple cell types play critical roles in the pathophysiology of human diseases, which cannot be easily recapitulated by *in vitro* screening. To overcome this limitation, *in vivo* functional genomics screening has been performed in murine disease models (Figure 2D).[54] The first CRISPR-based *in vivo* screening was performed by using a xenograft cancer model.[55] Chen *et al*. subcutaneously transplanted a non-metastatic cancer cell line infected with a genome-wide gRNA library into the back skin of nude mice. By comparing a metastatic cell population in the lung with primary tumors, they identified genes that promote lung metastasis. Since then, this screening design has been used to identify tumor suppressors, druggable oncogenes, and regulators of cancer immunotherapy.[56-59] Implantation of large numbers of cancer cells into mice, however, does not recapitulate the actual process of carcinogenesis nor reproduce the tumor microenvironment. To address this limitation, direct *in vivo* screening platforms have been developed that involve introducing gRNA libraries into the target organs via adeno-associated virus vectors in Cas9 mice.[60, 61] Tumor suppressor genes were identified with this platform in glioblastoma and liver

cancer. One of the future challenges for this technology is to improve direct *in vivo* screening approaches, which are are currently limited by technical issues, such as library size, coverage, and multiplicity of infection (MOI).

CRISPR-BASED IMAGING PLATFORMS

Chromatin conformation and localization regulate genome function and resulting cellular phenotypes. The comprehensive analysis of chromatin dynamics with spatiotemporal precision is important for fully understanding the regulatory mechanisms of genomic activities and subsequent biological processes. Although fluorescent *in situ* hybridization (FISH) methods have been widely used for investigating chromatin architectures, the need for cell fixation has prohibited the application of FISH for live cell imaging, which is critical for obtaining temporal information.

To overcome this limitation, CRISPR-based imaging platforms have been developed (Figure 3).[18] By using the fusion protein of dCas9 and EGFP (dCas9-EGFP), Chen *et al*. demonstrated that dCas9-EGFP with one sgRNA can efficiently visualize the highly repetitive elements of telomeres in living mammalian cells (Figure 3A).[62] In addition, non-repetitive regions of the *MUC4* gene were detected by using an array of at least 26 different gRNAs. Using this platform, Gu *et al*. studied the dynamics of the enhancer and promoter of the *FGF5* gene with 36 unique gRNAs.[63] Duan *et al*. successfully labeled highly-repetitive elements of chromosomal loci in dCas9-EGFP knockin mice.[64]

The dCas9-SunTag system has also been used to increase signal intensity at non-repetitive regions. Shao *et al*. demonstrated that in this system, non-repetitive regions of the *MUC4* gene can be tracked with fewer different gRNAs compared with the number required for dCas9-EGFP.[65]

Modified gRNAs have also been developed for genome imaging. Researchers integrated multiple RNA aptamers, such as MS2 and the Pumilio/Fem3 mRNA-binding factors (PUF)-binding sequences, into gRNAs (Figure 3B).[66-72] Using these modified gRNAs in combination with fusions of aptamer-binding proteins and fluorescent proteins, researchers labeled both repetitive and non-repetitive elements in the genome. Qin *et al*. demonstrated that the MS2-based system, using gRNA harboring 16 MS2 aptamers, can detect low-repeat-containing loci with a single gRNA and non-repetitive regions of the *MUC4* gene with only 4 unique gRNAs.[66]

One challenge with these technologies is high background arising from fluorescent signals from fusion proteins unbound to modified gRNAs. To overcome this limitation, Hong *et al.* used split fluorescent proteins in combination with both the dCas9-SunTag and a modified gRNA system (Figure 3C).[73] In their system, dCas9 is labeled by the carboxyl terminal fragment of the fluorescent protein Venus through SunTag, whereas MS2-based modified gRNA is labeled by the amino terminal fragment of Venus. Since fluorescent signals can be detected only when the split parts of the fluorescent protein are fused, this technology showed a higher signal-to-background ratio compared with other methods using modified gRNAs or fusion proteins consisting of dCas9 and fluorescent proteins.

The high modularity and scalability of CRISPR-based technologies have enabled multiple-color genome imaging (Figures 3D and 3E). Ma *et al*. visualized three genomic loci simultaneously by using three fluorescent protein-fused dCas9 orthologs [*Streptococcus pyogenes* (Sp) dCas9, *Streptococcus thermophilius* (St1) dCas9, and *Neisseria meningitidis* (Nm) dCas9], which have unique PAM sequences and gRNA scaffolds (Figure 3D).[74] By using this platform, the authors could obtain information on the distance between the targeted loci on the same chromosome or on different chromosomes in living cells. Alternatively, modified gRNAs have also been used for multiple genomic labeling. Ma *et al*. have developed a modified gRNA-based multiple-color imaging platform, termed CRISPRainbow (Figure 3E).[68] In this system, three types of RNA aptamers, such as MS2, PP7, and BoxB, were integrated into gRNAs. By co-expressing the proteins, each made by fusing a different fluorescent protein to each aptamer-binding protein, three colors could be labeled on the genome. In addition, by integrating two different aptamers into the same gRNA and overlaying the fluorescent signals, CRISPRainbow has enabled simultaneous visualization of 6 chromosomal loci, which clarified the differences in the dynamic properties among different chromosomes— and among different loci within the same chromosome— in living cells.

Recently, a molecular beacon (MB)-based platform has been developed for both single-color and multi-color genomic labeling.[75, 76] MBs are unique oligonucleotide probes with a fluorophore and its quencher. Fluorescent signals are activated when oligonucleotide sequences are bound to their complementary sequences, and subsequently, quenchers are separated from fluorophores. By using

modified gRNAs harboring unique MB target sequences, this platform efficiently visualized the genomic loci of interest with a high signal-to-background ratio.

