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CRISPR Tools for Engineering Prokaryotic Systems: Recent Advances and New Applications

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Abstract

In the past decades, the broad selection of CRISPR-Cas systems has revolutionized biotechnology by enabling multimodal genetic manipulation in diverse organisms. Rooted in a molecular engineering perspective, we recapitulate the different CRISPR components and how they can be designed for specific genetic engineering applications. We first introduce the repertoire of Cas proteins and tethered effectors used to program new biological functions through gene editing and gene regulation. We review current guide RNA (gRNA) design strategies and computational tools and how CRISPR-based genetic circuits can be constructed through regulated gRNA expression. Then, we present recent advances in CRISPR-based biosensing, bioproduction, and biotherapeutics across in vitro and in vivo prokaryotic systems. Finally, we discuss forthcoming applications in prokaryotic CRISPR technology that will transform synthetic biology principles in the near future.

INTRODUCTION

Synthetic biologists seek to understand and harness biological systems to engineer solutions in a wide variety of fields, ranging from chemical bioproduction to medicine, agriculture, and energy (1, 2). In the past decade, CRISPR-Cas tools have revolutionized biological research by simplifying the design and multiplexing of complex genetic functions. This ability to rapidly modify genetic material is streamlining reverse engineering of genotype–phenotype relationships and genetically encoding new desired functions. To expand beyond these applications, molecular tools that facilitate novel genetic manipulation are in great demand (3).

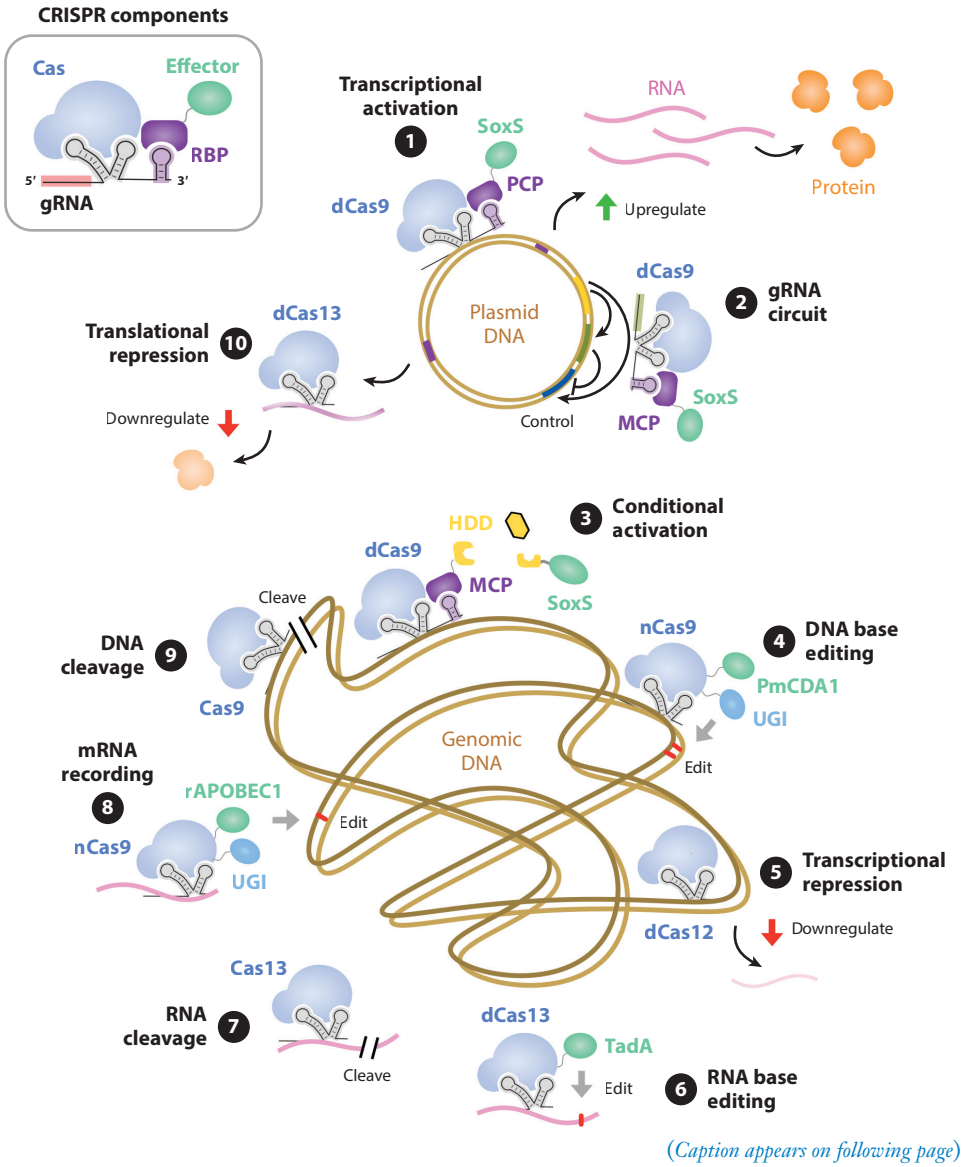
Sequence-specific DNA-binding domains, combined with different nucleases, transcriptional regulators, and base editors (BEs), have been successful in many genetic engineering applications (4–6). Protein-based tools consisting of DNA-binding domains derived from zinc-finger nucleases or transcription activator-like effector proteins offer robust and very precise behaviors (7). However, tuning their effect and designing these proteins to target multiple genes is relatively difficult. In contrast, CRISPR tools facilitate targeting of multiple sites simultaneously because targets are encoded through simple Watson–Crick interactions in short guide RNA (gRNA) sequences. In terms of designability, the discovery of new CRISPR systems, paired with a wide variety of tethered effectors, has greatly expanded the palette of genetic engineering tools (8–11).

Genetic engineering can be subdivided into (*a*) gene editing to change the underlying genetic information and (*b*) gene regulation to change how the information is processed. To date, a wide range CRISPR-Cas techniques have been developed for gene editing, from DNA breaks by Cas nucleases to large DNA integrations via CRISPR-assisted transposases (12–15). Base editing is another promising gene editing technique that introduces point mutations into DNA or RNA by using nickase or catalytically dead Cas proteins (nCas or dCas) to localize nucleobase editor enzymes to precise target sites (16–18). Additionally, many CRISPR-based tools are now available for up- and downregulation of gene expression by fusing dCas proteins to transcriptional or translational regulators. dCas9 was rapidly repurposed for programmable transcriptional repression, termed CRISPR interference (CRISPRi), by physically blocking the transcription initiation or elongation processes (13, 19). Other dCas proteins have also been implemented in the same fashion, including dCas9s from different species (20) and dCas12 (21, 22). The transcriptional activation counterpart, termed CRISPR activation (CRISPRa), was promptly developed in eukaryotes based on transcription factors widely applicable in any eukaryotic system, e.g., VP64 (23, 24). In contrast, prokaryotic CRISPRa lagged due to limited universal transcriptional activators and stringent design rules for implementation (25, 26). Nevertheless, these genetic engineering tools are now broadly applied in different bacteria (27).

The versatility of CRISPR gene regulation and gene editing tools has fueled many applications across diverse bacteria as well as cell-free systems (**Figure 1**). In bacteria, CRISPR tools are being used for rapid strain engineering in model organisms such as *Escherichia coli*, mainly for bioproduction applications (28, 29). More recently, CRISPR tools have also been implemented in non-model microbes such as *Cupriavidus necator*, *Rhodobacter sphaeroides*, and *Clostridium autoethanogenum* due to their unique metabolisms and wide range of substrate utilization, including CO_2 (30–33). Beyond metabolic engineering applications, biotherapeutic bacteria harboring CRISPR tools are now capable of recording changes in their environment and deploying novel antimicrobials (34–38). In cell-free systems, purified Cas enzymes and gRNAs are widely used as biosensors for in vitro diagnostics of nucleic acids, small molecules, proteins, and even bacteria (39, 40). Moreover, cell-free expression systems (CFES) that also contain the molecular machinery required for gene expression, such as PURE or lysate-based TXTL (41–43), have accelerated the characterization of new CRISPR systems and the development of CRISPR genetic circuits (44–47). In turn, these

advancements are paving the way for cell-free bioproduction platforms and biosensors that can multiplex input signals and launch appropriate CRISPR programs in response (42, 48–51).

This article reviews CRISPR-based genetic engineering tools developed to date and their cutting-edge applications across diverse bacteria and cell-free systems. For eukaryotic counterparts, several reviews have been published recently (18, 52–54). We first showcase the designability and modularity of CRISPR systems by covering the constituent components, namely the different Cas proteins, tethered effectors, and gRNAs, and how they can be designed for specific genetic engineering applications (**Figure 1**). We then examine how CRISPR tools can be integrated into genetic circuits, granting a new layer of programmability and a framework for genetic information processing. We describe how these different CRISPR systems are being used for



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

CRISPR tools for genetic engineering of prokaryotic systems. Schematic of different CRISPR tools and their modes of action. The top left panel depicts the main components of a CRISPR tool: Cas protein, guide RNA (gRNA), effector, and RNA-binding protein (RBP). For simplicity, different Cas proteins are depicted the same and labeled accordingly. Plasmid and genomic DNA can be used interchangeably. ① A dCas9-based transcriptional activation tool that recruits the SoxS activator through the RBP PP7 coat protein (PCP) and, once localized to a gene of interest, upregulates RNA transcription. ② A CRISPR circuit operating through the regulated expression of gRNAs that target other gRNAs and control a gene of interest. In this case, SoxS is recruited through the MS2 coat protein (MCP). ③ A transcriptional activation tool in which recruitment of the effector SoxS is mediated by heterodimerization domains (HDD) responsive to a small molecule. ④ An nCas9-based tool that uses *Petromyzon marinus* cytidine deaminase 1 (PmCDA1), which catalyzes the irreversible conversion of cytidine to uridine, and uracil DNA glycosylase inhibitor (UGI) for making DNA edits at a location specified by the gRNA. ⑤ A dCas12-based transcriptional repression tool capable of downregulating transcription of a gene of interest. ⑥ A dCas13-based tool tethered to transfer RNA adenosine deaminase A (TadA), which catalyzes the irreversible conversion of adenosine to inosine, capable of targeting specific RNAs for base editing. ⑦ A Cas13-based tool that cleaves target RNA and can trigger collateral cleavage activity of any RNA. ⑧ An nCas9 tool capable of recording the presence of a messenger RNA (mRNA) by making DNA edits with the tethered rat apolipoprotein B mRNA editing enzyme catalytic subunit 1 (rAPOBEC1). ⑨ A Cas9 system capable of making precise cuts of double-stranded DNA. ⑩ A dCas13 translational repression system that downregulates protein translation from a specific mRNA.

engineering complex biosensing, bioproduction, and biotherapeutics platforms. Lastly, we discuss the current challenges that CRISPR technologies encounter and explore next-generation CRISPR applications in prokaryotes.

ENGINEERING CRISPR COMPONENTS TO BUILD DIVERSE MOLECULAR TOOLS

The fast and wide adoption of CRISPR tools has been fueled mainly by two inherent properties: (*a*) designability of the CRISPR systems based on different Cas proteins, tethered effectors, and gRNA properties and (*b*) multiplexability of CRISPR programs based on orthogonal gRNAs. In this section, we describe the different CRISPR components, how they can be engineered, and how they can be combined in a modular fashion to suit specific applications.

