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REVIEW PAPER

Microbial CRISPRi and CRISPRa Systems for Metabolic Engineering

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Abstract Transcriptional regulation is essential for maintaining the natural cell metabolism of microbes and therefore important for metabolic engineering. The development of non-native transcriptional regulation tools in engineered microbes may change the gene expression and metabolic flux, and shall benefit the product titer and yield in bioprocess. CRISPR interference (CRISPRi), as an artificial transcriptor which may regulate any gene at different scales, has rapidly gained popularity for metabolic engineering strains. This article briefly describes the mechanism and development of CRISPRi, including inhibition and activation of two forms of action and several different sources of dCas9 protein. And we summarize the applications of CRISPRi in regulating the metabolic pathway, changing the physiological state of the host, and genomic screening. Finally, we analyze a few limitations of the CRISPRi system and summarize some ways to improve them.

Keywords: CRISPRi, transcriptional regulation, metabolic engineering, high-throughput screening.

1. Introduction

The development of metabolic engineering allowed us to produce a variety of compounds from the microbial cell factories, including chemicals, drugs, biodegradable plastics, and biofuels [1,2], and brought enormous economic, environmental, and social benefits [3-6]. In fact, the metabolic network of host cell is extraordinarily complex and possess a series of strict regulatory mechanisms, such as negative feedback inhibition by downstream metabolites, transcriptional activation by special signaling molecules, the balance of competitive pathway, cofactor, energy provision, *etc*. Transcriptional regulation of the metabolic network is widespread in microbial cells and plays an important role in different physiological processes. The transcriptional regulators able to activate or repress the expression of relevant genes through binding to the specific DNA operator sequences and altering the transcription of targeted operons. These mechanisms allow cells to respond to various intra- and extracellular signals and eliciting responses. Most transcriptional regulators response to a certain single and controlling the expression of a specific operon, such as LacI, TetR, AraR, *etc* [7-9]. However, global transcriptional regulators, such as CRP, ArcA and FNR, can simultaneously regulate multiple genes containing the corresponding DNA operator region [10]. The most representative CRP protein directly regulates the expression of 400 genes and able to affect the expression of more than half of the genes in *Escherichia coli* by indirectly regulating other transcriptional regulators [11,12]. These complex transcriptional regulatory mechanisms interact with each other and ultimately determine the metabolic properties of cells.

Therefore, to maximize the flux from the substrates to the final products, a variety of transcriptional regulation tools have been developed [13-18], such as RNA interference (RNAi), Metabolite-sensing riboswitches [19-21], Hfq-associated regulatory small RNA [22,23], Zinc-finger nucleases (ZFN), and transcription activator-like effector nucleases (TALEN) [24,25]. The RNAi-mediated regulation

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of gene expression has been wildly used in clinical research, drug discovery, disease detection, and crop pest control. Down-regulation of gene expression was achieved by synthetic small interfering RNA (siRNA) or short hairpin RNA (shRNA) that binding to the homologous region of mRNA and inducing degradation. However, these methods have serious off-target effects and limit its use in metabolic engineering [26]. ZNF and TALEN were composed of a sequence-specific DNA binding domain and a non-specific DNA cleavage domain. The DNA binding domain can be programmed by specific amino acid combination, but the entire construction process is time consuming and laborious [27,28].

Recently, the development of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (CRISPR/Cas) system have brought new opportunity to modify the cell genetic characteristics [29,30]. The most representative CRISPR-Cas9 system, only requires an endonuclease Cas9 protein and a chimeric gRNA to target DNA sequence of interest and create double-strands break (DSB). DSB can be repaired either by homologous recombination (HR) or non-homologous end joining (NHEJ), achieving the editing of target sequence [31-33].

A catalytically inactive version of Cas9 retaining only the ability to bind double-stranded DNA can be repurposed as a platform for RNA-guided transcription regulation without genetically altering the target sequence. This transcriptional regulatory tool is known as CRISPR interference (CRISPRi), and should have many advantages compared to other systems [34-36]. This review briefly introduces the basic working principle of CRISPRi, focuses on the application of CRISPRi in metabolic engineering, and discusses the perspective.

