TrAC Trends in Analytical Chemistry, Volume 190, May 2025, Article number 118297
DOI:10.1016/j.trac.2025.118297

CRISPR-enabled genetic logic circuits for biosensing

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Abstract

Synthetic biology aims to engineer genetic circuits for custom-designed behaviors in living systems, including sophisticated biosensing applications. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system has gained attention for its potential in genetic circuit design due to its modularity, programmability, precision, and orthogonality. Here we highlight the current CRISPR-based tools for gene regulation at both transcriptional and translational levels. We discuss how these CRISPR technologies facilitate the design and construction of complex genetic circuits that can perform customized logic computations within living systems. Furthermore, we summarize the applications of CRISPR-based genetic logic circuits in biosensing, emphasizing their potential for detecting diverse biological and environmental signals. Finally, we highlight the key challenges facing the development and application of CRISPR-enabled genetic logic circuits and propose directions for future research to overcome these bottlenecks.

Keywords: CRISPR-enabled gene regulation; Genetic circuit; Genetic logic gate; Biosensing; Biocomputing

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The final published version (version of record) is available online at DOI:10.1016/j.trac.2025.118297. Please refer to any applicable publisher terms of use.

1. Introduction

Cells in nature are capable of sensing extracellular and intracellular signals, utilizing their intrinsic signaling systems to initiate appropriate responses and adapt to the ever-changing environment. Such natural processes have inspired synthetic biologists to create synthetic gene circuits in a bottom-up approach by integrating naturally occurring or engineered genes and proteins. This approach enables the programming of cells to execute specific tasks, thereby facilitating applications in diverse fields such as medicine, biofuel production, commodity chemical manufacturing, and biosensing [1–5].

Synthetic gene circuits generally comprise three functional modules: the sensor, the processor, and the actuator [6,7]. The sensor is capable of monitoring environmental or internal signals. The processor subsequently integrates these signals through a synthetic gene regulatory network to facilitate logical decision-making. Ultimately, the actuator produces proteins or biochemicals that lead to final phenotypic changes as outputs. Since the design and construction of the genetic toggle switch [8] and the repressilator [9], synthetic gene circuits have made significant advancements, enabling the implementation of a variety of user-defined functions, such as Boolean logic computation [10–15], oscillations [16], memory [17,18], timing [19], pattern formation [20–22].

The motivating vision of synthetic biology is to modularize and standardize biological components, including proteins and RNA, thereby allowing their assembly in a "plug-and-play" manner akin to their electronic counterparts. This objective requires biological components to operate together with minimized crosstalk in the cellular environment. To date, numerous natural biological components, such as transcription factors [13,23], recombinases [24], riboswitches [25], and ribozymes [26], have been engineered and repurposed as regulatory parts to build gene circuits. Despite these advances, the shortage of well-characterized and orthogonal parts continues to constrain the large-scale design of robust genetic circuits.

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) regulatory systems, characterized by their high programmability, modularity, and orthogonality, hold immense

potential for constructing large-scale synthetic gene circuits and addressing many limitations associated with previous biological components. In this review, we introduce current CRISPR-based gene regulation tools, primarily including the CRISPR activation and interference systems. We then provide an overview of how the CRISPR tools enable the design of synthetic genetic circuits with various customizable functions. Ultimately, we review the potential applications of CRISPR-enabled genetic logic circuits in biosensing.

2. Repurposing CRISPR system for transcriptional and translational regulation

CRISPR was initially recognized as a defense mechanism in bacteria and archaea against invading genetic elements such as viruses and plasmids, and was once thought to be exclusive to these organisms [27–29]. Recent research has uncovered its presence in a wider range of biological systems, such as phages [30]. This finding suggests that CRISPR systems emerged as parts of a broader genetic arms race among various genetic elements rather than solely as the "immune mechanism of bacteria" [31].

Mechanistically, the CRISPR system is activated by the complementary pairing of guide RNA (gRNA) with its target DNA or RNA, allowing precise discrimination between self and non-self nucleic acids to defend against potential invaders. CRISPR/Cas9 and CRISPR/Cas12a (also known as Cpf1) are among the most widely used RNA-guided endonucleases, as these systems utilize a single effector for DNA targeting [29]. The gRNA of the CRISPR/Cas9 system consists of two parts: the trans-activating CRISPR RNA (tracrRNA) and the CRISPR RNA (crRNA), while CRISPR/Cas12a generally contains a single crRNA [32]. The tracrRNA pairs with the crRNA to form a chimeric complex that acts as a handle to bind Cas9. The crRNA includes a programmable spacer sequence, which directs the CRISPR complex to its target DNA by recognizing a specific PAM (protospacer adjacent motif, NGG for Cas9 from *Streptococcus pyogenes*) sequence. These two RNA components can be fused into a single-guide RNA (sgRNA), which is commonly used in CRISPR-based engineering applications [29]. Unlike the two CRISPR systems above, the CRISPR/Cas13 system naturally targets RNA, facilitating the advancements of synthetic biology technologies for targeted gene

manipulation on the translational level [33].

By mutating these Cas proteins into nuclease-null mutants (referred to as dCas) to abolish their endonuclease activities and subsequently fusing a functional domain (repressor or activator) to the dCas protein, the CRISPR can be repurposed to interfere with (CRISPR interference, CRISPRi) or activate (CRISPRa, CRISPR activation) with target genes expression on either transcriptional or translational level **(Table 1)**.

2.1 CRISPR interference (CRISPRi) system

The CRISPRi system employs dCas9 or dCas12a proteins as programmable "roadblocks" for transcriptional repression or termination (**Figure 1A**), collectively called the CRISPRi system. This system prevents RNA polymerase (RNAP) access to promoters or blocks RNAP progression during elongation [34–36]. The first dCas9-based CRISPRi system achieved up to 1000-fold repression in *E. coli* when targeting the non-template DNA strand, whereas targeting the template strand resulted in significantly lower effects [34,35]. The binding specificity and strength of the dCas9-sgRNA complex are primarily dictated by the ~20-nucleotide spacer sequence, wherein the PAM-proximal seed region (7–12 bp) plays a crucial role [37]. Mismatches in the seed region severely reduce binding efficiency, decreasing gene repression by 70%–90% in CRISPRi assays, while the PAM-distal region can tolerate more mismatches [38].