The Different Types of CRISPR-Cas Proteins

Natural CRISPR-Cas systems differ greatly in their nucleic acid recognition capabilities and additional functionality, such as collateral cleavage and transposase activity (55). Currently, CRISPR-Cas systems are grouped into class 1 and class 2, differentiated by the architecture of the interference module involved in target recognition and nucleic acid cleavage. Class 1 CRISPR systems contain interference modules encoded in multiple genes. In contrast, class 2 CRISPR systems have interference modules within a single multi-domain protein (56). Although less abundant in nature, class 2 systems are more amenable to engineering efforts due to their compactness and therefore are the focus of this review. Class 2 systems are further subdivided into type II (Cas9), type V (Cas12), and type VI (Cas13).

Type II: the Cas9 workhorse for DNA targeting. The DNA binding activity of type II CRISPR systems can be controlled easily by an engineered small gRNA, making them particularly well-suited for DNA manipulation applications. In general, type II systems consist of a Cas9 protein and two RNAs, a *trans*-activating RNA (tracrRNA) and a CRISPR RNA (crRNA). The tracrRNA serves as the binding scaffold for the Cas9 protein, whereas the crRNA is the complementary sequence or spacer for the target DNA. In native systems, the tracrRNA and crRNA hybridize; however, many engineered systems fuse the two into a single guide RNA (sgRNA) with similar

performance in vitro (12). Cas9 DNA targeting requires two key features: (*a*) DNA target and gRNA spacer complementarity and (*b*) a protospacer adjacent motif (PAM) directly flanking the DNA target, necessary for preventing self-digestion of the gRNA encoding sequence. Mechanistically, Cas9 first scans DNA for potential PAMs and then checks guide–target complementarity. Once guide–target complementarity is validated (57, 58), the nuclease domains of Cas9 generate double-stranded breaks (DSBs) in the target DNA. Although mismatched spacer sequences can be tolerated, strict PAM requirements often limit Cas9 targeting (58, 59). For instance, the density of PAM sequences from the most widely used Cas9, SpyCas9 from *Streptococcus pyogenes*, is only ∼1 PAM (5′-NGG-3′) every 10 bases in the regions upstream of endogenous promoters in the *E. coli* genome (50.5% GC) (25). PAM density for SpyCas9 is potentially higher in organisms whose genome has high GC content (e.g., *Pseudomonas putida* 61.5% GC) but will be lower in AT-rich bacterial genomes (e.g., *C. autoethanogenum* 31.1% GC). Therefore, engineering non-model organisms with low GC content could be challenging with SpyCas9 alone.

To increase the range of PAMs and associated loci for targeting, many groups have bioinformatically mined for new orthologs (60, 61). Other type II Cas9 systems were found to have completely different consensus PAMs, a wide range of sizes, and distinct tracrRNAs (**Table 1**). For example, SmacCas9 from *Streptococcus macacae* has adenine-rich PAM preference (5′-NAAN-3′) (62). Sth1Cas9 and Sth3Cas9 from *Streptococcus thermophilus* have more stringent PAMs, 5′-NNAGAAW-3′ and 5′-NGGNG-3′; however, longer PAMs offer significantly lower off-target effects (63, 64). Computational pipelines to predict functional PAM sequences of uncharacterized CRISPR-Cas systems were also constructed (65, 66). For instance, a type II CRISPR system from *Methylocystis heyeri* was found with flexible PAM sequences (5′-GSNN-3′). Additional details on the size and compatibility of PAMs from different Cas9 orthologs beyond the scope of this review can be found in Collias & Beisel's (67) recent review.

Protein engineering has also been employed successfully to increase PAM flexibility (**Table 1**). These efforts have ranged from chimera construction to random mutagenesis, structure-guided design, and high-throughput PAM-determination assays (68, 69, 100–102). Hu et al. (68) used phage-assisted continuous evolution of the α-helical recognition domain to evolve expanded PAM SpyCas9 variants capable of targeting 5′-NG-3′ (xCas9–3.7), 5′-GAA-3′, and 5′-GAT-3′. In pursuit of similar goals, Nishimasu et al. (69) screened structure-guided designs to develop another expanded PAM variant, SpCas9-NG, also targeting 5′-NG-3′ PAMs. Comparison of xCas9 and SpCas9-NG demonstrated that SpCas9-NG has superior 5′-NG-3′ PAM recognition (101, 103). Similar engineering efforts were also conducted in other Cas9 variants (104–106). Overall, near-PAMless Cas9 variants, whose specificity depends predominantly on target–spacer complementarity, have been shown to improve target accessibility and efficacy in both eukaryotic (101–103) and bacterial systems (106–108).

However, PAM expansion can lead to extended timescales for target recognition and increased off-target effects (58, 59, 109). To address off-target effects, both rational engineering and enzyme selection approaches have been used to develop high-fidelity SpyCas9 variants with reduced off-target activity (110–115). Introducing mutations that confer high-fidelity traits into a PAM-expanded variant could also mitigate the off-target effect instigated by PAM flexibility (102). However, most high-fidelity variants remain uncharacterized in prokaryotes.

In the case of nuclease-deactivated Cas9 (dCas9), SpydCas9 has also been shown to bind non-specifically to NGG PAM sites (109) and to off-target genomic loci with up to six mismatched nucleotides that differ from the spacer (116). dCas9 exhibits toxicity rooting from this promiscuous DNA binding effect when highly expressed in bacteria (117, 118). Although previous work has demonstrated that Cas9 off-target binding directly scales with PAM flexibility (69, 100, 102), we observed that increased dCas9 PAM-flexibility has no additional growth defect compared to

Table 1 Class 2 Cas proteins prevalent in prokaryotic applications

Type	Cas protein	Sources	Size (aa)	PAM/PFS	Target substrates	Reference(s)
Type II	SpyCas9	*Streptococcus pyogenes*	~1,400	NGG	dsDNA	12
	xCas9-NG	SpyCas9[a]	~1,400	NGN	dsDNA	68
	SpRYCas9	SpyCas9[a]	~1,400	NRN > NYN	dsDNA	69
	SmacCas9	*Streptococcus macacae*	~1,400	NAA	dsDNA	62
	Sth1Cas9, Sth3Cas9	*Streptococcus thermophilus*	~1,200	NNAGAAW, NGGNG	dsDNA	63, 64
	ScCas9	*Streptococcus canis*	~1,400	NNG	dsDNA	70
	Nme1Cas9, Nme2Cas9, Nme3Cas9	*Neisseria meningitidis*	~1,000	N4GYTT, N4CC, N4CAAA	dsDNA	71
	SauCas9	*Staphylococcus aureus*	~1,000	NNGRRT	dsDNA	72
	CjCas9	*Campylobacter jejuni*	~1,000	N4RYAC	dsDNA	73
	SauriCas9	*Staphylococcus auricularis*	~1,000	NNGG, NAAN	dsDNA	74
Type V	AsCas12a, LbCas12a	*Acidaminococcus* sp., *Lachnospiraceae bacterium*	~1,500	TTTV	dsDNA, ssDNA	22, 75
	AsCas12a -RR	AsCas12a[a]	~1,500	TYCV	dsDNA, ssDNA	76
	AsCas12a - RVR	AsCas12a[a]	~1,500	TATV	dsDNA, ssDNA	76
	enAsCas12a - HF	AsCas12a[a]	~1,500	TRTV	dsDNA, ssDNA	77
	Cas12b	*Bacillus hisashii*	~1,300	TTN	dsDNA, ssDNA	78
	Cas12c	Marine and gut metagenomes	~1,300	TG or TN	dsDNA, ssDNA	79, 80
	Cas12d, or CasY	Uncultivated natural microbial communities	~1,300	TA or TG	dsDNA, ssDNA	81
	Cas12e or CasX	Uncultivated natural microbial communities	~1,000	TTCN	dsDNA	82
	Cas12f, or Cas14	Archaea from DPANN superphylum	~600	-	ssDNA	83, 84
	Casμ	Ruminant microbiomes	~500	ATTA	dsDNA	85
	Cas12g	Hot springs metagenome	~800	-	ssRNA	86
	Cas12h	Hypersaline lake sediment metagenome	~900	RTR	dsDNA, ssDNA	87
	Cas12i	Freshwater metagenome	~1,100	TTN	dsDNA, ssDNA	87
	Cas12j, or CasΦ	Bacteriophage	~750	TBN	dsDNA, ssDNA	88, 89
	Cas12k	*Scytonema hofmanni*, *Anabaena cylindrica*	~650	GTN	dsDNA	90
	Cas12l	Wastewater metagenome	~850	CCY	dsDNA	91, 92
	Cas12m	*Mycolicibacterium mucogenicum*	~600	TTN	dsDNA	80
	Cas12n	Metagenome analysis for TnpB-like sequences	~500	AAN	dsDNA, ssDNA	93
Type VI	Cas13a	*Leptotrichia shahii*, *Leptotrichia wadei*	~1,200	3′ H	ssRNA	94, 95
	Cas13b	*Prevotella buccae*, *Bergeyella zoohelcum*	~1,100	5′ D, 3′ NNA, NAN	ssRNA	96
	Cas13c	*Fusobacterium perfoetens*	~1,100	-	ssRNA	97
	Cas13d, CasRx	*Ruminococcus flavefaciens*	~900	-	ssRNA	98
	Cas13X, Cas13Y	Metagenome analysis	~750	-	ssRNA	99

[a]These Cas proteins were engineered from widely used Cas proteins.

Abbreviations: dsDNA, double-stranded DNA; PAM, protospacer adjacent motif; PFS, protospacer flanking sequence; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.

original dCas9, possibly due to reducing CRISPRi-like effects (107). This finding suggests that adverse effects from Cas engineering might behave differently depending on the biological function of the Cas proteins.

Overall, PAM expansion has increased our targeting ability with Cas9-based genetic tools, but not without functional trade-offs. For instance, although PAM-flexible dCas9 variants exhibit greater CRISPRa efficiency, CRISPRi gene repression efficiency with the same variants is decreased (107). Hence, frameworks for choosing the most effective variant for a given set of targets and specific applications are necessary. A database with systematic characterization of Cas9 variants across bacteria will streamline the process of choosing the optimal Cas9 variant for a given set of target genes and specific applications (119).

Type V: a diverse Cas12 family. After the type II Cas9 CRISPR system, the second most broadly used class 2 systems are type V interference effectors, Cas12 proteins. Cas12 enzymes possess one nuclease domain that sequentially cleaves both strands of the target nucleic acid and, unlike Cas9, generates sticky ends (21, 22, 120). This makes Cas12 particularly useful for precise in vitro DNA assembly. Several Cas12 enzyme variants also contain a subdomain involved in pre-crRNA processing, whereas others encode a tracrRNA that, along with RNase III, processes the pre-crRNA (21, 121). Recent computational analysis has indicated that type V CRISPRi effectors evolved from transposon-encoded TnpB nucleases on multiple, independent occasions, resulting in a large pool of type V variants (56, 121). As of 2020, these variants are grouped into 12 subtypes that are highly diverse with respect to the size and architecture of the interference effector proteins as well as molecular mechanisms (122) (**Table 1**).