2. The Establishment and Working Mechanism of CRISPRi System

There are three essential components for the CRISPR-Cas9 system: a mature CRISPR RNA (crRNA), a partially complementary trans-activating crRNA (tracrRNA), and a Cas9 protein [32,33]. TracrRNA and RNase III are required for the mutation of crRNA, which has a spacer binding target DNA and a direct repeat portion complementary to tracrRNA. To further make the tool convenient, an engineered chimeric guide RNA (gRNA) containing a hairpin that mimics the tracrRNA-crRNA complex has been designed, which makes the system simple and easy. The Cas9 protein contains two endonuclease domains, HNH and RuvC [37,38]. HNH domain is responsible for complementary sequence cleavage of target DNA, while RuvC is responsible for the non-complementary sequence cleavage [33]. Under the guidance of the tracrRNA-crRNA complex or gRNA, the targeted dsDNA is cleaved by Cas9 protein.

CRISPRi was established by replacement of Cas9 with a catalytically inactive Cas9 (dCas9), which is obtained by introducing mutation (D10A/H840A) to inactivate HNH and RuvC. The binding of dCas9 protein on DNA could block the passage of RNA polymerase (RNAP) by physical collision, thereby preventing transcription and repress the expression of genes (Fig. 1A) [39,40].

In eukaryotes, it is difficult to block mRNA transcription via dCas9 complex binding to the specific DNA sequence [41-44]. In order to enhance the inhibitory efficiency, dCas9 protein is usually fused to various transcriptional effect domains, and the target gene is maximally interfered by the dual inhibitory effect. Transcriptional repressors include the myc-associated factor X (MAX)-interacting proteins 1 (MXI1), the Krüppel-associated box (KRAB) domain of Kox1, the chromo shadow (CS) domain of HP1α, the hairy-related basic helix-loop-helix repressor proteins (WRPW) domain of Hes1, and the SID4X domain (Fig. 1B) [28,45-50].

When the dCas9 protein was fused to a transcriptional activator, this system can promote the expression of the target gene [49], which is called CRISPRa (Fig. 1C). In this case, the dCas9-transcriptional activator complex targets the upstream of the specific target gene, and recruits RNAP to activate transcription, therefore, enhances the expression of the target gene. Transcriptional activators include the transactivator domain of the herpes simplex viral protein 16 (VP16), multiple copies of VP16 (VP64 or VP160), or the transactivator domain of nuclear factor kappa B (p65) [51-54]. Fusing multiple copies of the transactivator domains to dCas9 can increase the efficiency of activation. Based on this principle, new CRISPRa systems incorporating a multi-copy transactivator domain were designed, such as SunTag, SAM, VPR and SPH systems [55-59]. And scaffold RNA (scRNA) has also been constructed to achieve simultaneous activation of multiple genes. Contrary to eukaryotic cells, there are not many alternative transcriptional activators in prokaryotic cells, which limits the use of CRISPRa in prokaryotic cells [60]. Among bacteria, dCas9 can directly fuse RNA polymerase subunits such as the ω subunit, RpoZ, or fuse transcriptional activator such as SoxS to activate the complete RNA polymerase [40,60-64].

In addition, CRISPR-SpdCas9 system derived from *Streptococcus pyogenes* can be combined with other orthogonal CRISPR systems to regulate or knockout multiple genes simultaneously. The orthogonality of several Cas9 variants came from different microorganisms were compared, establishing the orthogonal CRISPRi and CRISPRko systems consisting of SpdCas9 and *Streptococcus thermophilius*

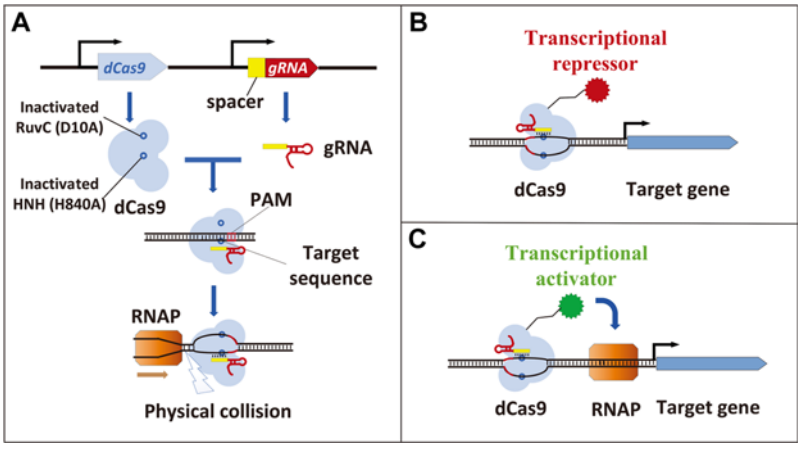

Fig. 1. The biological mechanisms of CRISPRi/a. (A) CRISPR/dCas9 which guided by gRNA regulates transcription through blocking RNAP. (B) The dCas9 fused transcriptional repressor (red) has a preferable inhibitory effect. (C) Transcriptional activator (green) can recruit RNA polymerase to activate gene expression. The CRISPRa system combining with transcriptional activator can upregulate the expression of genes.