Although there remain limitations such as off-target binding, variable efficiencies of on-target binding, and background fluorescence due to the characteristics of Cas9 variants and gRNA sequences,[18] CRISPR-based genome imaging platforms have great potential to elucidate the relationship between chromatin dynamics and genomic function.

CRISPR-BASED LIVING CELL COMPUTATION PLATFORMS

Conditional regulation systems of gRNAs or CRISPR-based transcriptional regulators have enabled the implementation of various computing platforms in mammalian cells (Figure 4). Nissim *et al*. designed novel synthetic gene circuits using a CRISPR transcription factor and an RNA polymerase II promoter-derived gRNA expression system.[14] The authors flanked gRNAs with 28 base pair (bp) recognition sites by the Csy4 RNA-modifying protein to generate functional gRNAs from RNA polymerase II promoters. In this architecture, transcribed RNAs are cleaved in the presence of Csy4 and release functional gRNAs. The authors also demonstrated that gRNAs flanked by cis-acting ribozymes, such as a hammerhead ribozyme and an HDV ribozyme, can be expressed by RNA polymerase II promoters and can operate as functional gRNAs in mammalian cells. Csy4-dependent gene activation circuits, such as cascades and switches, were designed with these toolkits and found to

work efficiently in mammalian cells.

Kiani *et al*. designed a modified U6 promoter containing two gRNA target sites.[15] In this system, dCas9 is directed to these sites to inhibit downstream gRNA expression; thus, the expression of one gRNA is regulated by that of another gRNA as a cascade. Liu *et al*. developed modified gRNAs with aptamers responsive to small molecules or protein ligands.[16] These gRNAs contain self-inhibitory anti-sense sequences. Conformational changes induced by aptamer ligands lead to exposure of the SDSs so that they can work as functional gRNAs. By integrating the MS2 or Rev aptamer into modified gRNAs and expressing both MS2-dCas9 and Rev-dCas9-VP64, the authors also demonstrated the simultaneous repression of one target gene and the activation of another target gene in mammalian cells, respectively. Using this architecture, they built an AND gate circuit that kills cancer cells expressing both NF-κB and β-catenin, but not normal cells, by activating the *BAX* gene and suppressing the *BCL2* gene simultaneously to induce apoptosis. Braun *et al*. also utilized MS2 aptamer-integrated gRNAs to create a rapamycin-inducible epigenetic regulation system by expressing MS2 bacteriophage coat proteins fused with FK506 binding protein (FKBP) and epigenetic regulators fused with FKBP rapamycin-binding domains (FRB).[77] Because FKBP dimerizes with FRB, this architecture regulated the transcriptional activities of the targeted genes in response to rapamycin, as demonstrated in human embryonic kidney (HEK) 293 cells and mouse embryonic stem (ES) cells. These studies provide the basis for the conditional regulation of gRNA expression and function in response to extracellular or intracellular inputs, capabilities that can be

used to build complex and multilayered gene circuits for computing in living cells.

Various conditional regulation systems for CRISPR-based transcriptional regulators have recently been developed. Gao *et al*. created a robust and flexible chemical-inducible platform by fusing chemically inducible heterodimerization domains, including abscisic acid-inducible ABI-PYL1 and gibberellin-inducible GID1-GAI, to dCas9 and transcriptional regulation domains, such as KRAB, VPR, Suntag-VP64, and Suntag-VPR.[78] This highly modular architecture, in which DNA-binding parts and transcriptional regulation parts can be swapped by chemical inputs alone, enabled the authors to design AND, OR, NAND, and NOR logic operators, and a regulator that activates gene expression with one inducer and represses with another, in HEK293T cells. This work showed the potential for CRISPR-based transcriptional regulators to enable multiple-input and multiple-output biological computation platforms.

In line with this concept, several split Cas9 variant architectures have been developed for inducible transcriptional regulation. Zetsche *et al*. developed a rapamycin-inducible split dCas9-VP64 architecture by fusing FKBP and FRB to C-terminal and N-terminal dCas9 fragments, respectively.[79] By linking each split Cas9 fragment with the ligand-binding domain ERT from an estrogen receptor, 4-hydroxytamoxifen-responsive split dCas9-VP64 and dCas9-VPR architectures were also constructed.[80] In addition to chemically regulated systems, photo-inducible split Cas9 systems were developed by fusing split dCas9 fragments and photo-inducible dimerization domains.[81] These architectures allow external control of CRISPR-based transcription regulator activity and synthetic

gene circuits in mammalian cells.

An intein-mediated split Cas9 system has also been developed. Using split inteins, Ma *et al*. designed multiple split dCas9 domains that can be fused by protein splicing.[82] In this system, transcriptional regulators fused to dCas9 can be swapped; thus, the CRISPR-based transcriptional activator and repressor are switchable by the conditional expression of functional domains without external control. The authors also developed an orthogonal split Cas9 pair that recognizes NGCG PAM sequences instead of NGG PAM sequences. Furthermore, they demonstrated that toggle switch circuits can be designed in combination with transcription activator-like effector (TALE) repressors and microRNA regulation systems. Their findings show the feasibility of complex and multilayered biological computers that respond to multiple inputs.

Recently, it was reported that the proteasomal degradation pathway can be used for the temporal regulation of CRISPR-based transcriptional regulator activity. Kuscu *et al*. fused a specific degron called AID, to dCas9-p300.[83] AID is recognized by the SCF-E3 ubiquitin ligase system via the TIR1 protein in the presence of a plant hormone, auxin. By expressing TIR1 in HEK293T cells, the AID-dCas9-p300 complex was depleted rapidly within 12 hours after induction with auxin, thus suppressing further changes in epigenetic marks. This work provides a strategy for the rapid termination of circuit activity.

CRISPR-BASED MEMORY PLATFORMS

DNA is an ideal data storage medium because of its ubiquitous presence, durability, and compatibility with biological functions.[84] Because nucleotides have a standard format across all cells and species, they provide a universal format for writing, reading, and processing data within living cells. Furthermore, this medium can readily be copied through biological means, thus facilitating amplification or sharing.