The corresponding type V CRISPR proteins can be categorized based on their properties and functions (**Table 1**). Cas12a, previously known as Cpf1, has dual-nuclease activity involved in both crRNA biogenesis and target DNA interference (21). Upon recognition of the 5′-TTTV-3′ PAM and an 18–23-nt seed region complementary to the crRNA sequence, Cas12a induces a staggered DSB in the target DNA (22). An intriguing feature of Cas12a is its collateral cleavage activity; after target binding and cleavage, Cas12a turns into an active nuclease that continues to nonspecifically cleave DNA sequences (123). Although Cas12a was the first discovered and extensively studied type V interference effector, other Cas12 variants offer unique properties for developing novel molecular tools (**Table 1**). Cas12e (or CasX) is substantially smaller than Cas12a and exhibits minimal *trans*-cleavage activity (82). Cas12g is the only type V RNA-guided ribonuclease that targets a single-stranded RNA substrate (86). Some Cas12 proteins retain DNA-targeting efficiency but exhibit different biological functions. Cas12c and the recently discovered Cas12m bind but do not cleave the dsDNA target, silencing transcription or replication (79, 80). Cas12k also lacks DNAse activity and associates with Tn7-like transposase subunits to mediate RNA-guided transposition (78). Even more type V effectors are actively being discovered across metagenome-assembled genomes and viruses (124). As with Cas9, there have been many efforts to engineer Cas12 variants to improve cleavage accuracy (125), abolish *trans*-activity (126), increase PAM flexibility (75–77), and increase thermostability for biosensing applications (127).

Type VI: targeting RNA with Cas13. Type VI Cas13 is the most well-studied CRISPR system for directly targeting and manipulating RNA (128). Cas13 is further divided into four subtypes: A (previously known as C2c2), B, C, and D (**Table 1**). Cas13 systems typically contain two RNase domains, which are active upon the crRNA base pairing with a matching target RNA (95). Once activated, Cas13 exhibits nonspecific cleavage activity, similar to Cas12a. This collateral RNA cleavage activity helps prevent bacteriophage infection by shifting the cells into dormant stage (129). Cas13 can also process its own crRNA arrays into mature crRNAs of roughly 60 nt in length (130–132). Natural crRNA spacer length has been reported to vary between 14 nt and

30 nt (94, 133). Instead of PAM, most Cas13 have a protospacer flanking sequence preference, which is usually less restrictive compared to the PAM requirements of DNA-targeting Cas proteins (94, 131). Although Cas13a and Cas13b were among the first discovered (128), recent research has shifted attention to Cas13d, which exhibited improved efficacy for RNA silencing and knockdowns (133–135). Several Cas13 proteins have also been repurposed for various applications beyond RNA cleavage, including in vitro RNA detection assays (136), RNA editing of mammalian cells and bacteriophage genomes (137–141), and RNA interference (133, 134).

The flexibility in nucleic acid recognition capabilities provided by the different Cas9, Cas12, and Cas13 variants has already fueled many applications in prokaryotic systems, from editing and regulating genes to biosensing nucleic acids and small molecules (39, 142). Nevertheless, the full repertoire of Cas variants developed for eukaryotic applications is yet to be characterized and exploited across different bacteria. Given that genomic GC content varies greatly from 13% to 75% between prokaryotic species (143), the diverse catalog of Cas variants will provide suitable options for implementing CRISPR systems in any microbe with specific traits for each application, including bioproduction, biotherapeutics, and bioremediation.

Expanding CRISPR Functionality with Tethered Effectors

Abolishing Cas nuclease activity generates nickase or catalytically dead Cas proteins (nCas or dCas, respectively), opening a new paradigm of CRISPR tools for sequence-specific nucleic acid modifications and gene expression regulation. Point mutations in one of the Cas nuclease domains generates a nicking variant that cleaves only one strand of DNA, whereas mutations in both domains form a catalytically dead variant with no cleavage activity. In prokaryotes, dCas9 was rapidly repurposed for programmable transcriptional repression by physically blocking the transcription initiation or elongation processes. Tethering effector domains to the CRISPR complex enabled sequence-specific localization of effectors to a DNA or RNA of interest, for applications such as transcriptional activation and base editing (6). Effector tethering has been achieved through covalent fusion to the Cas protein, protein–protein interactions (PPI), or recruitment of effectors fused to RNA-binding proteins (RBPs) that interact noncovalently with modified gRNAs (scaffold RNA, scRNA). Importantly, orthogonal effector recruitment can be programmed with multiple protein–protein interaction systems or RBP–RNA hairpin pairs (**Figure 2**; **Tables 1** and **2**). In contrast to eukaryotes, where CRISPRa can readily be achieved with a universal transcriptional activator (144, 145), CRISPRa across prokaryotes remains challenging due to the distinct transcriptional regulation mechanisms present in different bacteria (25, 26). Here we focus on the development of transcriptional activators for prokaryotic CRISPRa as well as recent advancements in tethering effectors for CRISPR gene editing.

Effectors for CRISPR-based gene regulation. Bikard et al. (146) showed that transcriptional activation could be achieved through recruitment of accessory proteins in prokaryotes, similarly to previous work in eukaryotes. They developed a CRISPRa system in *E. coli* by fusing an RNA polymerase (RNAP) omega subunit (RpoZ) to dCas9. One limitation of this system was the need to delete the endogenous *rpoZ* gene. Later work from our group found that bacterial CRISPRa could also function through an RBP-based recruitment using a bacterial transcription factor, SoxS, that interacts with the C terminus of an alpha subunit of RNAP (RpoA) (147). Notably, SoxS-based CRISPRa does not require modification of the genomic background, which streamlines the portability to other bacterial systems. The SoxS–RpoA interaction motif is conserved across diverse bacteria (147) and was demonstrated to be functional in *P. putida*, despite the absence of a SoxS homolog (148). Another approach by Liu et al. (149) achieved CRISPRa through

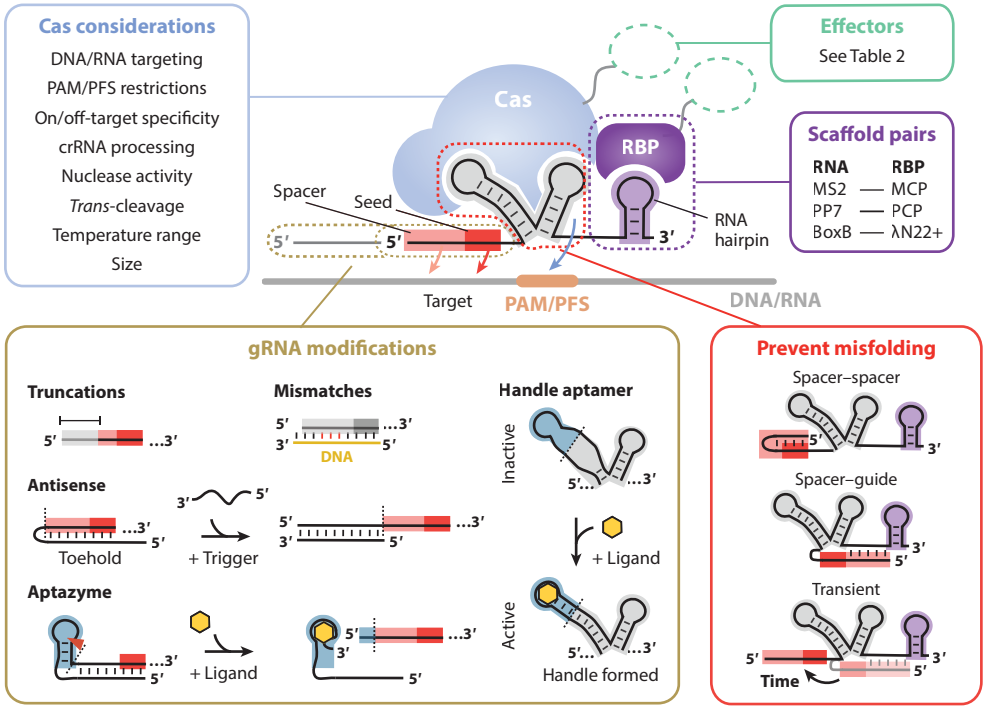


Figure 2

Design considerations when engineering CRISPR tools. Schematic of the different CRISPR components and how they can be engineered. Given the diversity of Cas proteins (**Table 1**), several properties, such as DNA or RNA targeting and *trans*-cleavage activity, must be considered based on the specific application. Effectors for transcriptional regulation (**Table 2*a***) or base editing (**Table 2*b***) can be covalently tethered to the Cas protein or recruited through a set of orthogonal scaffold pairs consisting of an RNA-binding protein and the cognate RNA hairpin encoded in the gRNA. When designing gRNAs, there are several techniques to tune their activity and computational tools to ensure proper folding (see the section titled gRNA Design Strategies). Abbreviations: crRNA, CRISPR RNA; gRNA, guide RNA; PAM, protospacer adjacent motif; PFS, protospacer flanking sequence; RBP, RNA-binding protein.

the RBP recruitment of the transcriptional activator PspF. Interestingly, PspF and SoxS could be implemented simultaneously through orthogonal RNA hairpin–RBP pairs—MS2/MCP for SoxS and BoxB/λN22 for PspF— and also work complementary to each other. SoxS can activate a broad range of σ^{70} superfamily promoters but is ineffective toward σ^{54} family promoters (25). On the other hand, the PspF system works specifically on the σ^{54} family promoters due to its unique ATP-dependent activation mechanism but cannot activate σ^{70} family promoters (149). In both cases, strategies to abolish the DNA binding of transcriptional effectors proved to be crucial because CRISPRa DNA targeting should be governed by dCas9 rather than by the transcriptional effectors. In PspF-based CRISPRa, this was achieved by truncating the helix-turn-helix DNA-recognition motif of PspF (149). In SoxS-based CRISPRa, we reduced the DNA-binding capacity of heterologous SoxS through multiple mutations, leading to key improvement in CRISPRa performance (25).

Through characterization of different CRISPRa elements and target genes, we have found that CRISPRa performance relies on (*a*) composition of the target promoter and (*b*) the position of the target sequence relative to the transcription start site (25, 48) (**Figure 3*a***). These design factors remain consistent across multiple transcriptional activation systems (25, 149). For

Table 2 Effectors for prokaryotic CRISPRa, their mechanisms, and design rules

CRISPRa systems	Mechanism	Optimal sites[a]	Strand	Organisms	Reference(s)
Effectors tethered directly to dCas					
dCas9-RpoZ	RNAP assembly	−60 to −100	NT	*Escherichia coli*, *Bacillus subtilis*, *Lysobacter enzymogenes*, *Myxococcus xanthus*	146, 156–158
dCas9-RpoA	RNAP assembly	−267 to −415	T	*B. subtilis*	156
dCas9-SYNZIP-αNTD	RNAP assembly	−60 to −100	NT	*E. coli*	151, 152
dCas9-RpoD	RNAP assembly	−199 to −216	NT	*Shewanella oneidensis*	159
dCas9-AsiA[b]	RpoD and RpoB binding	−182 to −252	T	*E. coli*, *Salmonella enterica*, *Klebsiella oxytoca*	150
dCas12a-SoxS	RpoA and RpoD binding	−50 to −150	T	*Paenibacillus polymyxa*	154
dCas12a-RpoZ	RNAP assembly	−183 to −328	T	*Corynebacterium glutamicum*	153
dCas12a-RemA	Unknown	−90	NT	*B. subtilis*	160
Cascade-αNTD	RNAP assembly	−100 to −120	T	*E. coli*	155
dCas13d-IF	Ribosome recruitment	5′ UTR	-	*E. coli*	161
Effectors tethered via guide RNA hairpin					
SoxS[b] (MCP or PCP or MCP + SYNZIP)	RpoA and RpoD binding	−60 to −100	NT	*E. coli*, *Pseudomonas putida*, cell-free expression system, hydrogel	25, 45, 48, 147, 148, 162
TetD (MCP)	RpoA and RpoD binding	−60 to −100	NT	*E. coli*	25, 147
RpoZ (MCP)	RNAP assembly	−91	NT	*E. coli*	25, 147
αNTD (MCP)	RNAP assembly	−60 to −100	T	*E. coli*	25, 147
PspF[b] (λN22 or MCP)	RpoN recruitment[c]	−91 to −131[d]	NT	*E. coli*, *K. oxytoca*, cell-free expression system	149, 163

[a]Relative to transcription start site (TSS).
[b]Engineered proteins.
[c]RpoN promoter is distinct to other bacterial promoters of RpoD-promoter superfamily.
[d]DNA looping is present in the promoter.
Abbreviations: CRISPRa, CRISPR activation; MCP, MS2 coat protein; PCP, PP7 coat protein; RNAP, RNA polymerase; SYNZIP, heterospecific coiled-coiled peptide.

instance, the dynamic range of CRISPR-based gene overexpression depends on DNA helical phases (**Figure 3*b***). We also found that these two systems exhibited consistent design rules in both *E. coli* and *P. putida* (148).