CRISPR1 (St1Cas9). This system was used to increase the production of succinic acid in *E. coli* [65]. Type III CRISPR/Cas-associated Csy4 endoribonuclease from *Pseudomonas aeruginosa* has the RNA processing capacity to generate multiple gRNAs from a single transcript. This property allows Cys4 to be introduced into *Saccharomyces cerevisiae* to achieve multi-gene editing and regulation of its genome [66].

CRISPRi other than CRISPR Cas9 system was also developed. Cpf1 is a single RNA-guided endonuclease of a Class 2 type V CRISPR-Cas system. Unlike Cas9, the maturation of crRNA requires only the action of the Cpf1 protein. Cpf1 forms a Cpf1-crRNA complex with the matured crRNA, recognizes and cleaves the target gene sequence, and does not require the participation of tracrRNA. Cpf1 recognizes PAM sequences which are predominantly T-rich, so in areas rich in AT, are easier to find suitable recognition sites than Cas9 [67]. CRISPR-Cpf1 system proves to be a highly efficient tool for genetic modification of some important industrial strains that cannot utilize the SpCRISPR-Cas9 system. Cpf1 has only one nuclease domain-RuvC, which can be mutated to obtain DNase-deactivated Cpf1 (dCpf1), such as FndCpf1 (D917A) [67] from *Francisella novicida* and AsCpf1 from *Acidaminococcus sp.* (E993A) [68]. A mature gene regulatory system based on FndCpf1-KRAB or FndCpf1 (from *Francisella novicida*) was established in *Yarrowia lipolytica* and *Streptomyces hygroscopicus* SIPI-KF, expanding the CRISPRi metabolic engineering toolbox [69,70].

After a preliminary understanding of the mechanism of CRISPRi, the convenient gene regulation technology established on this basis is rapidly applied in various fields of biology, including metabolic engineering (Table 1).

3. Regulating the Metabolic Pathway with CRISPRi

In order to obtain higher yields and productivity, regulating the expression of key enzymes to redirect the metabolic flux to desired products is pathway is one of the commonly used strategies. Recombineering has been broadly used to block the gene expression by direct gene knockout or replacement gene upstream cis-acting element. However, this method has a long experimental period and high-intensity workload. CRISPR based genome editing enables rapid knockout of the gene of interest, greatly reducing the experimental time. Nonetheless, many genes of interest are essential genes for cell survival and cannot be direct knockout. CRISPRi and CRISPRa system able to change the expression of the gene of interest at the transcriptional level, thereby optimizing the metabolic pathway. The degree of gene inhibition can be controlled by regulating the expression of dCas9 or regulating the binding position of gRNA. Those features have made CRISPRi widely used in the field of metabolic engineering and play an important role in regulating the metabolic pathway [89]. The *gltA* encodes the citrate synthase (CS). Repression of this gene by CRISPRi increased the production of L-lysine in *Corynebacterium*, while in *Synechocystis sp.* PCC 6803, the titer of biofuels such as ethanol and butanol were increased [71,72]. *PlsX* is a C18-specific phosphoacyltransferase which is essential for membrane lipid biosynthesis. The inhibition of *PlsX* activity by *Synechocystis* causes the flux of fatty acids to shift from membrane biosynthesis to fatty alcohols, and the production of fatty alcohols is increased by a factor of three [73]. The application of CRISPRa in metabolic engineering with the eukaryotic host is comparative maturity. The dCas9 fused with the