Unlike in bacteria, dCas9 alone is insufficient to repress target gene expression in eukaryotes, particularly in mammalian cells [35]. To address this limitation, repressor domains such as Krüppel-associated boxes (KRAB), Mxi1, or SRDX have been fused to dCas9 to enhance its repression capabilities in mammalian cells, yeast, and plants **(Figure 1B)** [39–41]. Despite the improved repression efficiency, this fusion strategy restricts the valid binding sites of the dCas9-repressor complex to a DNA region spanning -50 to +300 bp relative to the transcription start site (TSS) [40]. More advanced systems with additional functionality go beyond the direct genetic fusion of repressor domains to dCas9. Instead, the sgRNA can be engineered with RNA aptamers, such as MS2, PP7, com, or BoxB, which are specifically recognized by RNA-binding proteins to form a scaffolding RNA (scRNA) **(Figure 1C)** [42,43]. Furthermore, additional Cas proteins beyond Cas9 have been discovered over the

past decade, including Cas12a. By harnessing similar strategies used in CRISPR/dCas9, the CRISPR/dCas12a system has been repurposed for CRISPRi applications, either by using dCas12a alone or by fusing a repressor to dCas12a [44–47].

2.2 CRISPR activation (CRISPRa) system

Tethering a repressor to dCas functions as a gene "turn-off" tool, while activators can be either directly fused to dCas or recruited via an engineered sgRNA scaffold to serve as the CRISPR activation (CRISPRa) systems. Several activator domains based on sigma 70 factors, such as the omega subunit of RNAP, AsiA, TetD, and SoxS from *E. coli*, have been used in CRISPRa systems **(Figure 1D** and **1E)** [34,48,49]. Due to the "unclosed" property of sigma 70 dependent promoters, these systems generally suffer from low dynamic ranges in bacteria. Unlike these sigma 70-dependent promoters, the sigma 54-dependent promoters form a "closed" complex with sigma 54 factors, which blocks the transcription initiation until a sigma 54 activator unlocks the complex **(Figure 1F)** [50]. This unique mechanism leads to an inherent low leakiness from the sigma 54 promoter-based regulatory tools. Building on this, our group has developed a eukaryote-like CRISPRa system based on sigma 54 promoters in bacteria, which supports multi-input regulation with high dynamic ranges. This work fills the gap in bacterial CRISPRa systems, which heavily relied on sigma 70-dependent promoters [51].

Early studies in eukaryotes showed transcriptional activation by fusing VP64 (four copies of herpes simplex viral protein 16) or the nuclear factor kappa B (NF-κB) subunit p65 to dCas9 [39]. Effective activation often requires multiple copies of dCas9–VP64 or even dCas9–VP160, and strong activation of native genes typically needs three or more gRNAs per promoter [40,52–55]. Although incorporating multiple gRNA binding sites can address this issue, it limits the ability to regulate many genes simultaneously when constructing complex gene circuits [55]. Since then, several strategies have been developed to expand the eukaryotic CRISPRa system. For example, Chavez *et al.* fused a tripartite VPR domain, comprising VP64, p65, and Rta activation domains, to dCas9 **(Figure 1G)**. The three activator domains could interact with distinct subsets of regulatory proteins, thus synergistically improving the activation performance by over tenfold compared to that of VP64 alone [56]. A three-component SAM system was then developed to enhance the effectiveness of the

CRISPRa system further. This system consists of a VP64-fused dCas9, a scRNA incorporated with two MS2 aptamers, and an activator complex (MCP+p65+HFS1) that can be recruited by MS2 aptamers **(Figure 1H)** [57]. Furthermore, a repetitive peptide array that forms a protein scaffold referred to as SunTag, was harnessed to recruit multiple copies of antibody-fused VP64 to activate target promoters efficiently **(Figure 1I)** [58].

2.3 CRISPR-RNAi/a (interference/activation) system

The CRISPRa/i system mentioned above primarily functions via transcriptional regulation. In bacteria, multi-gene operons are susceptible to polar effects, whereby the repression or activation of genes can inadvertently lead to the silencing or enhancing of downstream genes, complicating the independent regulation of individual genes. To address this challenge, CRISPR/dCas13-enabled gene regulation at the translational level has been developed, referred to as CRISPR-RNAi (interference) and RNAa (activation). For example, dCas13 could act as a physical hindrance to block ribosomes during translation initiation without influencing mRNA stability **(Figure 1J)** [59]. By fusing a translation initiation (IF) factor to dCas13a, this system can serve as a gene activation tool on the translation level when targeting the 5' end of the untranslated region of mRNA of a reporter gene **(Figure 1K)** [60]. A CRISPR-RNAi system utilizing catalytically deactivated hfCas13X (dhfCas13X) was developed to regulate gene translation, accompanied by the design of mRNA–crRNA pairs to construct the DiCRISPRa (degradation-inhibited CRISPRa) and TsCRISPRa (translation-started CRISPRa)systems. The two systems can be harnessed to activate downstream gene translation by enhancing mRNA stability or initiating translation. Furthermore, the fusion of dhfCas13X with the RNA-binding chaperone BHfq significantly enhanced the activation efficiency of these systems by 43.2-fold [61]. By coupling CRISPRa and CRISPR-RNAi, individual genes within a synthetic multi-gene operon can be selectively activated [62]. To date, only a few CRISPR/dCas13-based regulatory systems have been developed and have not garnered significant attention.

3. CRISPR/dCas system-based genetic circuit design

To date, CRISPR/dCas system has been extensively utilized in constructing gene circuits,

including Boolean logic gates (Buffer, NOT, AND, NOR, OR, NAND, XOR, XNOR, NIMPLY gates), toggle switch, IFFL (incoherent feed-forward loop), oscillator, and half adder in bacteria, yeast, mammalian cells, and cell-free systems **(Figure 2)** [63–73].

The CRISPR regulation system consists of multiple biological parts, including a dCas9 protein, a crRNA, a tracrRNA, and an effector domain. This property naturally simplifies the construction of single-layer, multi-input genetic logic circuits. For example, dCas9 and MCP-VP64 were controlled under a galactose-inducible or estradiol-inducible promoter to construct an AND gate in yeast [64]. A readable output was only observed when both signals were present. Likewise, a NAND gate was developed based on the CRISPRi system, and the two components, a sgRNA, and a dCas9, were driven by two inducible promoters [73]. However, harnessing dCas9 and effectors as the input signals do not support these logic gates to be used as modules in constructing multi-layered circuits since they are required at each layer [64,74,75]. Further, this shortcoming was addressed by harnessing natural crRNA and tracrRNA as two-input signals [76] or reengineering sgRNA as a conditional scRNA [77].