Bacterial CRISPRa systems are effective in a narrow range of target positions covering 60 to 100 bp upstream of the transcription start site (**Figure 3** and **Table 2**). There have been several attempts to develop CRISPRa systems for targeting alternative positions and promoter contexts. Directed evolution of AsiA, an antisigma70 protein from T4 bacteriophage, covalently fused to dCas9 provides long-distance CRISPRa beyond previously reported systems (150). This system was tested in a massively parallel fashion by replacing a minimal promoter with a library of promoter sequences derived from a metagenomic data set. Although only 3% (248 out of ~8,000) of the promoters could be upregulated, these data do provide a proof of concept that targeting regions can be expanded through molecular engineering. Another strategy to overcome the stringent CRISPRa distance requirements has been to use circular permuted dCas9 variants to

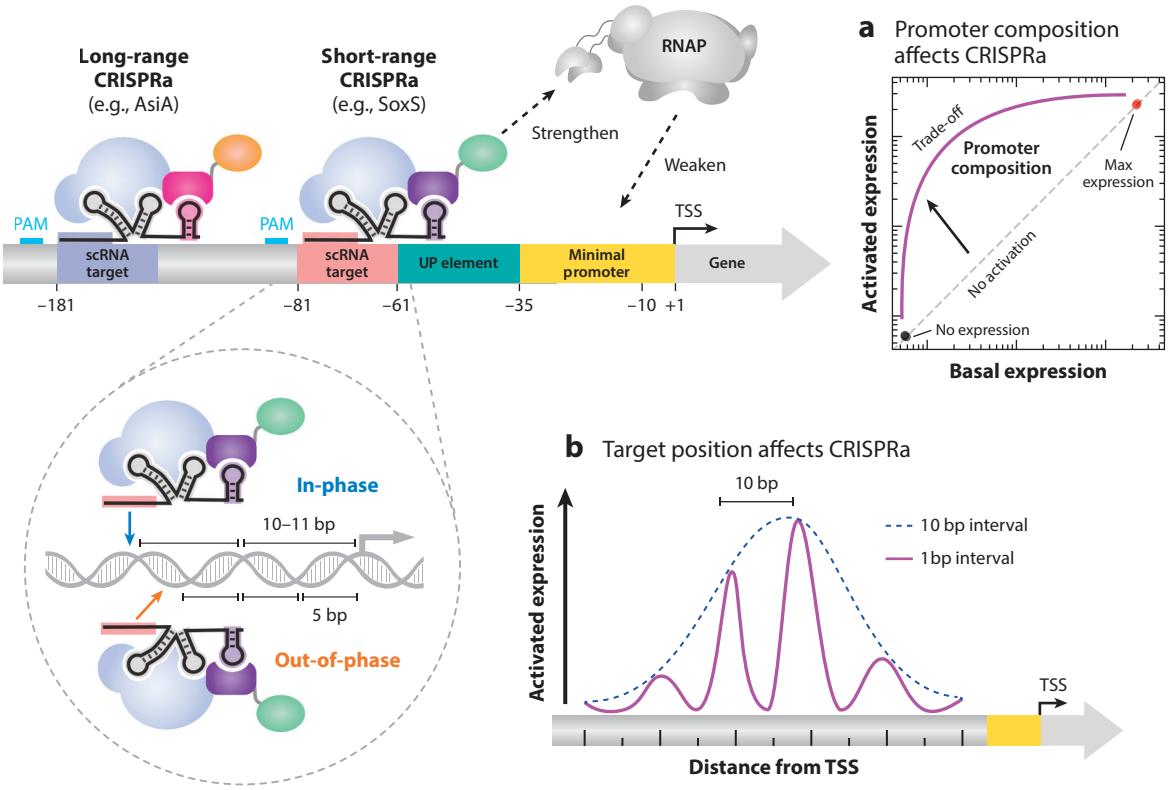


Figure 3

Prokaryotic CRISPRa sequence and distance requirements. Schematic of a prokaryotic promoter showing stringent requirements for bacterial CRISPRa. RNAP affinity for the minimal promoter and UP element determines basal expression levels. When CRISPRa is targeted to a promoter with a correct PAM and complementary scRNA target site, RNAP is recruited by the transcriptional activator. (*a*) Promoter composition determines RNAP–promoter and CRISPRa–promoter interactions and hence affects CRISPRa-mediated expression levels. (*b*) CRISPRa-mediated expression is highly dependent on the distance of CRISPRa from the TSS and exhibits a periodic dependence with peak activities every 10–11 bases, corresponding to a single DNA helix turn. Abbreviations: bp, base pair; CRISPRa, CRISPR activation; PAM, protospacer adjacent motif; RNAP, RNA polymerase; scRNA, scaffold RNA; TSS, transcription start site.

change the positioning of the tethered effector (151). They achieved increased targeting flexibility by screening a library of circular permuted dCas9s that recruited the N-terminal domain of RpoA (αNTD) of eight different bacterial species through recruitment of heterospecific coiled-coiled peptides (SYNZIPs) (151, 152). With the advent of new CRISPRa systems, multiple CRISPRa systems could be implemented simultaneously for more effective activation and more target sites.

Even though most of the CRISPRa systems in bacteria rely on dCas9 to recruit effector proteins, other Cas systems could also be implemented in a similar manner. Direct fusion of RpoZ or SoxS effectors to dCas12a was implemented for CRISPRa in *Paenibacillus polymyxa* and *Corynebacterium glutamicum*, respectively (153, 154). Type I-E Cascade-mediated bacterial CRISPRa was also demonstrated recently in a CRISPR-free *E. coli* using αNTD as an effector (155) (**Table 2**). Of note, CRISPRa could be improved from engineering efforts on other parts beyond the effector domain and could also be developed in parallel from effector-specific optimization, e.g., dCas9 engineering for PAM flexibility and reporter engineering to improve dynamic range (48, 107).

Table 3 Effectors for CRISPR-based gene editing and their editing window

Editing system	Effector	Cas protein	Organisms	Editing window	Reference(s)
Cytidine base editing	rAPOBEC1	Cas9n	*Escherichia coli*, *Brucella melitensis*	4–8	174
		Cas9n	*Corynebacterium glutamicum*	4–7	171
		Cas9n	*Salmonella typhimurium*	5–10	35
		enCas9	*Pseudomonas putida*	3–8	175
		dCas9	*Bacillus subtilis*	17–20	172
		dCas12a	*E. coli*	8–13, 10–12	176
		Cas12m/dCas12m	*E. coli*	25, 13–19	80
	PmCDA1	dCas9	*E. coli*	15	170
		dCas9	*B. subtilis*	15	173
		dCas9	*Streptomyces* spp.	15	177
		dCas9	*Paenibacillus polymyxa*	17–20	172
		dCas9	*Agrobacterium* spp.	16–20	178
Adenosine base editing	TadA	dCas9/nCas9	*E. coli*	48	179
		Cas9n	*Staphylococcus aureus*	48	180
		dxCas9/nxCas9	*P. putida*, *Pseudomonas* spp.	48	181
EvolvR	Poll3M	nCas9	*E. coli*	~60	182
Prime editing	M-MLV2	nCas9	*E. coli*	-	183

Effectors for CRISPR-based gene editing. CRISPR BEs with tethered nucleobase editor enzymes, or deaminases, can be used to introduce sequence-specific, single-nucleotide modifications, such as to abolish enzyme activity through precise point mutations or early stop codons (164) (**Figure 1**). In comparison to traditional CRISPR editing technologies that induce DSBs, BEs nick only one strand of DNA. In bacteria, given the species-dependent variations in editing efficiency (165) and the lethality of generating DSBs in unintended targets (166, 167), base editing is often favored over traditional editing through native Cas cleavage and DNA repair mechanisms (8, 9). Current prokaryotic BEs rely on direct tethering of the deaminase to inactivated Cas proteins, such as dCas9, nCas9, and dCas12a (**Table 3**). After Cas recruitment to the DNA, the deaminase recognizes and edits the target single-stranded DNA (ssDNA) in the R-loop structure formed between the gRNA and the target DNA. Each BE system targets a characteristic proximal stretch of ssDNA, termed the editing window, based on the deaminase and tethering approach (**Table 3**). Adenosine BEs generate A>G through A>I deamination, whereas cytidine BEs (CBEs) generate C>T substitutions through C>U deamination and subsequent DNA replication (**Table 3**). Most CBE systems also tether a uracil glycosylase inhibitor to prevent base excision repair and point mutation reversal (168). Notably, CBEs are used more widely, likely due to the higher editing efficiency and high GC content of host genomes (164). Developing RBP-tethered BEs could allow for simultaneous regulation of multiple genes and even opportunities for multiplexed applications with other effector proteins (169). The potential for multiplexing BE has been shown in multiple non-model organisms (11, 170–173).

In addition to deaminases, other effectors have been tethered to expand CRISPR editing capabilities beyond single-nucleotide substitutions. CRISPR-EvolvR uses an error-prone, nick-translating DNA polymerase (Poll3M) fused to nCas9 to continuously diversify nucleotides within short DNA regions (60 nt) (182). CRISPR prime editing (PE) uses a reverse transcriptase (M-MLV2) fused to nCas9 to introduce deletions, substitutions, and insertions encoded in the

3′ extension of the gRNA (183). Recently, serine integrases have been added to CRISPR-PE for targeted genomic recruitment and integration of desired payloads in eukaryotes (184). However, the low editing efficiency limits the application of these tools: approximately 40%, 20%, and 10% for PE-based plasmid deletions, substitutions, and insertions, respectively, and half of that for genomic edits. Editing efficiency is even lower (<2%) when combining modalities or multiplexing two gRNAs.

A major bottleneck for base and prime editing is that gRNA spacer design is more challenging than traditional spacer design because it must also factor in the constraints imposed by the editing effector (164). Spacers designed for BE must factor in deaminase type, editing window, target nucleotide position, and desired translation product. For PE, the 3′ gRNA extension must be designed carefully to enable reverse transcription without affecting DNA targeting. Hence, traditional gRNA spacer design tools often cannot be directly translated into designing spacers for base editing. Although a few tools exist to support BE- and PE-gRNA design in eukaryotes (185–188), their development in bacterial systems is fairly limited (189).

Even when gRNAs can be designed successfully, editing applications are still limited by the toxic effects of different combinations of effectors and Cas proteins (170, 190). Hence, most studies require tedious screening combinations of Cas9 variants, deaminases, and expression cassettes to find a balance between efficiency and toxicity (172, 175, 191). Moreover, sequencing-based BE efficiency analysis is currently expensive and time-consuming (192), and in vivo, real-time assessment of editing efficiency is available only in mammalian cells (193). Adaptation of these tools for bacterial systems would enable rapid comparison of Cas9 nucleases and deaminases across diverse genetic backgrounds, elucidating the design rules for predictable implementation.