Table 1. The applications of CRISPRi/a in metabolic engineering

Strain	System	Promoter (dCas)	Promoter (gRNA)	Target gene	Expression level	Product	Effect	References
Regulating the metabolic pathway with CRISPRi								
Corynebacterium glutamicum DM1919	CRISPRi-SpdCas9	aTc inducible promoter PtetA	A constitutive promoter	*gltA*	35 %	L-lysine	1.39-fold increase in production	[71]
Synechocystis PCC6803	CRISPRi-SpdCas9	aTc inducible promoter PL22	aTc inducible promoter PL22	*odhB*	<50%	Ethanol	2-fold higher carbon partitioning	[72]
Synechocystis PCC6803	CRISPRi-SpdCas9	aTc inducible promoter PL22	aTc inducible promoter PL22	*gltA*	<10%	Butanol	5-fold higher carbon partitioning	[72]
Synechocystis sp. PCC 6803	CRISPRi-SpdCas9	aTc inducible promoter PL22	A constitutive promoter	*plsX*	15%	Octadecanol	9.3 (SD 1.7) mg/g DCW	[73]
Yarrowia Lipolytica	CRISPRa-SpdCas9-VPR	An episomal plasmid	Hybrid Pol III promoter	*BGL1* *BGL2*	112-fold 20-fold		Growth with cellobiose as the sole carbon source	[74]
E. coli	CRISPRa-SpdCas9-scRNA-SoxS	aTc or arabinose inducible promoter	aTc or arabinose inducible promoter	*pdc adhB*		Ethanol	3-fold increase in production	[60]
B. subtilis	CRISPRi-SpdCas9	xylose-inducible promoter P*xylA*	A constitutive promoter	*zwf* *pfkA* *glmM*	2% 5% 8%	GlcNAc	103.1 ± 2.11 g/L in a 3-L fed-batch bioreactor	[75]
E. coli	CRISPRi-SpdCas9	aTc or IPTG inducible promoter	A constitutive promoter	*ppc* *gltA* *aceB*	10% 20% 80%	Malate	2.3-fold increase in titer	[76]
E. coli	CRISPRi-SpdCas9	An AND gate integrating the glucose and acetate sensors	An AND gate integrating the glucose and acetate sensors	*poxb*	Dynamic regulation		Reducing the final acetate concentration by half	[77]
E. coli	CRISPRi-SpdCas9	A circuit which responds in the presence of glucose AND NOT low oxygen	A circuit which responds in the presence of glucose AND NOT low oxygen	*pta*	Dynamic regulation		4-fold reduction in acetate	[77]
E. coli	CRISPRi-SpdCas9 + CRISPR-St1Cas9	aTc inducible promoter	A constitutive promoter	*ptsG* *ldhA* *pflB*	<30% <35% <40%	Succinate	Elevated the succinate titer ≈178% to ≈2.5 g/L	[65]
Yarrowia lipolytica	CRISPR-FndCpf1	A strong, endogenous TEFin promoter	Synthetic hybrid Pol III promoter SCR1′-tRNAGly	*vioA* *vioB* *vioE*		PVA	PVA relative absorbance reduced to 39%	[69]

Table 1. Continued

Strain	System	Promoter (dCas)	Promoter (gRNA)	Target gene	Expression level	Product	Effect	References
Streptomyces coelicolor	CRISPR-FndCpf1	An inducible promoter *tipAp*	A strong promoter *kasOp**	*redX*	29.40%	RED	The corresponding pigmented antibiotics are reduced in yield and the color of the strain is lighter.	[70]
				actI-orf1	32.60%	ACT		
				cpkA	30.10%	CPK		
Anabaena sp. PCC 7120	CRISPRi -SpdCas9	An AND gate integrating a riboswitch responsive to theophylline and an aTc inducible promoter	A constitutive promoter	*GlnA*	<20%	Ammonium	200 μM ammonium was excreted	[78]
Clostridium ljungdahlii	CRISPRi -SpdCas9	The lactose-inducible promoter pAH2	An engineered promoter P4	*pta*	3%	3-hydroxybutyrate	2.3-fold increase in titer	[79]
Kluyveromyces marxianus	CRISPRi -SpdCas9	A constitutive *S. cerevisiae TEF1* promoter	A synthetic polymerase III promoter	*ACO2b*	25%	Ethyl acetate	3.8-fold increase in ethyl acetate productivity	[80]
				SDH2	31%			
				MSS51	20%			
				RIP1	10%			
Changing the physiological state of the host with CRISPRi								
E. coli	CRISPRi -SpdCas9	aTc inducible promoter	aTc inducible promoter	*ftsW*	<50%	Poly-3-hydroxybutyrate (PHB)	PHB synthesis increased from 25% to 93% in CDW	[81]
E. coli	CRISPRi -SpdCas9	A constitutive promoter	A blue light repressible promoter	*wcaF*	Dynamic regulation		Control the biofilm thickness spatially	[82]
E. coli	CRISPRi -SpdCas9	aTc inducible promoter	A constitutive promoter	*pyrF*		Mevalonate	41% increase in yield	[83]
E. coli	CRISPR-lim	aTc inducible promoter	A constitutive promoter	T7 / lacO1 promoter			Attenuating leaky gene expression	[84]
E. coli	CRiPi	aTc inducible promoter	aTc inducible promoter	*murE*			Increased sensitivity of engineering strains to fosfomycin	[85]
CRISPRi as a tool for genomic screening								
Corynebacterium glutamicum	CRISPRi -SpdCas9	aTc inducible promoter	A constitutive promoter	Gene candidates for the unknown esterase		Esterase	The *Cg0961 (CgHle)* gene was successfully identified as esterase	[86]
E. coli	CRISPRi -SpdCas9	aTc inducible promoter	A constitutive promoter	Entire genome			Verifying the necessity of 79% of the previously reported essential genes and some phage host factors	[87]
Mycoplasma pneumoniae	CRISPRi -SpdCas9	aTc inducible promoter	A constitutive promoter	3 essential genes and 3 non-essential genes previously reported			Targeting of six selected endogenous genes with this system results in lowered transcript levels or reduced growth rates that agree with lack or shortage of data in previous transposon bombardment studies.	[88]