With the advent of genome editing technologies and DNA data acquisition with NGS, researchers have developed various platforms for data storage in DNA (Figure 5).[84] These platforms consist of stochastic and deterministic memory systems.[10, 12] Stochastic memory systems generate random mutations in defined DNA addresses. These mutations can be interpreted as information when they are analyzed in cell populations, though each mutation only contains information about the occurrence of a single event. Deterministic memory systems generate predetermined mutations in defined DNA addresses; thus, mutations themselves can correspond to defined events and can be analyzed either at the population level or at individual memory loci within single cells.

McKenna *et al*. developed a proof-of-concept CRISPR-based stochastic memory device with Cas9 and an unmodified array of CRISPR/Cas9 target sites, termed Genome Editing of Synthetic Target Arrays for Lineage Tracing (GESTALT).[85] In this system, multiple arrayed CRISPR target sites were integrated into the genome with lentivirus in Cas9-expressing cells. Over successive

cellular divisions, random indels were introduced into the target arrays through the NHEJ pathway. By analyzing mutation patterns obtained by DNA sequencing as barcodes, the authors could reconstruct lineage relationships and generate large-scale maps of cell lineages in cultured cells or zebrafish (*Danio rerio*). In line with this concept, Frieda *et al*. created a stochastic memory system that enables mammalian cells to record lineage information into the genome, termed Memory by Engineered Mutagenesis with Optical In situ Readout (MEMOIR) (Figure 5A).[86] MEMOIR consists of three components integrated into the genome of embryonic stem cells by the PiggyBac system: a distributed set of barcoded "scratchpads" targeted by a gRNA, a Wnt-inducible gRNA flanked with ribozymes for RNA polymerase II promoter-driven expression, and a small molecule-inducible Cas9 variant driven by a constitutive promoter. By using MEMOIR to analyze scratchpad collapse patterns by Cas9 in combination with endogenous gene expression patterns, the authors were able to reconstruct lineage information. They visualized these patterns with single-molecule RNA Fluorescence In situ Hybridization (smFISH) using barcode-specific oligo probe sets and a scratchpad-specific oligo probe set. In their approach, uncollapsed scratchpads produce co-localized barcode plus scratchpad signals, while collapsed scratchpads produce only a barcode-specific signal. The authors also showed the potential of the MEMOIR system for the simultaneous recording of cellular lineages and events by using multiple gRNAs expressed by inducible promoters and their targeted scratchpads. Their concept of distributed memory addresses, instead of an array of memory addresses in a single locus, provides a way to overcome problems associated with inter-target

deletions and loss of memory due to the multiple double-strand breaks observed in GESTALT.

As multi-address memory systems, these recording systems need a large number of loci to encode large amounts of information. To reduce required memory capacity, Perli *et al*. developed a novel system that targets one memory address for continuous recording. To create this system, the authors designed self-targeting gRNAs (stgRNAs) with the modified gRNA scaffold itself harboring a PAM sequence just downstream of the SDS (Figure 5B).[87] With this architecture, the DNA locus encoding the gRNA can be targeted by itself, which makes it possible to use the SDS as a memory address. Moreover, the mutated gRNA can recognize itself continuously as long as the PAM sequence remains unmutated, because the mutated gRNA remains a perfect match for the DNA locus expressing itself, which allows a single memory address to generate highly variable sequences. The authors infected Cas9-expressing HEK293T cells with the barcoded stgRNA library via lentiviruses, and demonstrated that this system, termed mammalian Synthetic Cellular Recorders Integrating Biological Events (mSCRIBE), could undergo multiple rounds of mutagenesis for at least 16 days. By using mSCRIBE, they developed inflammation-recording HEK293T cells expressing Cas9 under the control an the NF-κB-responsive promoter and the stgRNA under the control of a U6 promoter, and showed that, in mice, lipopolysaccharide (LPS)-induced acute inflammation events were recorded over time in these cells. Furthermore, these events could be reconstructed in an analog fashion by calculating the percent of mutated stgRNAs in the total stgRNAs with the intact PAM sequence. These findings provided a new architecture for CRISPR-based stochastic memory devices that can be

used to record biological events of interest.

By deploying independent stgRNA loci in parallel in the mouse genome, Kalhor *et al*. demonstrated the application of the stgRNA technology in combination with a multi-address memory system for *in vivo* lineage tracing.[88] The authors created a mouse line with a scattered array of sixty stgRNA loci in the genome. By crossing this mouse line with Cas9 knock-in mice, they created developmentally barcoded offspring that continuously introduce random mutations into a scattered stgRNA array in their cells. By analyzing mutant alleles of each stgRNA, they found that commitment to the anterior-posterior axis is established before commitment to the left-right axis in the development of the central nervous system (CNS). Interestingly, they also characterized the activity profile of each stgRNA and demonstrated different mutating activity depending on the sequence. Furthermore, Kalhor *et al*. demonstrated that the stgRNA technology can be used in combination with the Fluorescence In Situ Sequencing (FISSEQ) technique to preserve spatial information in mammalian cell lineage tracing.[89] Their work showed the advantage of the stochastic stgRNA-based multi-address memory system in cellular barcoding for its ability to create highly variable mutational signatures and to reconstitute ancestral relationships. However, this system loses recording ability over time because the PAM sequence is lost by random mutation, a drawback that could be overcome by deterministic memory systems.

The advent of base editors has enabled the creation of more deterministic memory platforms.[13] Using a base editor and gRNA targeting the established human safe-harbor gene CCR5, Tang *et al*.

developed a system termed CRISPR-mediated Analog Multi-Event Recording Apparatus (CAMERA), which can record the duration or amplitude of signals as single-nucleotide changes in HEK293T cells (Figure 5C).[90] They showed that either by placing a base editor under a doxycycline-controlled promoter or a β-catenin-responsive promoter or by placing gRNAs under TetR- and LacI-suppressed U6 promoters, CAMERA can record the extracellular or intracellular signal-dependent accumulation of the predefined mutation in mammalian cell populations. This accumulation is assumed to correlate with signal duration or amplitude. They also demonstrated multiple targeting of the CCR5 locus by expressing multiple gRNAs, which indicates the potential for multiplexed deterministic recording in mammalian cells.