The CRISPR regulation system inherently functions as one-input Buffer gates (CRISPRa) or NOT gates (CRISPRi). Theoretically, both the CRISPRi and CRISPRa systems can support multi-layered cascades. However, the CRISPRi system exhibits better signal layering and integration efficiency than the CRISPRa. In *E. coli*, a set of CRISPRi-based NOT gates was designed by harnessing reprogrammed sgRNA to repress its corresponding promoter [66,69,78]. Subsequently, these NOT gates were connected to construct muti-layered genetic logic gates, including a NOR gate and a 3-gate circuit consisting of four layered sgRNAs. Moreover, a non-toxic dCas9 variant, dCas9*_PhlF, was developed to achieve relatively high dCas9 expression and supports the simultaneous use of up to 15 sgRNAs, enabling the construction of NOT gates with a dynamic range exceeding 10-fold [72]. Furthermore, the same group discovered that designing sgRNA-binding regions can enable reversible repression through competitive binding, a process known as derepression [79].

Working with dCas9 presents unique challenges compared to protein-based gates. The lack of saturation in the response function causes signal degradation when layering logic gates. Without an RBS to tune, it is difficult to address this issue through gate modification [66]. This

leads to the degradation of signaling transduction at each layer while constructing a multi-layered circuit [66]. The lack of cooperativity and non-linearity prevents CRISPRi system circuits from achieving key behaviors, such as bistable (toggle) switches and oscillators [66]. Such a gap was filled by indicating that cooperativity is not an insurmountable obstacle for building CRISPRi-based circuits. A bistable toggle switch and an oscillator [80,81], a seven-layer NOR gate [65], and a half-adder circuit [69] were successfully constructed by harnessing the CRISPRi system. The CRISPR/dCas12-based synthetic oscillator exhibits much more regular fluctuations than the one built using CRISPR/dCas9 [80]. By combining CRISPRi and CRISPRa systems, multi-layered CRISPRa/i circuits were successfully constructed in bacteria, mammalian cells, and the cell-free system [71,82]. Others have reviewed more details about CRISPR-based genetic logic gates [83–85].

4. CRISPR-enabled genetic logic circuits for biosensing

While various methods for constructing gene circuits based on the CRISPR regulation systems have been successfully explored, applying these circuits in biosensing presents an exciting frontier, offering new possibilities for real-time monitoring and detection of biological signals [86–88].

Intracellular proteins are key signals for distinguishing normal cells from cancer cells. Fusing signal-responsive RNA aptamers to sgRNA can construct a protein-responsive CRISPR-based AND gate to identify and inhibit bladder cancer cells. This AND logic circuit has been used for cell-type specific classification by recognizing β-catenin (CTNNB1) and NF-κB (REL-A), which are typically highly expressed in cancer cells but not in normal cells. By sensing the two oncogenic signals, the AND gate was utilized to rewire the cellular signaling pathway to selectively trigger apoptosis by activating BAX expression and repressed BCL2 expression in cancer cells without affecting normal cells **(Figure 3A)** [67].

Furthermore, miRNAs/mRNA have emerged as promising biomarkers closely associated with disease development. Thus, detecting miRNAs/mRNAs is extremely important for tracking disease progression. CRISPR-based logic gates enable the detection of multiple miRNAs/mRNAs in a single test. For example, in eukaryotes, gRNAs transcribed by RNA

polymerase II are repressed by a 5′ cap and 3′ poly (A) tail, preventing CRISPR-based regulation. Introducing miRNA binding sites flanking the gRNA enables miRNA-triggered cleavage and further release of active gRNAs. Further, this strategy can be combined with strand displacement reactions to develop a CRISPR-based AND gate circuit. This AND gate is capable of detecting both miRNA and mRNA, thereby enhancing RNA sensing accuracy **(Figure 3B)** [89,90]. In the future, integrating the multipartite RNA self-assembly into sgRNA engineering will scale up the CRISPR-based genetic logic gates for multiple miRNAs/mRNAs detection [91].

Besides engineering a single sgRNA for biosensing, programming crRNA-tracrRNA hybridization could also realize a two-input AND gate in bacteria **(Figure 3C)** [76]. By redesigning the matching region of crRNA and tracrRNA, our group constructed an orthogonal library of crRNA-tracrRNA pairs, which can further be used to create CRISPR-based AND gates [76]. The high programmability of crRNA-tracrRNA hybridization was then leveraged to repurpose endogenous small RNAs/mRNAs as crRNAs, which were hijacked by tracrRNAs to trigger CRISPR functions. This finding allowed CRISPR/Cas9 to be implemented as RNA sensors to detect RNA molecules both *in vivo* and *in vitro* [76]. A similar observation was also reported in CRISPR/Cas9 from *Campylobacter jejuni* [92] and leveraged by sensing specific cellular transcripts as the RNA recording tool [93]. Recently, following the same design, reprogrammed tracrRNAs for biosensing were extended into multiple CRISPR/Cas12 systems, hinting that any CRISPR/Cas systems containing crRNA and tracrRNA have the potential to be repurposed as an RNA sensor for RNA detection.

Beyond detecting intracellular proteins and RNAs, sensing extracellular signals is critical for regulating biological processes in engineered cells, which is a fundamental aspect of remotely controlled, precision-guided medicine. By fusing complementary heterodimerization domains to dCas9 and a transcriptional effector, these components form a functional complex only in the presence of an external inducer **(Figure 3D)**. These heterodimerization domains can be induced by chemicals including abscisic acid (ABA) [94], gibberellin (GA)-inducible GAI-GID1 [95], and lights containing red-light [96,97] and blue light [98]. Combining this design with orthogonal dCas9, a range of genetic logic gates were constructed to sense signals

to alter phenotypes of mammalian cells [68]. Furthermore, split-dCas9 is another powerful approach providing spatiotemporal control of gene expression, and this strategy can be further harnessed to construct genetic logic gates for biosensing applications [99]. Our group has previously developed a library containing 15 pairs of orthogonal split inteins and could further expand the split strategy on constructing genetic circuits for sensing multiple signals [100].

Combining CRISPR regulation with specific inducible promoters for sensing cell-specific signals enables selectively identifying and treating bladder cancer cells by using a CRISPR-based AND gate **(Figure 3E)**. These promoters include the tumor cell-specific promoter hTERT driving dCas9 and the bladder cell-specific promoter hUP II driving a gRNA targeting a *lacI* transgene [75]. In their design, the dCas9–gRNA complex inhibited *lacI*, thereby alleviating lacI-mediated repression of the output transgene. Consequently, the output transgene was expressed exclusively in cells where both hTERT and hUPII promoters were active, specifically in bladder cancer cells. Likewise, a tumor-targeted immune-rewiring genetic circuit was developed by leveraging a AND-NOT logic gate **(Figure 3F)** [74]. This circuit enables the detection of specific tumor signals for tumor cell identification, followed by the activation of the immune response. Although CRISPR logic gates for biosensing are still in their infancy and primarily based on "simple" logic gates, these circuits have demonstrated significant potential for detecting biomarkers or specific RNA in diagnostic and therapeutic applications.