synthetic tripartite activator VPR able to active the native β-glucosidase expression, and promote the growth of *Yarrowia lipolytica* with cellobiose as the sole carbon source [74]. In prokaryotes, CRISPRa has not been wildly applied due to the lack of suitable transcriptional activators. Chen Dong *et al.* compared 11 potential activators and constructed an efficient CRISPRa system using SoxS. SoxS can recruit RNA polymerase to activate gene expression under the oxidative stress environment. The SoxS-based CRISPRa system has been used to upregulate the expression of a heterologous *pdc adhB* gene cassette from *Zymomonas mobilis*, and improved the yield of ethanol three times than that of the control *E. coli* [60].

The microbial metabolism is an extremely complex network, so that regulating a single intermediate reaction often has an unsatisfactory effect. CRISPRi only requires 20 bp of complementary RNA sequence to target the specific site, which has significant advantages in high-throughput gene regulation on the genome scale. Simultaneous regulation of multiple genes can be achieved simply by the concatenation of designed multiple gRNAs with different targeting sequences. A xylose-induced inhibition system was established that represses the expression of three genes, *zwf, pfkA,* and *glmM*. These genes belong to the pentose phosphate pathway (PPP, also referred to as hexose monophosphate shunt (HMP shunt)), the glycolytic pathway and the peptidoglycan synthesis pathway (PSP) respectively. By inhibiting the expression of these genes, carbon catabolite repression (CCR) was broken, and *Bacillus subtilis* could utilize xylose to produce N-acetylglucosamine (GlcNAc) [75]. Using multiplexed CRISPR interference in *E. coli* which targeting three key enzyme (pyruvate carboxylase (PC), citrate synthase (CS), and malate synthase (MS)) genes simultaneously, the metabolic constraints were eliminated by rationally assigning an optimal gene expression pattern for each pathway module. Then the production of malic acid was increased by a factor of 2.3 [76]. By combining CRISPRi with genetically encoded sensors, the dynamic multigenes regulation can be implemented in metabolic engineering. These sensors could respond to various signals such as external environmental signals (O_2, temperature, pH), the internal cell state (metabolites, growth phase, stress response, redox), the depletion of carbon feedstock (glucose), cell density, or the accumulation of products and by- products (acetate). Dynamic regulation is an important regulation strategy that may improve product titer. The dynamic regulation of the metabolic pathway by CRISPRi can be achieved by coupling these sensors to the expression of different gRNAs. Three *E. coli* sensors that respond to the consumption of feedstock (glucose), dissolved oxygen, and by- product accumulation (acetate) were constructed and optimized to establish a combinatorial logic circuit which can dynamically regulate endogenous metabolism in *E. coli* [77]. CRISPRi systems except dCas9 have also been established for metabolism pathway modification. Multi-gene repression based on SpdCas9 was also used in combination with other orthogonal Cas proteins, allowing the CRISPR and CRISPRi systems to be established simultaneously in the same host strain. Cas9 variants from *Staphylococcus aureus* (SaCas9) and *Streptococcus thermophilius* CRISPR1 (St1Cas9) as well as Cas12a derived from *Francisella novicida* (FnCas12a) were introduced in *E. coli*, and assessed the ability to induce DSB by a death assay. ScCas9 and St1Cas9 are able to achieve 99% lethal efficiency, and FnCas12a is only 60%. Further test results indicated that SpCas9, SaCas9, and St1Cas9 were orthogonal without mutual interference in *E. coli*. Then the St1Cas9 system to integrate SpdCas9 and sgRNA arrays were harnessed for constitutive knockdown of three genes (*ptsG*, *ldhA*, and *pflB*), knock- in *pyc* and knockout *adhE*. The combination of orthogonal CRISPR/CRISPRi for metabolic engineering enhanced succinate production while inhibiting byproduct formation [65]. Cpf1 system is also established as a multi-gene transcriptional regulation tool in different organisms. In *Yarrowia lipolytica*, four forms of CRISPRi systems, such as DNase-deactivated Cpf1 (dCpf1) from *Francisella novicida*, deactivated Cas9 (dCas9) from *Streptococcus pyogenes*, and two fusion proteins (dCpf1-KRAB and dCas9-KRAB), were used to operate single gene and multiple genes repression. Taking the PVA pathway as an example, the expression of gRNA arrays targeting three enzymes was designed, and the PVA relative absorbance was reduced by 61% (dCpf1) and 75% (dCas9) [69]. Cpf1 and dCpf1 have also been successfully introduced in *Streptomyces* for gene editing and transcriptional regulation. Lei Li *et al.* verified the ability of dCpf1 to simultaneously regulate multiple endogenous genes in *Streptomyces coelicolor* by using three pigmented antibiotic production genes *redX*, *actI-orf1*, and *cpkA*. The expression levels of the three mRNAs were reduced by 70.6%, 67.4%, and 69.9%, respectively [70].