Tethering different effector domains to different Cas proteins has enabled a wide range of functions beyond nucleic acid cleavage. These CRISPR gene regulation and base editing tools have already been implemented individually across different microbes (27, 194–196). Moving forward, simultaneous integration of multiple CRISPR tools in the diverse prokaryotic hosts should be within reach (26). Discovery and engineering of orthogonal hairpin–RBP pairs will enable the multiplexed recruitment of different effectors to specific sites. Moreover, multi-Cas systems for combined DNA and RNA targeting could be achieved with a better understanding of which Cas variants are better suited for each host as well as expression mechanisms to minimize burden effects (197, 198). Taken together, these advancements will fuel the next generation of prokaryotic CRISPR tools capable of concerted editing and regulation (199).

Tuning CRISPR Function Through gRNA Engineering

RNA-guided Cas proteins make engineering prokaryotic systems more straightforward than previously possible because gRNAs are genetically compact, metabolically inexpensive, and relatively simple to design (200). gRNAs can be engineered through chemical functionalization, sequence modifications, and fusion of other RNA scaffolds. Here we pay special attention to approaches that rely on engineering intrinsic gRNA properties because they are more relevant for prokaryotic applications in which the gRNA(s) are transcribed.

gRNA design strategies. Several gRNA design strategies have been proposed to tune CRISPR activity, mainly through spacer mismatches, truncations, and extensions (26, 201–203) (**Figure 2**). Mismatches in the Cas9 spacer region have been used to achieve graded levels of gRNA activity (204–206). Generally, mismatches at the PAM-distal end of the spacer are better tolerated than mismatches at the PAM-proximal, or seed region, of the spacer. Mismatches within the seed region exacerbate R-loop destabilization, whereas PAM-distal mismatches reduce the lifetime of the Cas9–DNA complex (207). Mismatches in the non-seed region can be used to tune gRNA activity,

but at the cost of gRNA specificity, because off-target activity at complementary sites is increased (203).

Truncating the gRNA spacer from the PAM-distal region up to the seed region has also been shown to reduce the level of gRNA activity (45, 208). A possible concern when using truncated gRNAs is an increase in off-target activity due to lower target complementary constraints. However, in a test set of orthogonal, synthetic, CRISPR-activatable promoters, we found that truncated (11–20-bp) scRNAs did not induce higher expression in their noncognate, unactivated promoters (208). Moreover, evidence from eukaryotic gene editing experiments with truncated gRNAs points to reduced binding energy as a leading factor in increased sensitivity to mismatches (209). These observations have been rationalized in terms of an excess energy model, postulating that decreases in binding energy beyond a certain threshold destabilize binding to both correct and incorrect targets (112, 209–211). Similarly, introducing a proper 5′ extension that folds back to cover the spacer region has been shown to increase gRNA specificity as the designed hairpin becomes more stable (212). However, long 5′ extension gRNA spacers could also lead to decreased activity (212, 213).

Researchers have implemented 5′ and 3′ gRNA extensions to exert conditional control over CRISPR activity (**Figure 2**). The general mechanism involves triggering occlusion or reveal of the gRNA spacer or handle in response to a nucleic acid or small molecule. Several gRNA with 5′ toeholds have demonstrated the ability to respond to single DNA or RNA trigger strands (214–216). Aptazymes, or ligand-responsive self-cleaving ribozymes, have been used to cleave a repressive 5′ extension from the gRNA upon the addition of the target ligand (217). Aptamers have also been added into the handle or hairpins to stabilize the gRNA in response to a specific ligand (218). Although 3′ end aptamers have been demonstrated in sgRNAs (219), 3′ extensions could be challenging in a scRNA due to the presence of the effector-recruiting hairpin. Finally, even though most gRNA engineering efforts have been on Cas9 gRNAs, some of the principles should be extendible to gRNAs for Cas12 and Cas13 (212, 220–222).

Computational design of gRNAs. Designing gRNAs to target arbitrary sites requires simply designing the complementary sequence into the spacer. However, accurately predicting gRNA performance in terms of activity and specificity is still challenging (223). One reason is that gRNA activity is highly influenced by target sequence context, with respect to both the sequence surrounding the PAM site and the local target site structure (224). CRISPR complex binding and activity at endogenous target sites can be hindered by other DNA-binding effectors regulating transcription or chromatin accessibility (26, 225, 226). Even the inherent resilience of endogenous gene regulatory networks containing feedback or feedforward loops can limit the degree of CRISPR activity. Machine learning models trained on large gene editing or CRISPRi data sets in eukaryotes have been used successfully to extract gRNA design rules (227, 228). However, the applicability of these models for prokaryotic gRNAs is rather limited due to the differences across both domains of life.

The degree of gRNA secondary structure, whether internal to the spacer or between the spacer and the rest of the guide, is also a key determinant of gRNA activity (**Figure 2**). Although some models do account for the energetics of nucleic acid interactions, these have focused primarily on the thermodynamics of spacer–DNA target interactions, ignoring other aspects of gRNA folding. Ensuring correct gRNA folding is crucial when designing gRNAs with modifications such as extra hairpins or aptamers. To this end, computational RNA folding packages such as ViennaRNA have been used in conjunction with deep learning models to predict on- and off-target activities for bacterial sgRNAs based on RNA folding parameters, melting temperatures, and potential off-target scores (229).

Only very recently have computational models started to combine these effects to more accurately predict gRNA performance in prokaryotic systems (230). Developing models that incorporate biophysical aspects of gRNA folding and target sequence context with heuristics from large-scale functional screening is expected to further advance our gRNA design capabilities. Importantly, these models should recapitulate the gRNA performance effects associated with modifications such as spacer truncations and aptamer extensions. Such an advance, if it can minimize or eliminate the current need for experimental verification of new gRNAs, would accelerate the design of CRISPR tools for editing or regulating specific genes to precise levels.

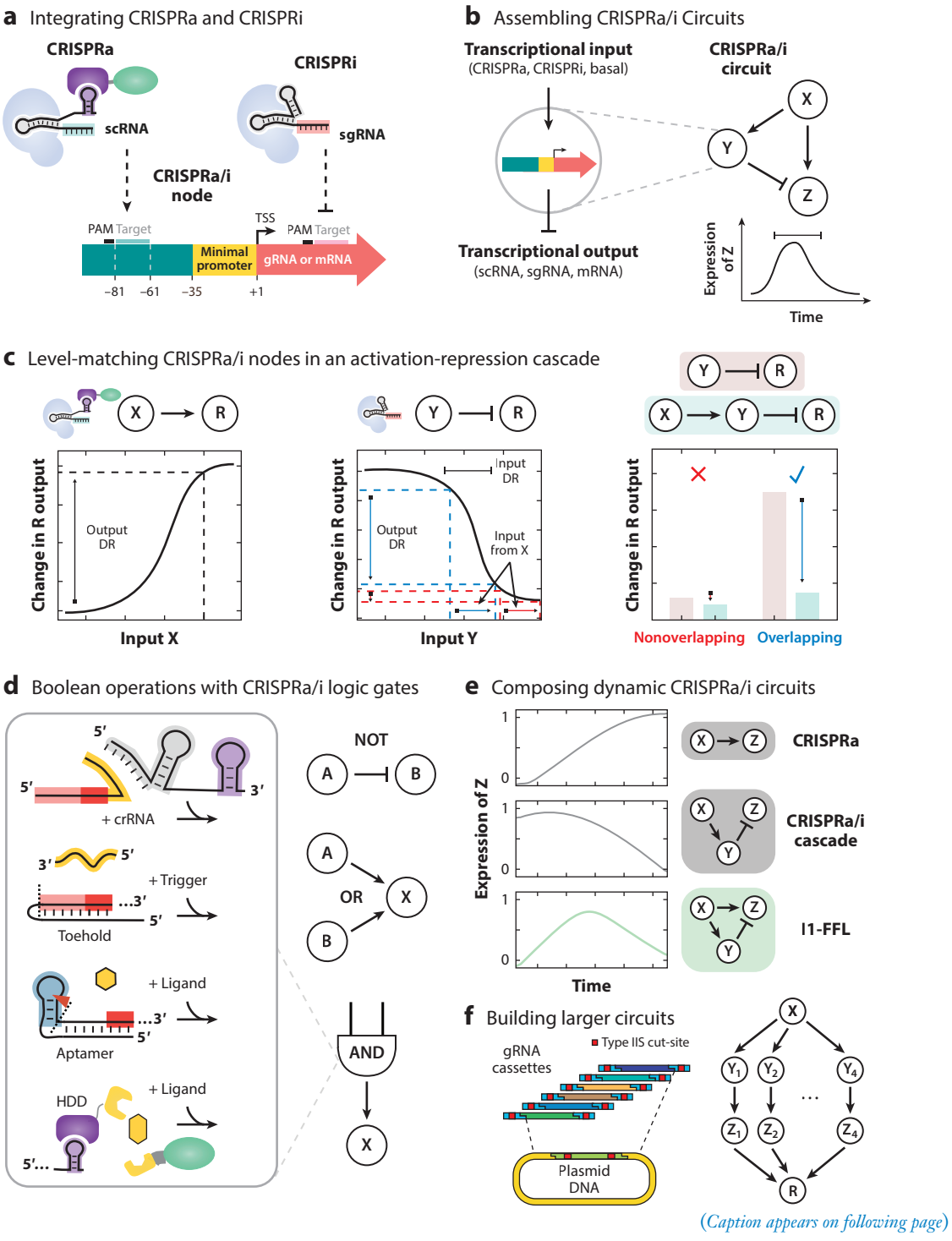
BUILDING CRISPR-BASED GENETIC CIRCUITS

Genetic circuits enable natural biological systems to respond to different input stimuli with complex, time-dependent behaviors by regulating the expression of multiple genes. Harnessing these capabilities is fundamental for engineered systems that need to monitor, compute, and respond to internal or external environment changes, as demonstrated by the hallmark designs of genetic circuitry (231, 232). However, examples of engineered genetic circuits capable of multiplexing stimuli and regulating multiple genes are limited. This paucity can be attributed to the limited number of suitable components for implementing scalable circuitry (1, 233–235) and to the difficulty of sequentially combining components into larger, high-level circuits (236–239). Due to the ease of designing and tuning new components that can be interconnected, CRISPR-based tools have rapidly become a suitable framework for building complex genetic circuits. In this section, we focus on how CRISPR tools have been assembled into genetic circuits in bacteria and CFES.

CRISPR circuits consist of a series of CRISPR nodes, which are discrete transcriptional or translational units containing target sequences for CRISPRa or CRISPRi (**Figure 4*a***), that are interconnected through gRNAs. Circuit topology is therefore specified by the set of connected nodes and gRNAs and can encode logic gates and network motifs, such as incoherent feedforward loops (**Figure 4*b***), to perform precise functions. Importantly, more complex functions can be achieved by combining several network motifs into a larger circuit.

CRISPR Logic Gates and Small Network Motifs

Theoretically, any computational operation could be performed with transcriptional repression nodes alone. Hence, the first examples of prokaryotic CRISPR circuits relied on transcriptional repression to create Boolean logic gates (e.g., NOT, AND, OR, NAND, NOR) (240). However, in practice, it is advantageous to combine CRISPR repression and activation to cover a larger circuit design space with fewer components (45, 48) (**Figure 4**). Whereas a CRISPRi OR gate requires four sgRNAs (237), a CRISPRa OR gate can be assembled with just two scRNAs. In addition, many variations of CRISPRa AND gates can now be constructed easily: through conditional expression of a scRNA and activator, conditional folding of the scRNA, or conditional recruitment of the activator through ligand-induced heterodimerization domains (48, 147, 163) (**Figure 4*d***). Circuits combining both CRISPRa and CRISPRi can be constructed through the regulated expression of sg/scRNAs and have been demonstrated on multiple genes (147, 148, 160) and in multilayer circuits (45) (**Figure 4*e***). Importantly, by interconnecting multiple gRNAs into multilayer circuits, more complex network motifs such as toggle switches that can serve as memory units and incoherent feedforward loops for programming spatiotemporal patterns can now be achieved. Notable examples are a recently developed CRISPRi-based synthetic oscillator, termed CRISPRlator (241), and a CRISPRi-based synthetic inverter for antibiotic-free selection plasmid maintenance (242).