5. Challenges and future perspectives

CRISPR-assisted biosensing has emerged as a cutting-edge analytic approach, enabling sensitive, specific, and accurate detection of diverse biomarkers. Currently, CRISPR-enabled biosensing platforms, such as the SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) system [101], the CARMEN (Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids) system [102], and the AGATHA (Atypical gRNA-activated Transcription Halting Alarm) system [76], have developed for *in vitro* RNA detection with great potential for commercial applications. To date, CRISPR-enabled genetic logic circuits for biosensing within living systems remain in proof-of-concept demonstrations. CRISPR

regulation system is distinguished by its straightforward programmability, wide dynamic range, and excellent compatibility with other transcriptional regulation methods [29,103,104]. With these properties, nearly all types of genetic logic gates were constructed based on CRISPR in bacteria [66,71], mammalian cells [69], yeast [65], plants [105] and even cell-free systems [71]. CRISPR-based logic circuits have been applied in biosensing for diagnostics and therapeutics, as mentioned above. Despite the promise, implementing multi-layered CRISPR gene circuits for complex signal detection and further application is still hindered by significant challenges.

One of these challenges in devising CRISPR-based genetic circuits for biosensing is "retroactivity" [72,106–108]. In a multi-layered CRISPR gene circuit, several sgRNAs need to compete for a shared pool of dCas9, which leads to an unpredictable change in the performance of the circuits. Therefore, building multi-layered gene circuits requires relatively high expression levels of dCas9, which may cause toxicity in cells, particularly in bacteria [72,109,110]. As previously mentioned, a fusion protein, dCas9*_PhlF, was developed to reduce the non-specific binding of dCas9 and alleviate toxicity. To date, it has only been applied in the CRISPRi systems in bacteria, requiring both the PhlF operator and the sgRNA binding site to repress a target promoter. These limitations hinder the scalable design and construction of CRISPR-based genetic circuit design. We believe that mining the extensive collection of sequencing data to discover novel CRISPR or CRISPR-like systems will be beneficial for designing both CRISPRi- and CRISPRa-based genetic circuits.

Another potential limitation of bacterial CRISPRa systems is their low dynamic range compared to CRISPRa systems in eukaryotes. Currently, most bacterial CRISPRa systems are developed based on the sigma 70 promoter, with the reporter controlled by a weak but constitutive promoter. This design results in low dynamic activation output [34,48]. To address this, we constructed a eukaryote-like CRISPRa system based on sigma 54-dependent promoters, exhibiting a dynamic range exceeding 1000-fold. Although this high dynamic range was achieved, we observed that dCas9 is highly sensitive to sgRNA, leading to leakiness when inducible promoters are used to drive the two components. This leakiness can be further reduced by using a PAM-flexible dxCas9, enabling the construction of a two-layer

CRISPRa circuit.

Currently, CRISPR-enabled genetic logic gates for biosensing are primarily limited to two-input designs, which restrict their ability to detect multiple signals simultaneously. The development of multi-input logic gates could enable the creation of more sophisticated biosensors capable of detecting several biomarkers at once, significantly enhancing their diagnostic capabilities. For example, Xie *et al*. developed a multi-input RNAi-based logic circuit that can sense the expression levels of a customizable set of endogenous microRNAs. By detecting these endogenous signals, this system can identify HeLa cancer cells and trigger cancer cell apoptosis without affecting non-HeLa cells [111]. The high sensitivity of the Cas9 protein to sgRNA, combined with the ability to repurpose sgRNA or utilize specific inducible promoters for detecting diverse signals, such as RNAs, proteins, or chemicals, enables the development of highly sensitive and multifunctional biosensors. The coupling of split intein and split intron with CRISPR regulation tools will enable the construction of multiple-input logic gates with enhanced specificity for biosensing [100,112]. These advanced genetic logic gates can further be applied in myriad fields, including cellular computation, disease diagnosis, and agricultural engineering (**Figure 4**).

Whole-cell biosensors have proven effective for environmental monitoring by detecting various toxic and pathogenic contaminants and these biosensors combine user-friendly operation, cost-effectiveness, and enhanced analytical performance, delivering exceptional sensitivity and high specificity in target recognition, making them particularly advantageous for accurate molecular diagnostics across diverse settings. For example, we have previously developed ultrasensitive bacterial sensors capable of detecting arsenic and mercury contaminants in aquatic environments [113]. However, current whole-cell biosensing platforms predominantly rely on transcriptional or translational regulation of gene expression, resulting in inherent limitations in detection speed. Furthermore, the integration of CRISPR-based nucleic acid recognition systems with these bacterial biosensors to achieve multiplexed detection capabilities remains unexplored in the field. We believe that combining these technologies could significantly enhance the sensitivity and versatility of biosensors for multi-target environmental detection. Currently, CRISPR-based genetic logic circuits are

being developed and tested across different chassis, resulting in variability in precision and accuracy. This inconsistency complicates direct comparisons. Quantitative characterization of these biosensors could provide systematic performance evaluations across various analytical methodologies. Moreover, adopting a standardized chassis would facilitate these evaluations, helping researchers identify the most effective methods for real-world applications, similar to standard practices for *in vitro* detection [114,115]. CRISPR-enabled genetic logic circuits have demonstrated exceptional capabilities in intracellular detection of nucleic acids and biomarkers. Additionally, electrochemiluminescence (ECL) biosensors offer high detection sensitivity while minimizing background interference [116,117]. This characteristic makes ECL an ideal platform for integrating with CRISPR systems, leading to the development of ultrasensitive and portable diagnostic platforms. Despite ongoing challenges related to multiplexing capabilities, clinical translation, and cost-effectiveness, recent advancements in nanomaterial design, microfluidic integration, and Cas protein engineering are propelling the evolution of CRISPR-powered electrochemical biosensors as transformative solutions for decentralized healthcare. Extensive discussions on the mechanisms and applications of CRISPR-ECL have been thoroughly documented in specialized reviews by other research groups [118,119].