The composition of CRISPRi system is simple and easy to establish in different microorganisms. As a multicellularity model for prokaryotic cells, *Anabaena sp.* A. 7120 has important research value. The CRISPRi system was constructed in *Anabaena sp.* and successfully inhibited the gene *glnA* which encodes glutamine synthetase. *GlnA* is the initial enzyme of the nitrogen assimilation pathway, the inhibition of this gene leads to ammonium efflux, which increases the yield of ammonium [78]. *Clostridium ljungdahlii* has emerged as an attractive candidate for the bioconversion of synthesis gas (CO, CO_2, H_2) to a variety of fuels and chemicals through the Wood-Ljungdahl pathway. CRISPRi has used to inhibit the expression of

aor2 and *pta*, transferring carbon flux from producing acetic acid to 3HB [79]. *Kluyveromyces marxianus* is a nonconventional yeast, which can tolerate high temperature and low pH, and produce volatile esters at a high rate. CRISPRi system has been established in this strain to the production of acetoacetate [80].

4. Changing the Physiological State of the Host with CRISPRi

The capacity of CRISPRi to regulate essential genes makes it easy to fine-tune cell physiological traits. Therefore, CRISPRi is very useful for investigating the relationship between genotype and physiological traits of cells. CRISPRi represses the expression of *murE* (encoding an ligase that catalyze the addition of the third amino acid to the peptide moiety of the monomer unit of peptidoglycan), *murD* (encoding UDP-N-acetylmuramoyl-L-alanine: D-glutamate ligase) [90], *mraY* (The expression of encoding phosphor-N-acetylmuramoyl-pentapeptide transferase) [91] and/or *ftsW* (encoding a lipid II flippase acting to transport lipid-linked peptidoglycan precursors across the inner membrane) [92] to weaken cell wall synthesis and reduce the mechanical strength of the cells, making the engineering strain hold more PHB particles. At the same time, cell wall synthesis competes with PHB production on carbon source glucose was reduced. Repressing the peptidoglycan synthesis increases the flow of glucose to the product synthesis pathway, which increases the titer of PHB. In addition, the excessive accumulation of PHB in weakened cell walls generated larger or even broken bacterial cells, which simplifies the process of PHB purification in industrial production [81]. Modification of biofilm is important for wastewater treatment and biological production. Therefore, a synthetic gene circuits based on CRISPRi has been designed to control the formation of biofilm by inhibiting the expression of *wcaF* in *E. coli* [93]. The expression of