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Building CRISPRa/i circuits for Boolean logic and dynamic control. (*a*) Schematic of a CRISPRa/i node, consisting of a transcriptional unit with target site for CRISPRa/i. (*b*) CRISPR circuits can be assembled by interconnecting CRISPRa/i nodes expressing gRNAs. (*c*) Successful circuit function requires level-matching the output DR of an upstream node with the input DR of a downstream node. (*left*, *middle*) Dose-response characterization of CRISPRa and CRISPRi, respectively, with overlays of input and output DR. (*right*) Functional characterization of CRISPRi and an activation–repression cascade when the DRs are matching or mismatched. (*d*) Schematics of different techniques for incorporating Boolean logic into CRISPR circuits via gRNA programming. (*e*) Dynamic gene expression profiles resulting from different CRISPRa/i circuits. The composability of CRISPR circuits enables building larger circuits from small motifs. (*f*) Large CRISPR circuits can be achieved by assembling small gRNA cassettes into multi-guide arrays. Abbreviations: CRISRPa, CRISPR activation; CRISPRi, CRISPR interference; crRNA, CRISPR RNA; DR, dynamic range; FFL, feed-forward loop; gRNA, guide RNA; HDD, heterodimerization domain; mRNA, messenger RNA; PAM, protospacer adjacent motif; scRNA, scaffold RNA; sgRNA, single guide RNA; TSS, transcription start site.

Prospects and Challenges of Building Larger CRISPR Circuits

Experimental and theoretical work indicate that the CRISPRa/i system is well suited to designing complex genetic circuits with many internal nodes (45, 243). Akin to natural transcriptional regulatory networks, interconnected CRISPR circuits could be used for parallel biocomputing and multigene regulation (208, 244). Crucial to building these circuits is the ability to level-match the input/output dynamic ranges between sequential nodes (45, 245). That is, the output transcription levels of upstream nodes must be matched to the relevant transcriptional input range of downstream nodes (**Figure 4*c***). Compared to other circuitry systems, the CRISPRa/i framework facilitates the circuit design and level-matching process because many orthogonal and high-performing gRNAs and promoters can be readily constructed (208), and it has already been shown to enable deep and wide circuits with up to ten nodes of regulation (48, 118, 237) (**Figure 4*f***).

Several factors must be considered when assembling multi-gRNA circuits for CFES or bacteria. In cell-free systems, multiple gRNAs can be readily expressed from individual plasmids or linear fragments (45, 246). In living cells, however, the repetitiveness of multiple gRNA parts could be difficult to synthesize chemically and prone to recombination (53). Expression of gRNA arrays from a single transcriptional unit is possible when using self-processing Cas, such as Cas12a and Cas13, or incorporating gRNA processing proteins (Csy4) (130, 247, 248). However, the order of gRNAs in the array also affects their activities, likely due to early termination in gRNA biogenesis (249). Additionally, incorporation of orthogonal CRISPRa/i targets in the gRNA expression cassette is required for gRNAs to regulate the expression of one another (48). Hence, expressing one gRNA per transcriptional unit is more appropriate for programmable gRNA circuits. Several studies have demonstrated the construction of large gRNA circuits using nonredundant parts of promoters, terminators, and gRNA scaffolds (118, 250). As the number of regulatory nodes increases, tools to design high-performing, orthogonal promoters and gRNAs will become increasingly important. With a recent workflow for promoter and gRNA design, circuits with up to three and six layers of activation have been achieved in *E. coli* and *E. coli*–based cell-free lysates, respectively (48) (**Figure 4*f***).

Given that natural CRISPR immunity systems contain ∼50 spacers, and some use multiple Cas proteins, more complex CRISPR circuits should be within reach (251). Currently, the main challenge in implementing larger CRISPR circuits is the retroactivity effects observed when expressing multiple gRNAs due to competition for the same pool of Cas proteins (243). For wide circuits regulating many genes in parallel, these effects have been minimized by tuning dCas9 expression levels through feedback control and RBS engineering, enabling simultaneous repression of up to 13 genes (252). For deep circuits with many internal layers, upstream gRNAs outcompete downstream gRNAs for dCas9 binding, mainly due to timing of expression. For these

circuits, strategies to dynamically regulate upstream gRNA expression, such as reversing CRISPRa complex binding or implementing negative autoregulation motifs, may be necessary. Moreover, incorporation of orthogonal CRISPR systems, such as alternative dCas9 variants or even dCas12a and dCas13 systems, could provide additional nodes and layers for CRISPR-based biocomputing.

FOREFRONT APPLICATIONS OF CRISPR-BASED TOOLS

Engineering CRISPR Systems for Biosensing

Coupling molecular recognition to the diverse biochemical functions of the CRISPR toolbox has enabled new avenues for biosensing across various biochemical systems, including cell-free systems and bacterial hosts (**Figure 5*a–c***). In cell-free systems, the native nucleic acid recognition and cleavage activity of Cas enzymes have fueled in vitro detection of diverse biomarkers, from nucleic acids to small molecules (39, 40). Addition of gene expression capabilities to cell-free systems (41–43) has enabled more sophisticated biosensors. In bacteria, CRISPR-based biosensors are being used to record transcriptome changes and screen bioproducing strains. In this section, we recapitulate how different CRISPR systems have been engineered to detect nucleic acids, small molecules, and other environmental cues.

Nucleic acid detection. Nucleic acid detection is critical in applications such as infectious disease diagnosis and transcriptomic monitoring. The different class 2 systems have been adapted for the specific, sensitive, and portable sensing of nucleic acids (39). One approach involves detecting a specific DNA/RNA sequence in vitro through gRNA complementarity, which triggers cleavage of the surrounding quenched probes through collateral activity (mainly Cas12a and Cas13a) to produce a fluorescent readout (136, 253–255) (**Figure 5*a***). When paired with target amplification processes, the limit of detection can be as low as 10 aM (attomolar, 10^{-18} M) (253, 256–259). This detection approach has also been paired with CFES harboring different enzyme-based reporters for higher amplification-free signal and even packaged into a cell-free wearable biosensor (260, 261). For a more in-depth review of CRISPR systems for in vitro nucleic acid detection assays, we point the reader to Tian & Zhou's recent review (40).

In a similar fashion, engineered Cas genes and gRNAs targeting antibiotic resistance or essential genes can be packaged into bacteriophages or nanoparticles to detect and lyse specific bacteria (262–267), with efficacy equal to that of high-dose antibiotics in in vivo models (263). Given the multiplexability of CRISPR tools, these systems could readily be engineered to simultaneously target different species or multiple sequences in the same bacteria (268) or be interfaced with genetic circuits for logic control.

Engineering tracrRNAs to hybridize with target RNAs as if they were crRNAs creates another approach for detecting nucleic acids (163, 269). This strategy has been paired with base editing to develop a messenger RNA (mRNA) recording platform (35) and with CRISPRa to construct a positive feedback loop for signal amplification (163). This platform has been used to record infection-induced small RNAs in the intracellular pathogen *Salmonella*. Similarly, strand displacement of 5′ toeholds covering the gRNA spacer or handle has also been used to detect nucleic acids, from endogenous mRNA and small RNA to synthetic ssDNA that can be used for complex computation (214, 220, 221).

Small molecule quantification. Detection of small molecules enables diverse applications, such as monitoring environmental contaminants in vitro and quantifying cellular metabolites in vivo. Several CRISPR-based small molecule detection systems rely on combining ligand-responsive DNA aptamers or transcription factors with the collateral cleavage activity of Cas proteins. In these systems, the DNA target site is originally occluded by either the aptamer secondary structure

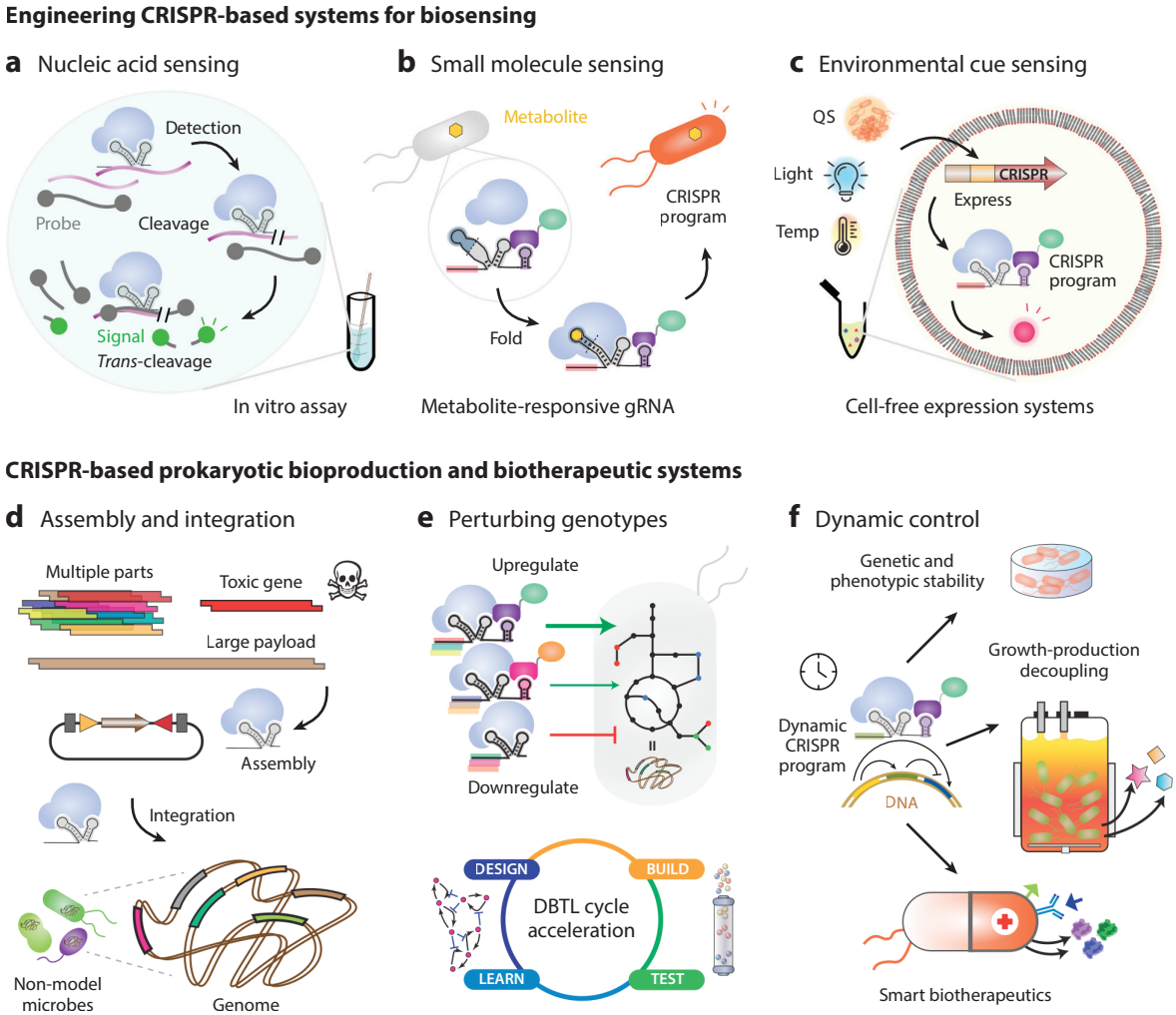


Figure 5

Forefront applications of CRISPR tools. (*a*) CRISPR systems engineered for detecting nucleic acids in in vitro assays. The gRNA spacer is designed to be complementary to the nucleic acid sequence, and the *trans*-cleavage activity of Cas12 or Cas13 is used to cleave the surrounding quenched fluorescent probes, leading to rapid fluorescent readout. (*b*) Example of a small molecule detection in vivo scheme in which a metabolite-responsive gRNA triggers CRISPRa of a fluorescent gene. (*c*) Schematic of a cell-free system harboring CRISPR components expressed from inducible promoters. Multiplexed response to different environmental cues triggers expression of a fluorescent gene through CRISPRa. (*d*) CRISPR tools for accelerating heterologous gene assembly and integration; these are particularly useful for assembly of large or toxic payload and delivery into non-model microbes lacking genetic tools. (*e*) Schematic of genome-wide CRISPR perturbations to alter prokaryotic metabolism. Coupling gRNA programmability and multiplexability with high-throughput measurements and computational models could accelerate DBTL cycles. (*f*) Examples of dynamic CRISPR programs enabling improved genetic and phenotypic stability, dynamic bioproduction, and smart therapeutics capable of sensing specific microenvironments and launching the appropriate therapeutic action. Abbreviations: CRISPRa, CRISPR activation; DBTL, design–build–test–learn; gRNA, guide RNA; QS, quorum sensing.