While the construction of CRISPR-based genetic logic gates at the transcriptional level has been extensively explored, CRISPR-RNAi/a systems remain underutilized tools for designing genetic circuits at the translational level. Recently, CRISPR/Cas13 was harnessed to construct an AND gate for detecting multiple biomarkers, enabling the evaluation of pathological states and the autonomous implementation of responsive therapies [120]. To date, no study has reported the development of genetic logic gates based on CRISPR/dCas13 at the translational level. Thus, exploring CRISPR/dCas13-based systems to develop translational genetic logic gates could offer new opportunities for precise gene expression control and constructing more sophisticated synthetic circuits. Despite the existing challenges, CRISPR-based tools have demonstrated significant potential in designing synthetic genetic circuits for new cell-based therapeutics, diagnostic devices, and biosensors. The growing accumulation of data from CRISPR-based genetic circuit design enables the training of artificial intelligence models,

which will facilitate predictable integration and performance optimization of CRISPR system components for real-world applications.

Acknowledgements

This work was supported by National Natural Science Foundation of China (32320103001, 32271475), "Pioneer" and "Leading Goose" R&D Program of Zhejiang (2024C03011), National Key R&D Program of China (2023YFF1204500), Beijing Life Science Academy (2024200CA0070), Kunpeng Action Program Award of Zhejiang Province, and China Postdoctoral Science Foundation (2022M722780). ZY thanks Leverhulme Trust Research Leadership Award (RL-2022-041) and Visiting Professorship grant (VP1-2024-030). JC thanks the BioBit grant by Zhejiang Lab.

Authorship contribution statement

Xiyan Wang: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Yuanli Gao**, **Nan Zhou**, **Zhugen Yang** and **Jonathan M. Cooper**: Writing – review & editing. **Baojun Yang**: Conceptualization, Writing – review & editing, Project administration, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures and Table with legends

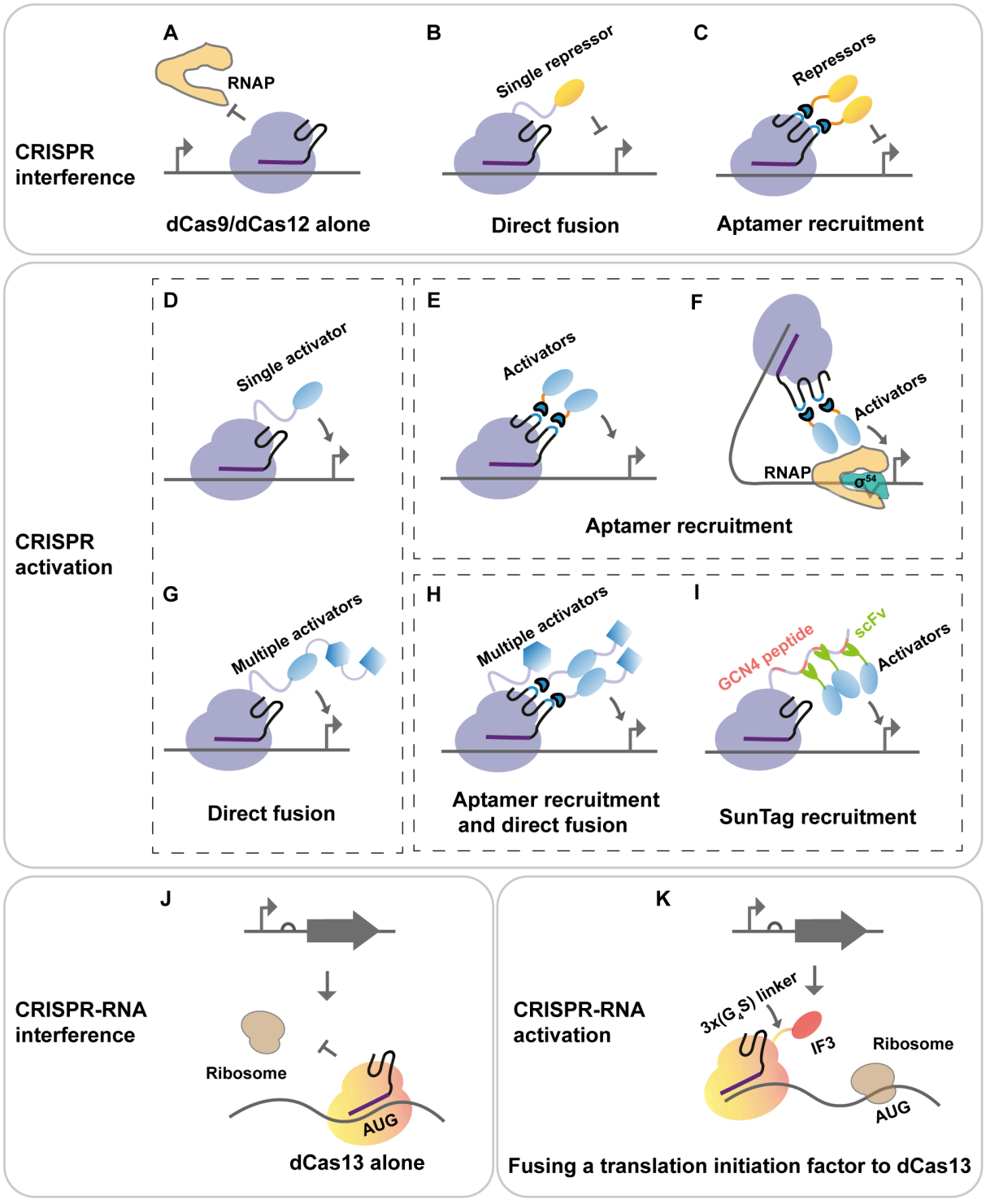


Figure 1. CRISPR-enabled gene regulation tools. Examples of the CRISPR interference (CRISPRi) system are shown in **(A-C)**, CRISPR activation (CRISPRa) in **(D-I)**, CRISPR/RNA interference in **(J)**, and CRISPR/RNA activation in **(K)**. **(A)** dCas9/dCas12 serves as 'roadblocks' for transcriptional repression [34–36]. **(B, C)** Direct fusion to dCas9 **(B)** [39] or sgRNA aptamer recruitment **(C)** [41] is necessary for sufficient repression in eukaryotes. **(D-F)** The CRISPR activation (CRISPRa) system in both prokaryotes and eukaryotes was built by direct fusion of activator(s) **(D)** [34,35] or sgRNA aptamer recruitment **(E** and **F)** [41,63]. **(G-I)** Multiple strategies were developed to enhance the efficiency of the CRISPRa system in eukaryotes, including the fusion of multiple activators to dCas9

(G) [56], the combination of direct fusion and sgRNA aptamer recruitment **(H)** [57], and a SunTag based recruitment **(I)** [58]. **(J)** By binding to mRNA, dCas13 can block the movement of ribosomes, referred to as CRISPR-RNAi (interference) system [59]. **(K)** Tethering a translational initiation factor to dCas13, this system can enhance gene expression referred to as CRISPR-RNAa (activation) system [60].