Farzadfard *et al*. developed a deterministic recording system based on DNA base editing to create computing circuits (Figure 5D).[91] The authors created a highly scalable, robust, and compact platform with single-nucleotide resolution for encoding both memory and computing operations, termed DNA-based Ordered Memory and Iteration Network Operator (DOMINO), by carefully designing gRNA and memory address nucleotide sequences in bacteria and mammalian cells. In the DOMINO system, logic operations, such as order-independent or order-dependent AND gates, are executed based on mutations introduced into the output DNA locus targeted by two different input gRNAs. For example, in an order-independent AND gate, each input gRNA introduces a predefined mutation independently. The output DNA locus executes its function only when both operations by two gRNAs are completed. In an order-dependent AND gate, the recording event by one gRNA is

required for the next recording event by another gRNA; thus, the output DNA locus is activated only when each operation is executed in the predefined order. By using the coding sequence of a fluorescent protein or its promoter as the output DNA locus or its final target, the DOMINO system can convert information stored as mutated sequences into visualizable fluorescent signals. Furthermore, the authors developed a synthetic self-reinforcing circuit that works in mammalian cells; in this circuit, predefined mutations are introduced in a targeting array that is written from downstream to upstream as a sequential cascade over time. This was achieved by placing an array of overlapping repeats targeted by the same gRNA (each array being composed of 4 wild-type repeats and a downstream mutant repeat harboring a dC-to-dT mutation) upstream of a minimal Adenovirus Major Late Promoter (MLP) that drives green fluorescent protein (GFP) expression. By fusing a transcriptional activator (VP64) to a base editor, the authors were able to convert arrayed mutation information into GFP signal intensity, because the number of fusion proteins that can bind to a targeting array for the activation of GFP expression depends on the number of introduced mutations. This information can be analyzed as both digital and analog data by calculating the total percentage of mutated arrays or the respective percentage of each memory state. This study represents a significant stride forward in developing artificial learning gene circuits and non-destructive monitoring of DNA computing and memory in living mammalian cells.

DISCUSSION and PERSPECTIVES

Mammalian cells maintain homeostasis by continuously sensing and responding to their extracellular environment and intracellular conditions. In the body, cells maintain homeostasis by processing vast amounts of past and present information in complex biological networks. To monitor this information non-invasively in real-time, as well as the myriad ways in which cells respond to it, powerful tools are required. These could be provided by scalable, robust, multiplexed, and high-throughput programmable platforms that function in living cells. Current strategies are still limited in their ability to capture large-scale information in real time in mammalian cells. However, CRISPR-based technologies are significantly more scalable than other previously used toolkits, such as recombinases or non-RNA-guided transcription factors.[10] Continuing advances in CRISPR technologies include the development of Cas13-directed A-to-I RNA base-editing technology, CasRx-based transcriptome engineering technology, and CRISPR-based epigenetic regulation.[26-30, 77, 83, 92, 93] These advances will enable ever greater insights into and control over how living organisms function.

CRISPR-based computation and memory platforms have the potential to confer new functions on mammalian cells for diagnostic or therapeutic applications in clinical settings by encoding artificial biological programs into the genome. Although substantial progress has been made in building these platforms, a number of technical challenges still need to be addressed. One of the ways that CRISPR-based computing and memory circuits can be interfaced with endogenous biological networks is through the activity of promoters that are linked to signals of interest. Many of the promoters that have been used for the control of these circuits have been identified empirically and thus they may not

exhibit optimal performance in terms of kinetics or dynamic range. Systematic approaches to identifying and engineering robust and efficient promoters, such as via synthetic promoter library screening and bioinformatics analysis of natural promoters, would enable tighter coupling of biological signals to CRISPR-based circuits.[94] So far, computing platforms have been designed such that small molecules, e.g., rapamycin and tamoxifen, control the gene circuits externally. These molecules may not be ideal in clinical settings due to poor pharmacokinetic properties or side effects.[95, 96] External regulation systems with minimal adverse effects will need to be developed for real-world applications in the future, especially in human medicine.

stgRNA technology-based stochastic memory systems rely on double-strand breaks and NHEJ to generate highly variable mutation signatures. The NHEJ pathway often results in DNA deletions rather than insertions, however. Deletion-based recording erases a part of previous memory information to generate new memory states, thus making it difficult to provide robust information on ancestral relationships with only a single memory address. In addition, the prevalence of deletions in NHEJ might cause the loss of the entire memory locus over time, which would limit recording ability. Using additional enzymes, such as terminal deoxynucleotidyl transferases (TdTs) and non-template polymerases, might overcome these limitations.[97-101] These enzymes can sustainably add random nucleotides to the 3' terminus of DNA; thus, the sequential addition of nucleotides to a memory address, where a double-strand break is introduced repeatedly by a stgRNA, might enable stochastic memory platforms to record temporal information in addition to event occurrence in a single memory

address.[101] Alternatively, base editors with wide editing windows and broader mutational activities, in combination with C- or A-rich stgRNAs, could be used for stochastic memory systems to generate highly variable sequences without the need for double-strand breaks, as such breaks may cause unwanted chromosomal arrangements and cellular toxicity as well as the loss of previous memory information.[13]

Encoding self-sufficient biological programs that function at the single-cell level will be key to the robust control of cellular behaviors. However, currently available CRISPR-based recorders are limited to population-based memory platforms, even in deterministic memory systems, because their base-editing is not efficient enough. Directed evolution, mutagenesis, or metagenomic mining may enable the creation of highly robust and efficient base editors for single-cell recording and computation that can perform erase and overwrite functions in a deterministic fashion.[102-105] Although non-invasive technologies to transform DNA data into visualizable outputs have been developed, these technologies can handle only a limited amount of data. New technologies to detect recorded sequences themselves without disrupting biological systems might be able to obtain detailed information about complex biological networks. Recently, circulating free nucleotides (DNA or RNA) or microvesicles containing nucleotides have been reported to be secreted from mammalian cells.[106-108] By harnessing their mechanisms and sequencing these circulating nucleotides, we may be able to detect recorded information in a non-invasive fashion.