or transcription factor binding (142, 270). The presence of the cognate ligand reveals the target DNA and subsequently initiates collateral cleavage of a fluorophore quencher-labeled ssDNA probe. Similarly, ligand-responsive transcription factors have also been used to induce transcription of Cas12a crRNA arrays and Cas13a target transcripts, followed by *trans*-cleavage of quenched

probes (271, 272). For a more in-depth review of Cas12 and Cas13 systems for in vitro small molecule assays, we point the reader to recent reviews (273, 274).

Small molecules have also been detected through conditional recruitment of the effector to the CRISPR complex. Many ligand-induced heterodimerization domains have been used in eukaryotes for modulating CRISPRa activity by conditionally recruiting the activator (275). In prokaryotic systems, recent advancements in CRISPRa have enabled conditional gene activation with abscisic acid– and gibberellic acid–responsive heterodimerization domains (48). Interestingly, the abscisic acid– and gibberellic acid–inducible CRISPRa systems possessed similar design rules, suggesting that novel CRISPRa-based biosensors could be developed with other ligand-induced heterodimerization pairs. The repertoire of detectable small molecules could be further expanded through membrane-augmented CFES (276, 277) harboring CRISPRa/i systems coupled with bacterial two-component systems and G protein–coupled receptors (278).

Aptamers can be incorporated into sgRNAs to stabilize or destabilize the sgRNA structure in response to ligands such as theophylline, 3-methylxanthine, and thiamine (217–219). The observed CRISPRi effect with these sgRNAs was concentration dependent and ligand specific, allowing simultaneous repression of two heterologous genes in *E. coli* using different ligands. Similar approaches, especially 5′ extension and conditional Cas9 handle folding, can be used to generate ligand-responsive scRNAs for CRISPRa by incorporating existing or novel aptamers (**Figure 5*b***). Combining our expertise in computational RNA design (279) and aptamer engineering (280), we successfully developed ligand-responsive scRNAs acting through a well-characterized theophylline aptamer and a novel human milk oligosaccharide (HMO) aptamer (I. Faulkner, D. Sparkman-Yager, A. Walls, J. Gershon & J.M. Carothers, manuscript in preparation). Because human milk oligosaccharide was produced intracellularly via a CRISPR program, metabolite sensing could be coupled to the CRISPR circuit to dynamically regulate the metabolic pathway. Further improvement of computational design techniques capable of adhering to sequence and structure constraints imposed by both aptamer and gRNA should accelerate construction of novel small molecule–responsive CRISPR circuitry.

Environmental cue sensing. For bioproduction, bioremediation, and biotherapeutic applications in which bacteria or CFES are deployed into changing environments, the ability to sense and respond to environmental cues is crucial for carrying out desired functions (281–283). The most general approach for responding to environmental cues relies on signal transduction modules, such as inducible promoters to conditionally express CRISPR components. In fact, in many bacterial genomes, CRISPR systems are already regulated by external stimuli such as nutrient availability, stress factors, and quorum sensing (QS) (284–286). Successful implementation of engineered inducible systems requires precise tuning of the expression levels to minimize leaky expression while maintaining high enough inducible expression to carry out downstream functions (48, 287). In eukaryotes, many conditional CRISPR expression systems have been used to interface with endogenous and engineered regulatory networks and signaling pathways (288, 289). In bacteria, there are only a few examples of biosensing CRISPR circuits capable of responding to environmental stimuli, such as light and heat (290–293). Some notable environmental conditions capable of gene regulation in bacteria could also be coupled with CRISPR systems, including pH and oxygen levels (281, 294, 295) (**Figure 5*c***). Recent advancements in inducible promoter engineering in bacteria and CFES should accelerate the design of other inducible systems with large output dynamic ranges that can be readily connected with downstream signal processing circuits (48, 296, 297).

As seen in this section, different CRISPR system components have been engineered to modulate CRISPR activity in response to diverse stimuli. Signal transduction modules based on gRNAs, through either aptamer fusions or expression from inducible promoters, are particularly

interesting for more complex applications for two reasons. First, biosensing through gRNAs provides a streamlined and scalable framework for integrating multiple signals into one CRISPR circuit (48, 298, 299). And second, gRNA engineering approaches can be used to modulate the response level of each signal transduction module, potentially without engineering the module itself (45, 48). In this fashion, many orthogonal signal transduction modules could be designed to interface with downstream CRISPR circuits capable of signal modulation, pattern formation, and computation. This framework highlights how CRISPR-based biosensors can not only be used for sensing different molecules and producing measurable readouts but also be readily engineered to launch any type of CRISPR program.

CRISPR Engineering of Prokaryotic Systems for Bioproduction and Biotherapeutics

CRISPR tools are accelerating the development of bacteria and CFES for bioproduction and biotherapeutic applications (29, 266, 300–303) (**Figure 5*d–f***). Engineering microbial chassis for programmable bioproductions and biotherapeutics often involves assembling multiple heterologous genes, fine-tuning their expression levels, and perturbing the endogenous metabolic pathways (304, 305). CRISPR systems streamline the development of microbial chassis by providing versatile toolboxes for manipulating multiple genes simultaneously during DNA assembly and strain engineering (27, 194). When optimizing strains, genome-wide CRISPR perturbations have proven to be a powerful tool for mapping the genotype–phenotype landscape, enabling discovery of new gene functions, and selecting desirable bioproduction and biotherapeutic traits (199, 306). In lysate-based CFES, precise control over the enzymatic composition is usually required to maximize product yield (301). CRISPR tools can be used to alter lysate composition through strain engineering and to coordinate multi-enzyme programs through genetic circuits. Additionally, dynamic CRISPR programs enable microbes to adapt and respond to environmental stimuli or exert metabolic control to maximize flux to the desired product. In this section, we focus on how CRISPR tools enable engineering of complex prokaryotic systems for bioproduction and biotherapeutic applications.

Assembly and integration of engineered pathways. Engineering prokaryotic bioproduction and biotherapeutic platforms often involves manipulating and assembling several heterologous genes. The programmable targeting of CRISPR tools has been repurposed for molecular cloning and introduction of large heterologous pathways into attractive microbial chassis (307–309) (**Figure 5*d***). The engineered near-PAMless SpRY Cas9 variant has been used as a versatile restriction enzyme for molecular cloning due to its programmable targeting of any DNA sequence (310). CRISPR nucleases have also been applied to accelerate genome engineering procedures spanning from gene disruption (knockout) to gene integration (knock-in) (311–314). For insertion of large genetic payload, several CRISPR systems were developed to enable rapid cloning of biosynthetic gene clusters of up to 145 kb, resulting in the discovery of novel natural products (307, 315–317). In the case of biosynthetic genes that exhibited toxicity in cloning hosts, CRISPRi could be used as an auxiliary system to suppress expression of harmful genes (318). Recent efforts to develop CRISPR-assisted recombineering have led to improvements in multiplexed recombineering efficiency (319, 320), streamlining engineering of bioproducing *E. coli* (321–323).

CRISPR tools have been explored intensively in model organisms like *E. coli* and *Bacillus subtilis* (324) and also have accelerated DNA manipulation in broad ranges of industrially relevant microorganisms, which were challenging via conventional approaches (27, 194, 325). The broad applicability of CRISPR nuclease systems has accelerated genome integration pipelines in attractive microbes, such as *Pseudomonas* and *Acinetobacter* species, known for their broad

applications in both chemical bioproduction and therapeutic discovery (311, 313, 314). Highly efficient chassis-independent recombinase-assisted genome engineering was employed to deliver CRISPR programs regulating biosynthetic gene clusters in unconventional hosts (326). Similarly, recent CRISPR transposase studies have demonstrated programmable insertion of DNA into various bacterial hosts, including the Gram-negative *Alphaproteobacteria* (*Agrobacterium fabrum*), *Betaproteobacteria* (*Burkholderia thailandensis*), *Gammaproteobacteria* (*E. coli*, *P. putida*, and *Klebsiella oxytoca*), and Gram-positive bacteria (*B. subtilis* and *C. glutamicum*) (14, 15, 327, 328). Nevertheless, the lack of basic genetic parts, such as expressing vectors, selectable markers, and functional promoters, creates a barrier to implementing CRISPR tools in other non-model chassis (305).

Perturbing genotypes to explore of phenotypic landscapes. Multigene CRISPR perturbations targeting endogenous and heterologous genes for editing or regulation can be used to explore bacterial phenotypes (199, 306). Bacterial phenotypes map ruggedly onto the genetic design space due to the complex underlying metabolic and regulatory networks (329, 330). In principle, combinatorial gene expression programs could be constructed to explore the genetic design space and identify desired phenotypes (205, 331, 332). Compared to traditional *cis*-acting approaches, *trans*-acting CRISPR perturbations can dramatically reduce the time needed to engineer and fine-tune multigene programs because no additional direct engineering of the heterologous pathway or of the endogenous metabolism is necessary (208).

CRISPR perturbations include up- or downregulating gene targets as well as editing (164, 333) (**Figure 5*e***). Both Cas9 and Cas12 gene editing have been implemented in metabolically versatile *Rhodobacter* species (32, 334), photosynthetic cyanobacteria *Synechococcus* and *Anabaena* (335–337), and obligate anaerobes such as *Clostridia* species (338), demonstrating improved chemical bioproduction. Importantly, multiple base-editing events can be instituted in parallel and sequentially, allowing for rapid genome-wide rewiring of the microbial metabolism (11, 31). For example, multiplexed base editing in *R. sphaeroides* improved bioproduction of antioxidant coenzyme Q10 (31), whereas sequential base editing in *P. putida* can introduce auxotrophy toward NADPH (11). Similarly, multiple CRISPRi repression has been implemented in various non-model organisms suitable for CO_2-utilizing organisms for chemical productions, including cyanobacteria and acetogen (30, 339–341). For CRISPRa, various systems have been applied in *E. coli*, *P. putida*, *P. polymyxa*, *Streptomyces venezuelae*, and *Myxococcus xanthus*, enabling bioproduction of compounds ranging from commodity chemicals like ethanol, butanediol, and mevalonate to therapeutics like biopterins, jadomycin, and epothilone (147, 148, 152, 154, 342). Notably, through CRISPRa of three heterologous genes, we have demonstrated production of biopterin in *E. coli* and *P. putida* (148, 162) and valuable oligosaccharide products in *E. coli* (208). Crucial to this task was the ability to rapidly explore the bioproduction landscape by generating 64 combinatorial heterologous pathway expression programs, tuned with designed gRNAs (208). Multiple studies across different prokaryotic systems have also demonstrated simultaneous CRISPRa and CRISPRi perturbations to achieve phenotypes inaccessible at the single-gene perturbation level (45, 147, 148, 160, 343).