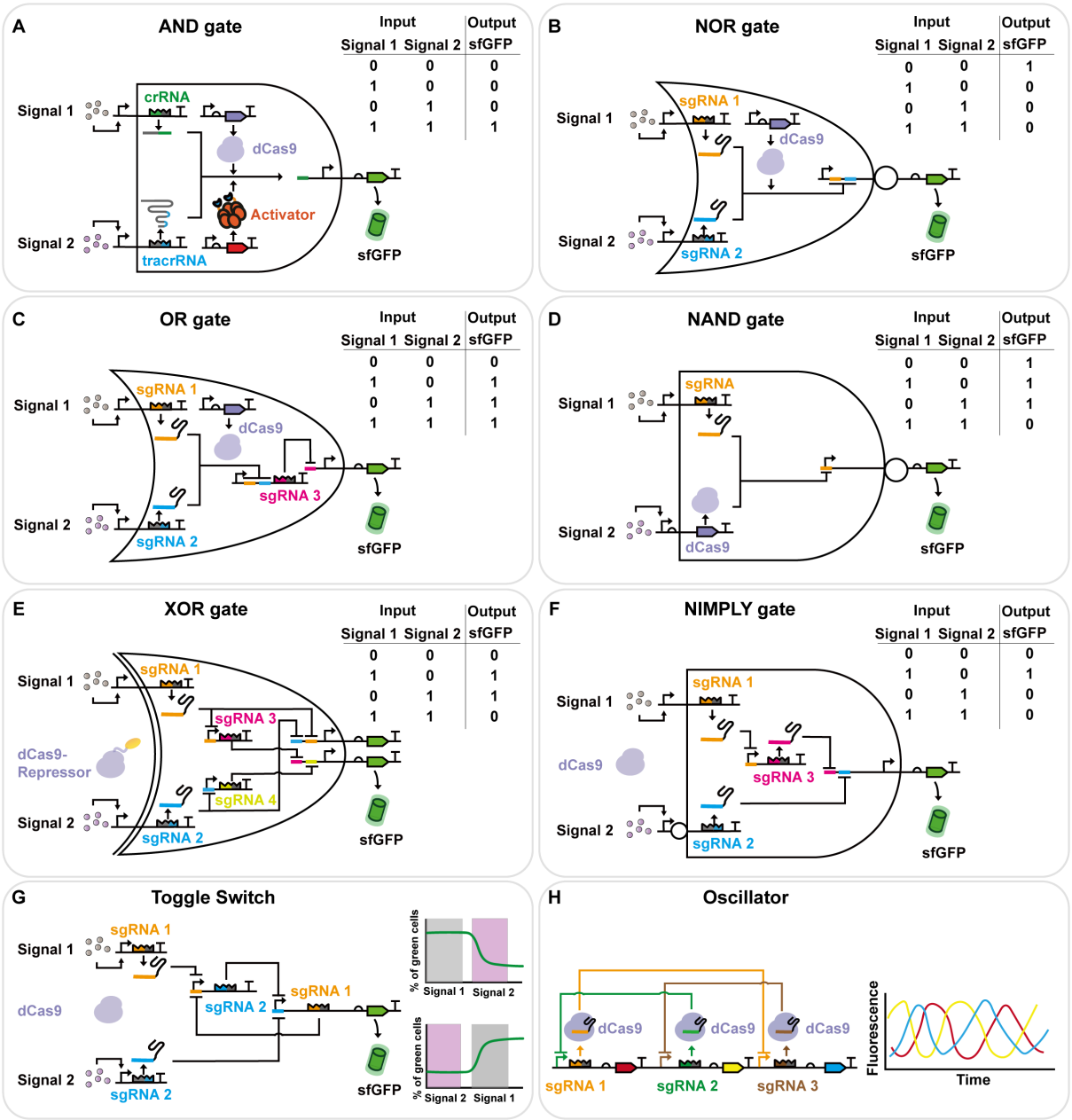


Figure 2. Examples of CRISPR-enabled genetic circuit design. (A) An AND gate is developed by harnessing natural crRNA-tracrRNA as two inputs [76]. The expression of the two RNAs can be induced only in the presence of both signals, triggering the CRISPRa system to activate sfGFP expression. **(B)** Two sgRNAs are driven by inducible promoters to build a NOR gate, and both of the sgRNAs can drive dCas9 to repress sfGFP expression [66]. **(C)** An OR gate is constructed using two sgRNAs (1 and 2) driven by inducible promoters. sgRNA 3 is controlled by a constitutive promoter. sgRNA 3 can guide dCas9 to repress sfGFP expression, while both the expression of sgRNA 1 and 2

can remove the repression [65]. **(D)** The dCas9 and a gRNA are driven by two inducible promoters to construct a NAND gate [65]; the expression of sfGFP is repressed when both components are present simultaneously. **(E)** An XOR gate is constructed using four sgRNAs and two reporters, with sgRNA1 and sgRNA2 regulated by inducible promoters and sgRNA3 and sgRNA4 by constitutive promoters. The design ensures output signals are produced only when a single input is active, while no output occurs when both or neither inputs are present [69]. **(F)** A NIMPLY gate is constructed using three sgRNAs, with sgRNA1 and sgRNA2 regulated by inducible promoters and sgRNA3 controlled by a constitutive promoter. sgRNA1 represses sgRNA3, enabling a detectable output signal when sgRNA1 is expressed [69]. **(G)** A toggle switch is designed using two sgRNAs regulated by both inducible and constitutive promoters. Inducible sgRNA1 inhibits constitutively expressed sgRNA2, and inducible sgRNA2 inhibits constitutively expressed sgRNA1. The circuit toggles between two stable states via inducer addition, with cells maintaining their state even after inducer removal [81]. **(H)** An oscillator is constructed using the CRISPRi system, wherein a dCas9-mediated closed-loop repression topology enables the generation of periodic oscillations in *E. coli* cells [81].