In conclusion, CRISPR technologies have enabled new and powerful strategies for engineering

living machines in mammalian cells. Although most of these technologies are at the proof-of-concept stage, the continuing development of creative architectures will provide a broad range of real-world applications not only for basic science but also eventually for clinical practice.

AUTHOR INFORMATION

Corresponding Author

*E-mail: tommy-h@tc4.so-net.ne.jp or timlu@mit.edu

Conflict of Interest Statement

T.K.L. is a co-founder of Senti Biosciences, Synlogic, Engine Biosciences, Tango Therapeutics, Corvium, BiomX, and Eligo Biosciences. T.K.L. also holds financial interests in nest.bio, Ampliphi, and IndieBio.

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FIGURE LEGENDS

Figure 1. CRISPR-based transcriptional regulators and base editors. (A) DNA repair mechanisms for double-strand breaks (DSBs) introduced by Cas9. NHEJ, non-homologous end joining; HDR, homology-directed repair. (B) Fusion proteins of dCas9 and transcriptional regulators can modulate transcriptional activities at the target loci by gRNAs. (C) A CRISPR-based base editor can introduce substitution mutations at the target locus by a gRNA in combination with the endogenous DNA repair and replication mechanisms.

Figure 2. CRISPR-based phenotypic screening platforms. (A) A schematic of CRISPR-based phenotypic screening using a pooled gRNA library. (B) CRISPR-based combinatorial screening platform. Multiple gRNA combination with a unique barcode can be transduced into a single cell. (C) CRISPR-based screening platform in combination with single cell RNA-seq. (D) CRISPR-based *in vivo* screening platform using a xenograft model or a direct *in vivo* transduction model.

Figure 3. CRISPR-based imaging platforms. (A) dCas9-EGFP can efficiently visualize the target DNA locus by gRNAs. (B) gRNAs modified by the addition of RNA aptamers can efficiently recruit fusion proteins with fluorescent proteins and aptamer-binding proteins at their target loci. (C) Split fluorescent proteins, in combination with both the dCas9-SunTag and the modified gRNA systems,

can be used to increase signal-to-background ratios. (D) CRISPR-based multi-color imaging platform using multiple fluorescent protein-fused dCas9 orthologs. (E) Modified gRNA-based multiple-color imaging platform, termed CRISPRainbow. Multiple combinations of different RNA aptamers in the same gRNA can increase color variation by overlaying the fluorescent signals.

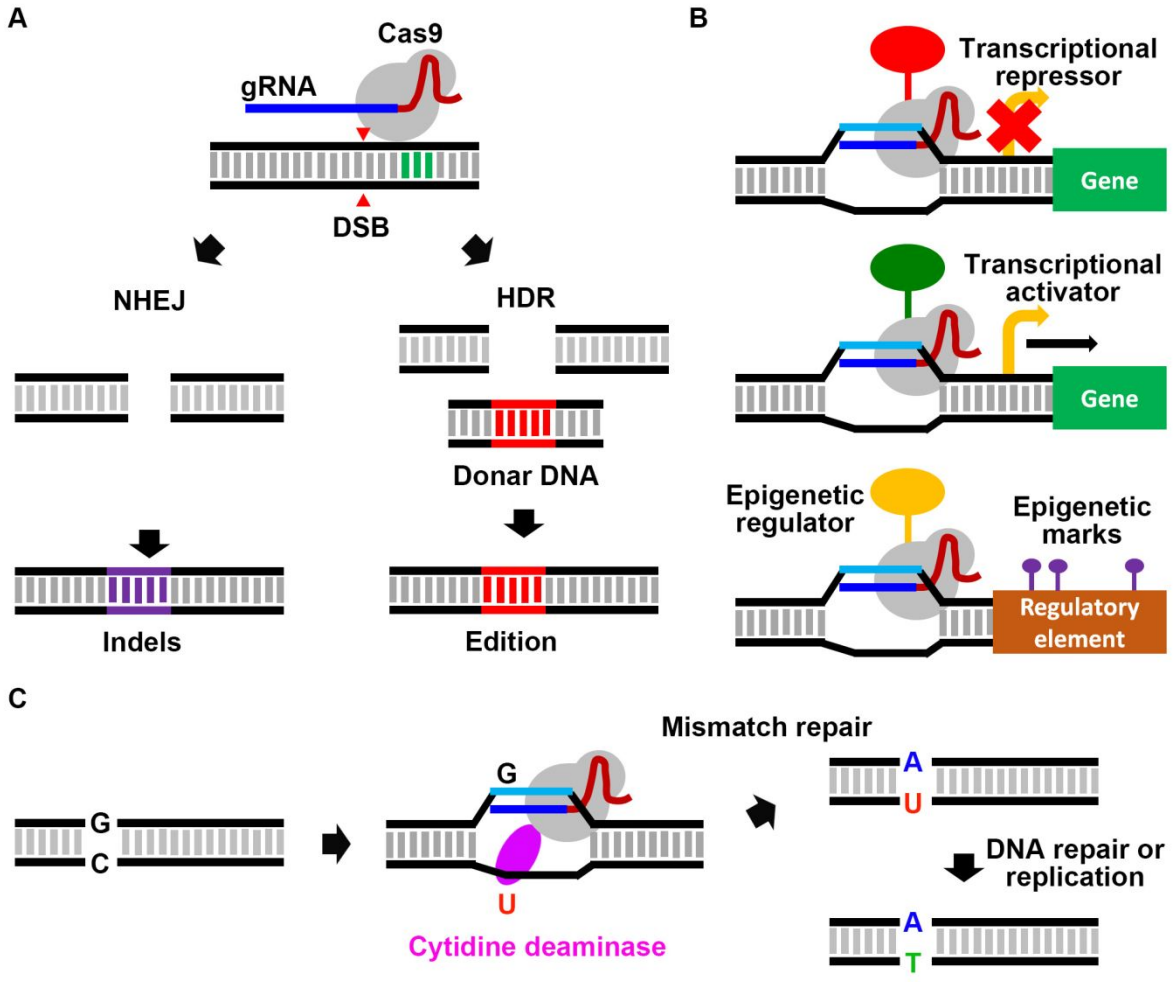
Figure 4. CRISPR-based computation platforms in mammalian cells. Various computing circuit architectures have been developed by using dCas9 variants and synthetic gRNAs in mammalian cells. In these architectures, transcriptional regulation activity or DNA-binding ability can be controlled by fusion or conformational change of split dCas9-transcriptional regulators (TRs) or synthetic gRNAs in response to chemicals or endogenous proteins. In combination with inducible promoters, complex and multi-layered computation platforms can be designed.