Multiple groups have implemented genome-scale CRISPR programs for improved bioproduction. Multiplexed CRISPRi programs have been shown to improve production of isopentenol, free fatty acids, and succinic acid in *E. coli* (250, 344, 345). Particularly, co-expression of 20 sgRNAs to simultaneously downregulate six genes (*ackA*, *iclR*, *poxB*, *pta*, *sdhC*, and *sdhD*) led to a 150-fold increase in succinic acid production (250). Simultaneous CRISPR base editing of up to 10 genes has been used to optimize glycerol use in *B. subtilis* and increase protocatechuate and lycopene productions in *P. putida* and *C. glutamicum*, respectively (11, 346). With high programmability and ease of design via short RNA sequences, CRISPR systems are permissive toward automated robotic platforms, which could revolutionize the deployment of the Design–Build–Test–Learn cycle (347). In combination with advancements in massive DNA library synthesis, high-throughput

sequencing (348), and metabolite-responsive biosensors, genome-wide screening for gene targets that improve productivity could be achieved at a single-cell level (349–351).

Similarly, CRISPR tools can be used to improve bioproduction in in vitro systems. Cell-free systems, ranging from purified enzymes (352–354) to lysate-based expression systems, have proven to be viable bioproduction platforms for a wide range of products, such as small molecules, vaccines, and even recombinant phages (301, 355–357). For more in-depth analyses on various cell-free bioproduction systems, we refer to other reviews (358–360). Because lysate-based CFES maintain some of the cellular enzymes and metabolites (361, 362), recent efforts have engineered lysates with a more favorable proteome by knocking out, knocking down, or upregulating target genes in the original strain (301, 363–365). Taking advantage of the full suite of CRISPR tools would enable rapid strain engineering and screening of different lysate compositions for improved CFES bioproduction. Paired with novel lysates from non-model organisms with more desirable endogenous metabolism for bioproduction (366, 367), CRISPR tools offer a promising route for engineering more efficient CFES bioproduction platforms.

CRISPR perturbations could also be used to identify genes with desirable traits for biotherapeutic applications. Living therapeutics, or live biotherapeutic products (LBPs), as defined by the US Food and Drug Administration, are becoming an exciting method for diagnosis and treatment (368). Recombinant LBPs are being explored currently to treat infectious diseases, inflammatory bowel diseases, and cancer (369). LBPs harboring CRISPR tools are already capable of recording changes in their environment and deploying novel antimicrobials (34, 35, 37, 370). One advantage of LBPs is that they can colonize solid tumors and activate an immune response. However, the immune system rapidly targets and depletes bacteria. Hence, CRISPRa/i could be used to discover genes that affect tumor colonization and improve resistance to the host immune system.

CRISPR tools are undoubtedly accelerating our ability to explore bacterial phenotypes. CRISPRa/i at the transcriptional and translational level in particular have proved to be powerful techniques for engineering metabolic pathways without altering the underlying genetics and regulation. Although CRISPRa/i perturbations could be hardcoded into the final strain, there are advantages in having genetic programs to dynamically control phenotype, for instance, to induce lysing only once a biotherapeutic strain is localized to the tumor or to decouple cell growth and product synthesis in a bioproducing strain.

Exerting dynamic control over multiple genes. CRISPR tools can be used to dynamically sense multiple stimuli and respond through the coordinated expression of multiple genes (**Figure 5*f***). In biotherapeutic applications, LBPs must sense characteristic features of their target microenvironment, such as low O_2 and high lactate levels, when targeting tumors. CRISPR circuits could be readily designed to sense these microenvironment signals, process them, and launch the appropriate therapeutic action. Additionally, engineered LBPs should possess biocontainment circuits that both enable selective removal from the host and prevent proliferation in the wild (371, 372). Recently, Rottinghaus et al. (292) demonstrated a CRISPR-based kill switch in the *E. coli* Nissle strain for in vivo use. The system induces cell death through multiplexed CRISPRi targeting of essential genes in response to a chemical inducer as well as temperature.

In engineered bioproduction systems, dynamic CRISPR control of metabolic pathways is a highly effective approach to maximize flux and minimize the accumulation of harmful intermediates, optimizing overall product yield (373). Recently, implementation of a CRISPRi dynamic feedback control increased the production of *N*-acetylglucosamine from 81.7 g/L to 131.6 g/L in *B. subtilis* (374). Crucial to the function of these circuits is the biosensing of a pathway intermediate, GlcN6P, to trigger negative feedback control, which leads to optimized flux and reduced metabolic burden. Furthermore, gRNAs could be expressed from native stress- or

stationary phase–responsive promoters to allow autonomous regulation, mitigate growth burden, and achieve greater total protein and metabolite production (198, 375).

In addition to flux and burden control, decoupling cell growth and product synthesis is another approach to maximize overall production (376). QS has been used to monitor cell density to prioritize biomass accumulation early and product synthesis later. QS-based CRISPRi circuits have been used to improve production of various chemicals in *E. coli* (e.g., naringenin and xylitol) (377–379) and rapamycin in *Streptomyces* (380). Importantly, QS-based CRISPR circuits also improve bioproduction robustness across industrially relevant culturing conditions and enable dynamic programming of *E. coli–P. putida* consortium composition (381). Installing dynamic CRISPRi programs into *P. putida* boosted acetyl-CoA levels (382, 383). In the cyanobacteria *Synechocystis*, inducible CRISPRi of citrate synthase *gltA* at low culture densities increased CO_2 partitioning to *n*-butanol (384). To maintain high carbon partitioning into the desired product and increase overall CO_2 fixation rate, a control strategy to dynamically cycle between growth and production phases has been proposed. In lactate-secreting *Synechocystis*, intermittent CRISPRi growth arrest significantly increased cumulative lactate titers and allowed stable lactate production for one month (385).

An emerging approach to maintain stable bioproduction genotypes and phenotypes during prolonged culture times involves encapsulating bioproduction microbes in biocompatible materials, forming engineered living materials (ELMs) (386). We recently fabricated *E. coli* ELMs carrying dynamic CRISPR programs for long-term bioproduction of biopterin (162). These multigene CRISPR programs could be used to switch bioproduction on and off in several cycles for at least 19 days, indicating that on-demand programming of ELMs could be signaled with CRISPR circuitry. With implementation of a CRISPR circuit at the material–living microbe interface, more complex programming, such as spatially compartmentalized microbial consortia of multiple bioproduction modules (387–389), could be constructed.

In CFES, dynamic CRISPRa/i circuits have also enabled precise temporal control of gene expression through delays, pulses, and filters (45, 46, 48). These CRISPRa/i circuits could provide an efficient mechanism for implementing dynamic multigene control programs for bioproduction applications. For instance, circuits capable of delaying gene expression could be useful when enzymes consume protein expression resources or produce toxic intermediates. In membrane-augmented CFES, dynamic CRISPRa/i circuits could be implemented to investigate and optimize how membrane enzyme expression impacts membrane insertion and function (276). In lysate engineering, if the desired proteome is detrimental to cell growth, dynamic CRISPRa/i programs could be used to switch into the desired proteome once high biomass levels have been achieved.

Diverse CRISPR tools are being adopted widely to accelerate the engineering of prokaryotic systems. By further coupling new CRISPR tools for rapid strain and lysate engineering with artificial intelligence and machine learning approaches to recommend promising candidates (390–392), it could be possible to assess fewer genetic programs and achieve optimal strain in less time and with cheaper cost. Together with advancements in genome-scale modeling and computational gRNA design, these approaches will greatly accelerate the development of sophisticated bioproduction and biotherapeutic platforms.

FUTURE PROSPECTS

CRISPR tools have proven invaluable for engineering prokaryotic systems across many fields, from biosensing to bioproduction and biotherapeutics. The ability of CRISPR tools to precisely modify genetic material and encode new desired functions is unparalleled among traditional genetic engineering tools. The vast repertoire of different Cas proteins, tethered effector domains,

and gRNA design strategies grants flexibility in engineering tools with different capabilities for specific tasks. Although CRISPR transcriptional regulation and base editing are already well established, novel tools are being developed to engineer prokaryotic systems through other molecular processes, such as DNA looping, DNA replication, RNA translation, and protein degradation (161, 393–396).

Recently, dCas9-based CRISPR tools have been repurposed for modulating DNA replication. Plasmid copy-number manipulation in *E. coli* has been achieved by controlling concentrations of DNA replication machinery (395, 396). Targeting the genomic DNA replication parts also provides programmable control of growth rate of individual bacteria in a co-culture of *E. coli* and *P. putida* (381). Implementation of similar strategies with broad-host-range CRISPRa tools could enable further replication control in non-model bacteria with a more limited set of copy-number plasmids. Such tools could be integrated with dynamic CRISPR circuits to enable growth arrest for allocating metabolic resources toward bioproduction or population control in a microbial consortium.

CRISPR-based translational regulation in prokaryotes has also been developed recently (161), and its potential remains largely unexplored. With the RNA-targeting property of Cas13, translational control over a specific gene in a multigene operon could be possible. In *E. coli*, 68% of all genes are contained in operons, with some containing as many as 14 genes (397). Precise regulation of this significant number of genes with current dCas9/dCas12-based tools is limited because transcription of all genes in the operon is affected. Hence, combining dCas13- and dCas9/dCas12-based gene regulation would greatly expand our ability to precisely regulate genes within bacterial genomes (135).

Similarly, Cas13-guided RNA editing has been demonstrated in mammalian cells by tethering the deaminase domain to dCas13 (139) but is yet to be implemented in prokaryotic systems. Given that some effectors of the DNA base editors target RNA natively in bacteria (398), programmable prokaryotic Cas13-based RNA-editing systems should be within reach. Similar to CRISPR DNA-targeting tools, realizing the potential of CRISPR RNA-targeting in prokaryotes will require learning the rules to effectively and predictably repress, activate, and edit genes at the mRNA level.

The flexibility granted by CRISPR tools to localize different effectors to many targets has enabled the transition from a disarray of one-off molecular tools to a framework under which multiple tools can be orchestrated to engineer more complex systems (101, 399). Moving forward, simultaneous integration of multiple CRISPR tools in the diverse prokaryotic hosts should be immediately feasible considering that the distinct gRNA structures of Cas variants allow each CRISPR system to function orthogonally (26). The diverse catalog of Cas variants will provide suitable options for implementing CRISPR systems in any microbe with specific traits for each application. Given the number of orthogonal hairpin–RBP pairs, different effectors can be programmatically recruited to specific gRNAs, and therefore to specific nucleic acid targets. The designability of orthogonal CRISPR systems enable further complex circuitry such as conditional gene activation (400), and synergistic gene activation (C. Kiattisewee, A.V. Karanjia, R. Cardiff, K. Olander, P. Leejareon, S.S. Alvi, J.M. Carothers & J.G. Zalatan, manuscript in preparation). Overall, harnessing the diversity of natural and engineered CRISPR-Cas systems will enable development of the next generation of prokaryotic biosensors, dynamic bioproduction platforms, and smart microbial biotherapeutics.

DISCLOSURE STATEMENT

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