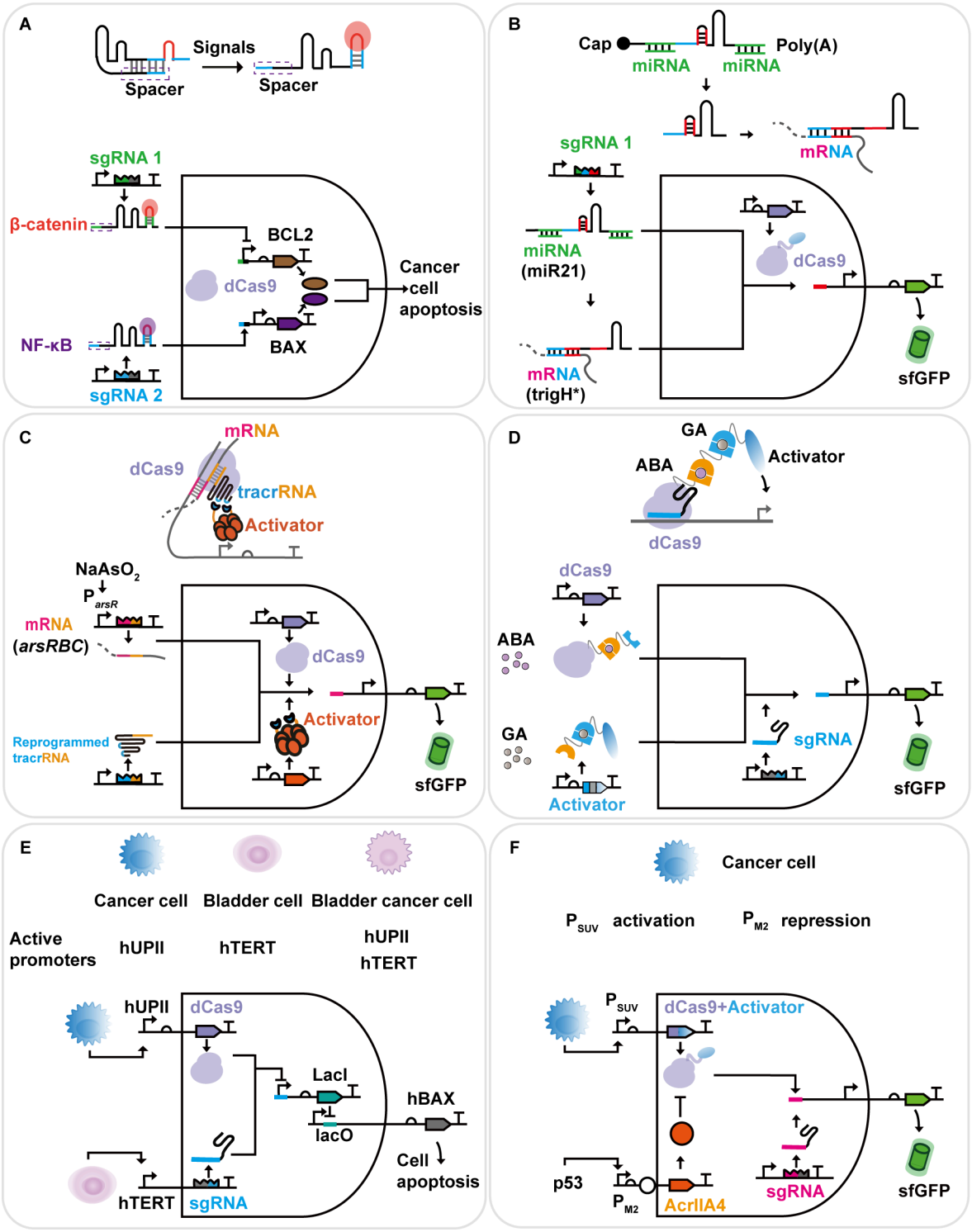


Figure 3. CRISPR-enabled genetic logic circuits for biosensing. **(A)** A CRISPR-based AND gate is designed to sense oncogenic signals, β-catenin (CTNNB1) and NF-κB (REL-A), which are overexpressed in bladder cancer cells but not normal cells. By detecting these signals, the circuit selectively induces apoptosis in cancer cells by activating *BAX* and repressing *BCL2*, sparing normal cells [75]. **(B)** Including miRNA binding sites flanking RNA polymerase II-transcribed gRNAs enables miRNA-triggered cleavage, releasing active gRNAs. This strategy enhances RNA sensing accuracy and facilitates the construction of a CRISPR-based AND gate capable of detecting both miRNA and mRNA [89]. **(C)** Reprogramming the tracrRNAs to hijack endogenous small RNAs/mRNAs as crRNAs

enables the repurposing of the CRISPR/Cas9 system as sequence-specific RNA biosensors [76]. **(D)** By fusing heterodimerization domains to dCas9, the CRISPR/dCas9 system can be repurposed to detect multiple extracellular signals for biosensing applications [68]. **(E)** Combining tumor-specific promoters with the CRISPR system, a CRISPR-based AND gate is harnessed to sense tumor-specific signals and to identify bladder cancer cells [75]. **(F)** The AND-NOT gate is built based on two tumor-specific promoters (P_{SUV}: tumor-specific promoter of survivin gene, P_{M2}: p53-responsive promoter) driven by the expression of CRISPR components and this design can be used to identify tumor cells, such as H1299 and Lewis lung carcinoma (LLC) [74].