Figure 5. CRISPR-based memory platforms in mammalian cells. Stochastic (A and B) and deterministic (C and D) memory platforms have been developed using CRISPR technologies in mammalian cells. (A) A stochastic memory system with multiple memory addresses, termed Memory by Engineered Mutagenesis with Optical In situ Readout (MEMOIR). MEMOIR enables mammalian cells to record lineage information into the genome. (B) A stochastic memory system with a single memory address, termed Mammalian Synthetic Cellular Recorders Integrating Biological Events (mSCRIBE). By using self-targeting gRNA (stgRNA) harboring a PAM sequence just downstream of

the SDS, mSCRIBE can continuously record information on signal intensity or duration into the stgRNA itself, until it loses the PAM sequence. (C) A deterministic memory system using a base editor, termed CRISPR-mediated Analog Multi-Event Recording Apparatus (CAMERA). In combination with responsive promoters, this system can record extracellular or intracellular signals into a defined locus of the genome as a predefined single nucleotide mutation. By analyzing the mutations introduced into cell populations, information on signal intensity or duration can be assessed. (D) A deterministic memory system interconnected with logic operation systems, termed DNA-based Ordered Memory and Iteration Network Operator (DOMINO). By designing an array of overlapping repeats targeted by the same gRNA, a synthetic self-reinforcing circuit was created in mammalian cells. Predefined mutations are introduced by a base editor in a targeting array such that the mutations cascade from downstream to upstream. By fusing a transcriptional activator (VP64) to a base editor, information in this array can be converted into fluorescent protein signals driven by a minimal promoter downstream of the array. GFP, a green fluorescent protein.