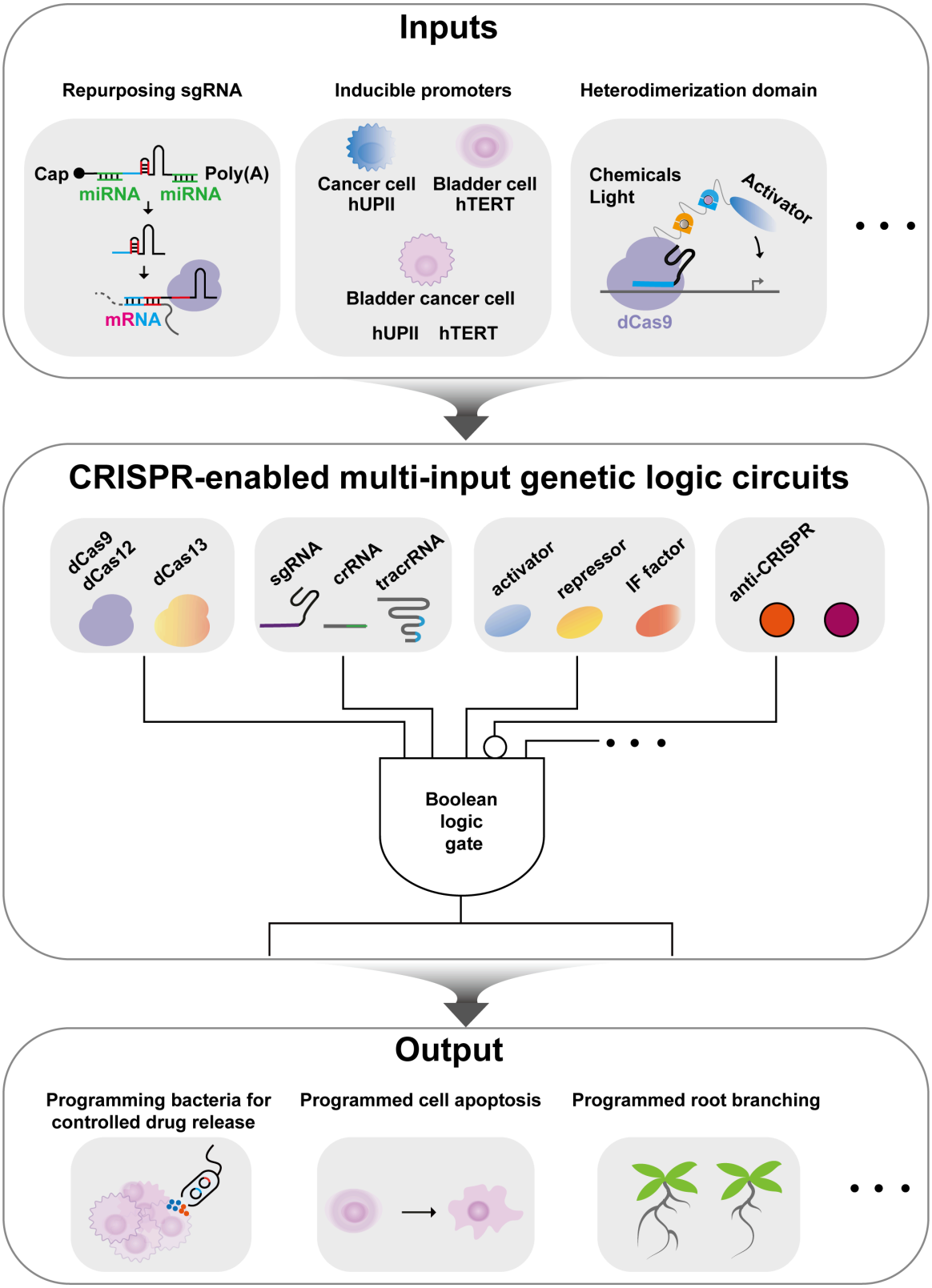


Figure 4. Design of CRISPR-enabled multi-input genetic logic circuits for advanced biosensing applications. Each component of the CRISPR regulatory system can be controlled by individual signals to construct multi-input genetic logic gates, which can then be harnessed for various output cellular controls including precision disease diagnosis, cancer therapy, and agricultural engineering.

Table 1. CRISPR-enabled gene regulation at transcriptional and translational levels.

Regulation level	Function	Cas protein	Functional domain	Fusion strategy	Chassis	References
Transcriptional regulation	CRISPRi	dCas9	-	-	Bacteria, yeast, mammalian cells	[34,39,40]5/29/2025 10:35:00 AM
	CRISPRi	dCas9/dCas12	KRAB	Directly fuse to dCas	Yeast, mammalian cells, arabidopsis	[41,64,121–123]
	CRISPRi	dCas9	+Com-KRAB	Fuse a com in sgRNA to recruit Com-KRAB	Yeast	[41]
	CRISPRi	dCas9	Mxi 1	Directly fuse to dCas	Yeast	[39,65,124]
	CRISPRi	dCas9/dCas12	SRDX	Directly fuse to dCas	Tobacco, arabidopsis, rice	[125]
	CRISPRi	dCas12	-	-	Bacteria, mammalian cells	[47,126]
	CRISPRa	dCas9	RNAP omega subunit	directly fuse to dCas9	Bacteria	[34]
	CRISPRa	dCas9	RNAP subunits	Fuse specific protein interaction domains to dCas9 and RNAP subunits	Bacteria	[127]
	CRISPRa	dCas9/dxCas9/dSpRY	MCP-SoxS	An aptamer (MS2)-modified single guide RNA (sgRNA) to recruit SoxS-MCP	Bacteria	[43,48,128]
	CRISPRa	dCas9/dxCas9	λN22plus-PspFΔHTH NorR WtsA	An aptamer (BoxB)-modified single guide RNA (sgRNA) to recruit λN22plus-PspFΔHTH	Bacteria	[63,76]5/29/2025 10:35:00 AM
	CRISPRa	dCas9	AsiA	Directly fuse to dCas9	Bacteria	
	CRISPRa	dCas9/dCas12a	VP64	Directly fuse to dCas9	Yeast, mammalian cells,	[35,52–54,129,130]

					arabidopsis, tobacco	
	CRISPRa	dCas9/dCas12a	p65	Directly fuse to dCas9	Mammalian cells	[39,131]
	CRISPRa	dCas9/dCas12a	VPR	Directly fuse to dCas9	Yeast, fruit fly, mouse and mammalian cells	[47,56]
	CRISPRa	dCas9	p300Core	Directly fuse to dCas9	Mammalian cells	[132]
	CRISPRa	dCas9	$SunTag_{10x_v4}$ -VP64	$SunTag_{10x_v4}$ was fused to dCas9 to recruit scFv-GCN4-VP64 complex	Mammalian cells	[58]
	CRISPRa	dCas9	MCP/PCP/Com-VP64	An aptamer (MS2/PP7/com) -modified single guide RNA (sgRNA) to recruit VP64	Yeast	[41]
	CRISPRa	dCas9	MCP-p65-Hsf1/MCP-VP64	An aptamer (MS2) -modified single guide RNA (sgRNA) to recruit VP64	Mouse and mammalian cells	
	CRISPRa	dCas9	MCP-6TAL–VP64/128	An aptamer (MS2) -modified single guide RNA (sgRNA) to recruit MCP-6TAL–VP64/128	Arabidopsis, rice cells, mammalian cells	[133,134]
Translational regulation	CRISPR-RNAa	dCas13/dCas13X	IF1/IF2/IF3/RNA chaperone protein	Directly fuse to dCas13	Bacteria	[60,61]
	CRISPR-RNAi	dCas13	-	-	Bacteria	[59,62,135]
	CRISPR-RNAi	Cas13d	-	-	Mammalian cells	[136,137]
	CRISPR-RNAi	dCas9	-	Insertion of sgRNA in 5’UTR of a reporter gene	Mammalian cells	[138]
Dual regualtion	CRISPRa/i & CRISPR-RNAi	dCas9& dCas13	MCP-SoxS	An aptamer (MS2)-modified single guide RNA (sgRNA) to recruit SoxS-MCP	Bacteria	[62]



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2025-09-01
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https://doi.org/10.1016/j.trac.2025.118297
Wang X, Gao Y, Zhou N, et al., (2025) CRISPR-enabled genetic logic circuits for biosensing. TrAC Trends in Analytical Chemistry, Volume 190, May 2025, Article number 118297

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