Figures

Figure 1



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Figure 2

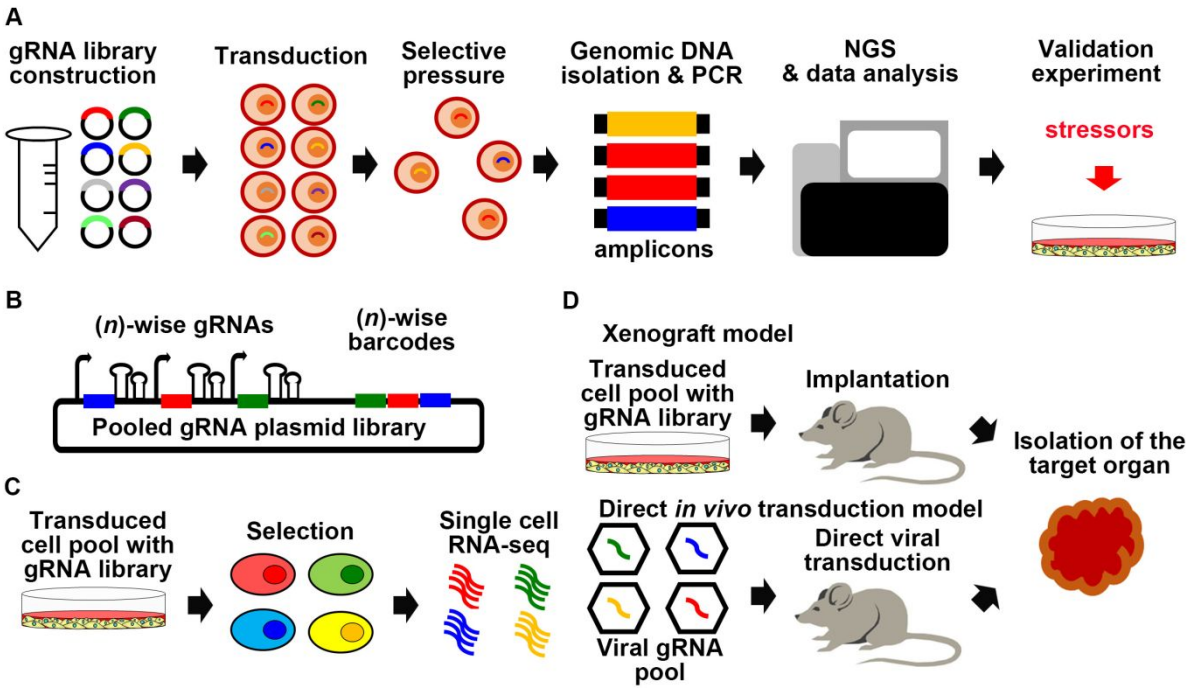
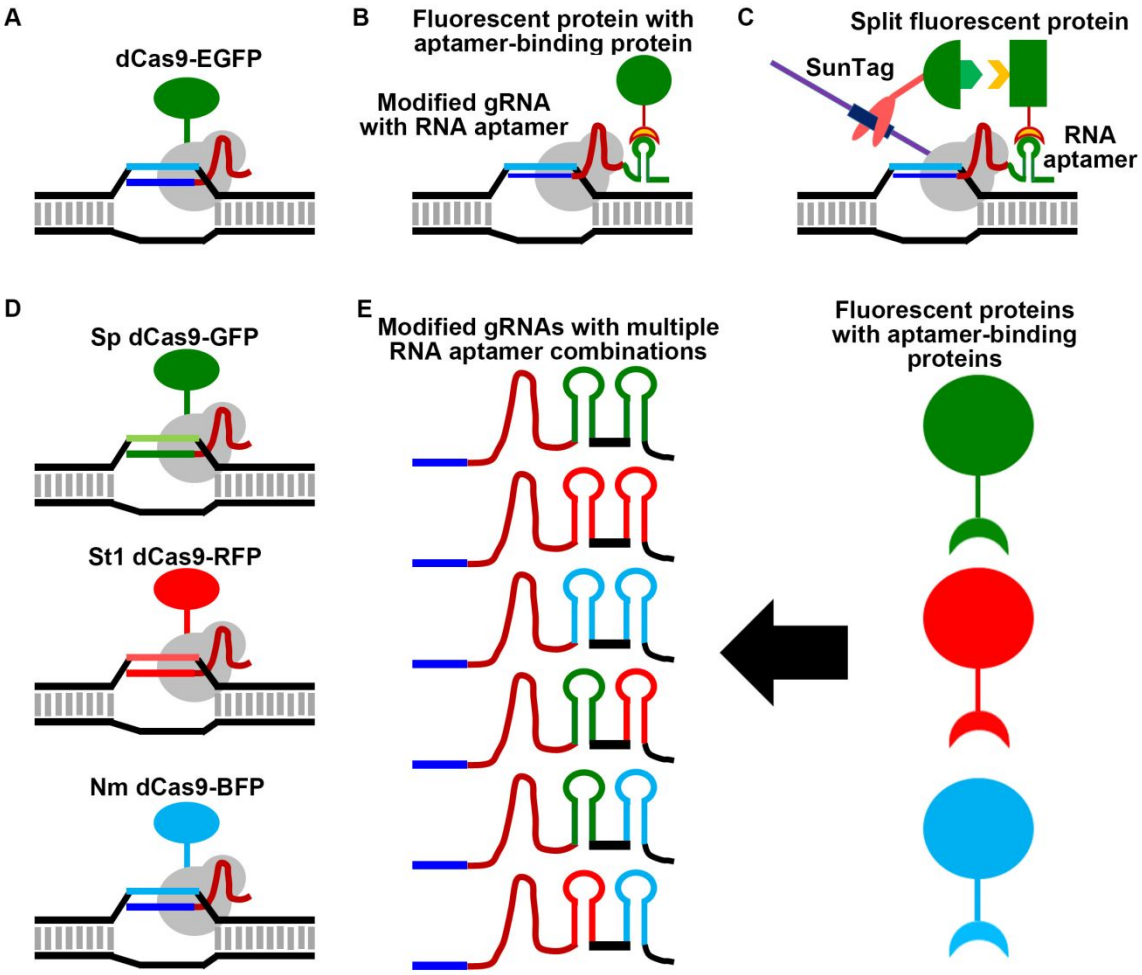
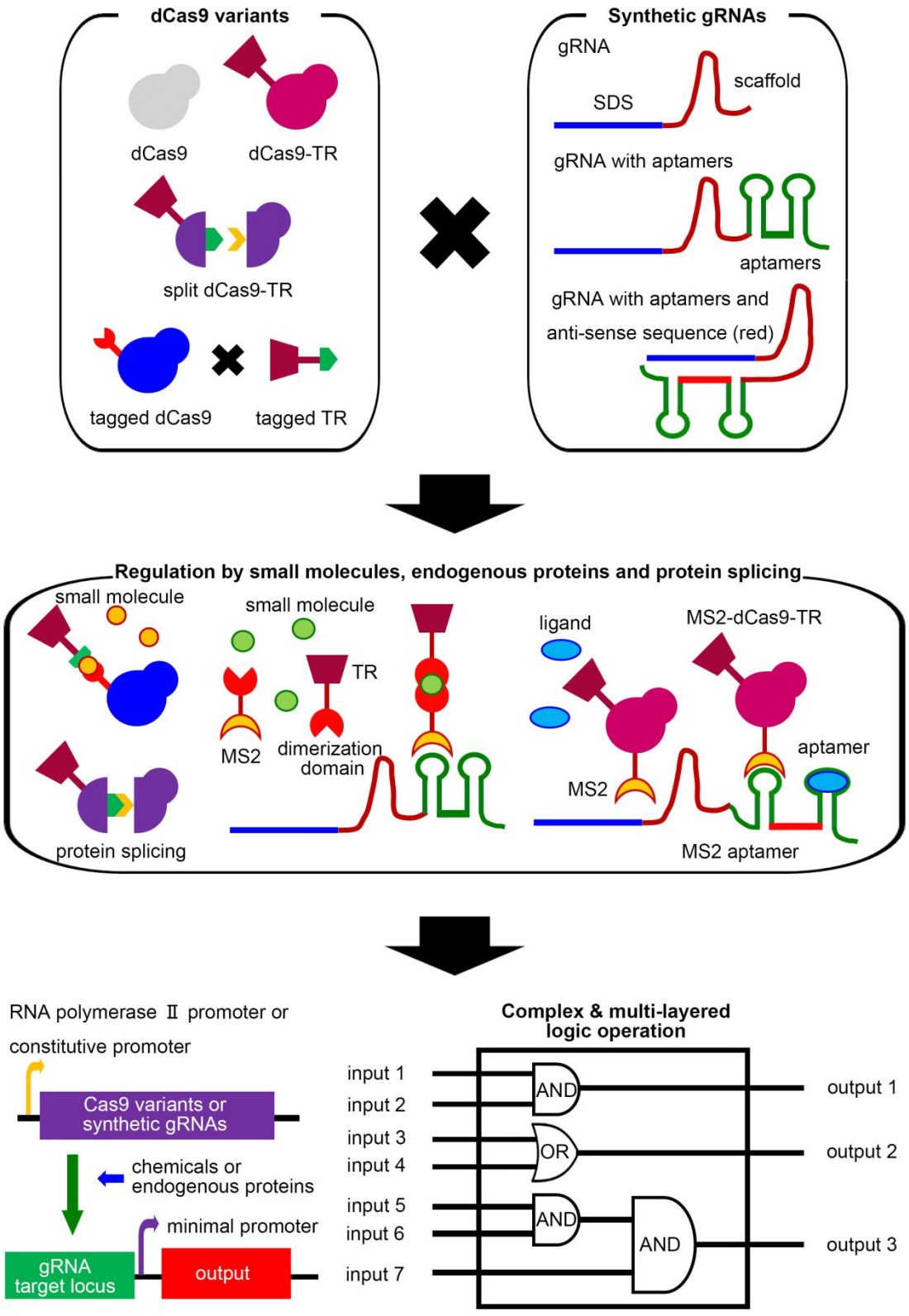


Figure 3


A
dCas9-EGFP
B
Fluorescent protein with aptamer-binding protein
Modified gRNA with RNA aptamer
C
Split fluorescent protein
SunTag
RNA aptamer
D
Sp dCas9-GFP
St1 dCas9-RFP
Nm dCas9-BFP
E
Modified gRNAs with multiple RNA aptamer combinations
Fluorescent proteins with aptamer-binding proteins

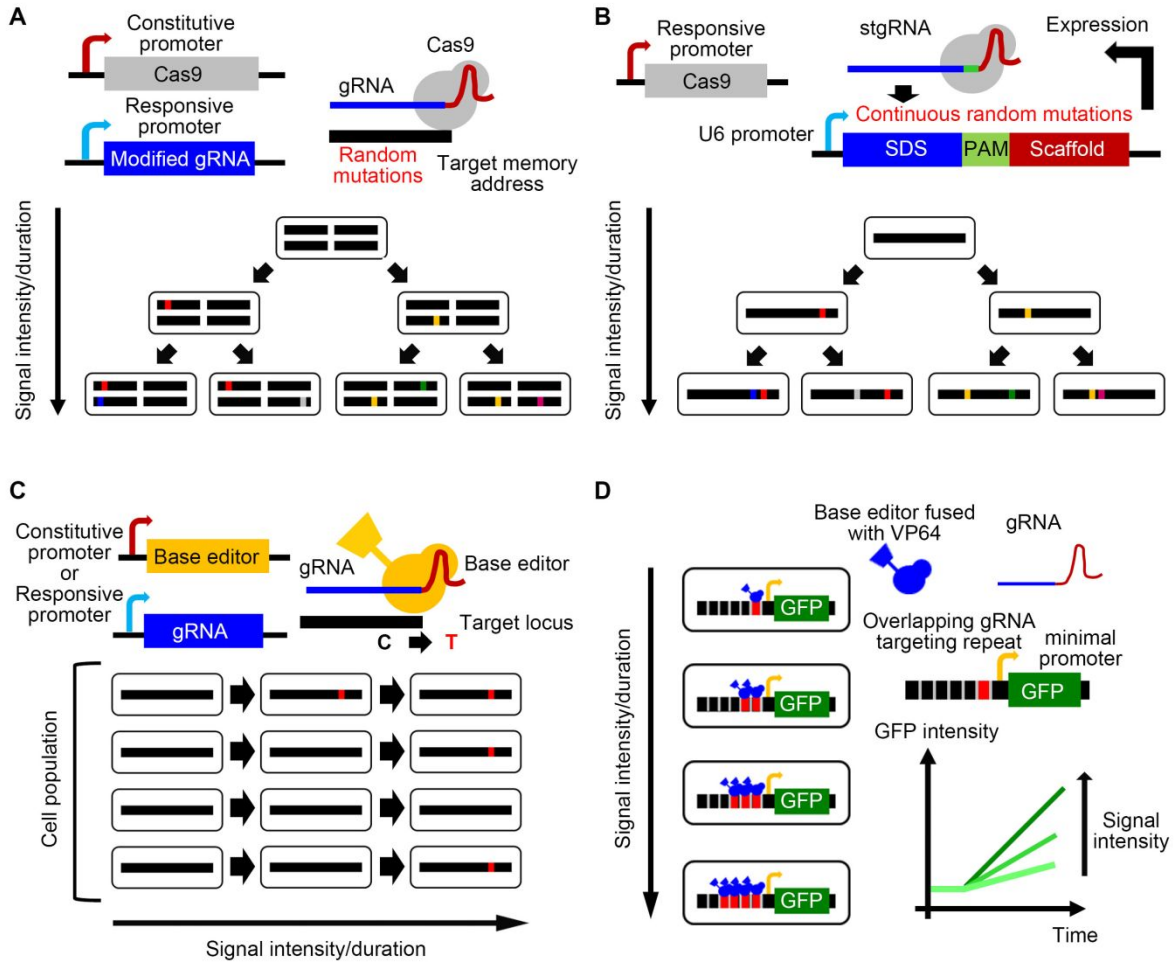
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Figure 4



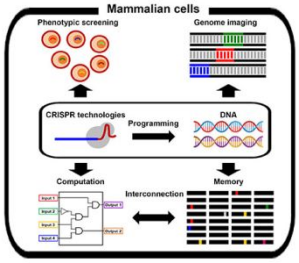
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Figure 5



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Advancing CRISPR-Based Programmable Platforms beyond Genome Editing in Mammalian Cells

Yasutomi Higashikuni, Timothy K. Lu