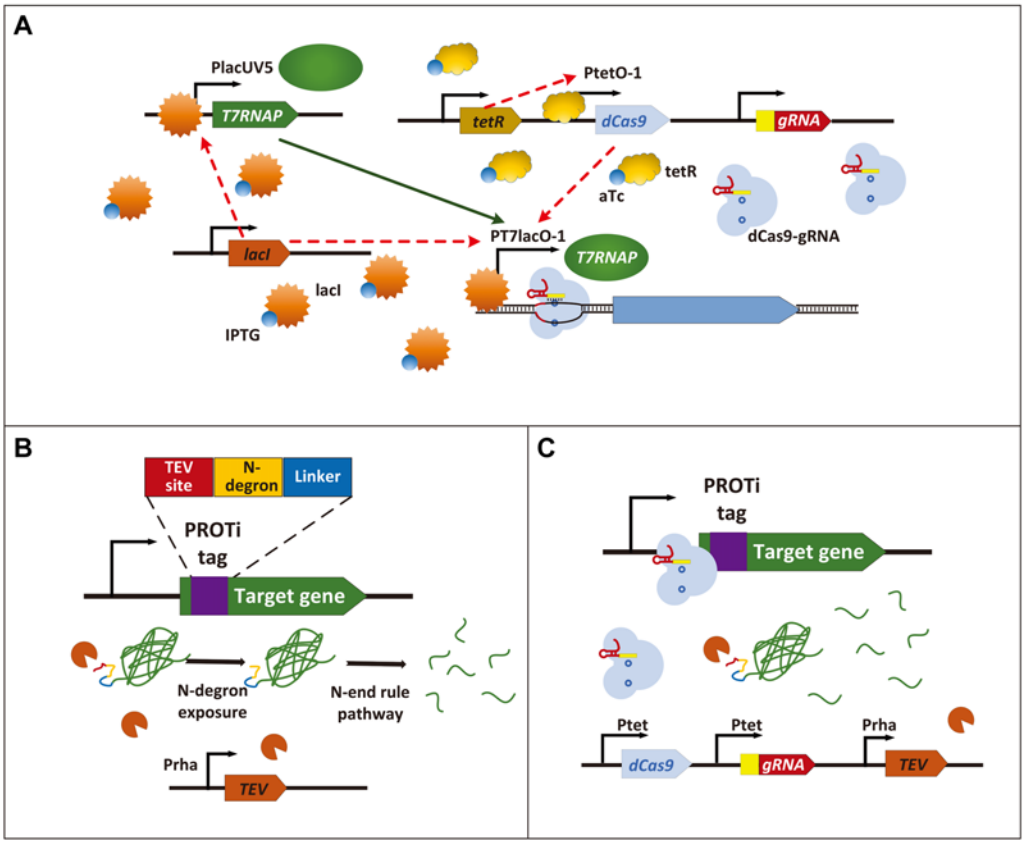

Fig. 2. The CRISPR- Lim, PROTi and CRiPi systems. (A) CRISPR- Lim consists of two parts, the aTc-induced CRISPRi system and the IPTG-induced T7 expression system. The repressor lacI regulates the expression of T7 RNAP and the T7 promoter $P_{T7lacO-1}$. Then CRISPRi targets to the T7 promoter so that the T7 promoter is dually inhibited by the lacI repressor and dCas9 protein, increasing the stringency of the T7 expression system. (B) The PROTi tag (purple) which integrated into the N-terminus of the target protein using CRMAGE contains the N-degron (dark yellow) shaded by the TEV site (red). When rhamnose is added to induce expression of TEV (brown), the TEV site is degraded, exposing N-degron. Then the target protein is degraded by the N-degron-mediated N-end rule pathway. (C) The CRiPi system combines PROTi and CRISPRi. The expression of the target protein is reduced by targeting CRISPRi to the coding sequences. And PROTi system accelerates the degradation of the target protein.

wcaF can be regulated on the time (regulating the time of addition of inducer) and space (bluelight-mediated gene circuit) dimension [82]. Industrial production strains need to consume a large portion of feedstock to produce biomass. Dynamically regulation of cell growth through CRISPRi could increase production yield. Cell growth is inhibited, while protein and compound yields are increased by designing gRNA that target DNA replication (*dnaA* or *oriC*) or nucleotide synthesis (*pyrF* or *thyA*) related genes. The promotion of yield was the most remarkable when targeting *pyrF*, and the GFP fluorescence intensity and MVA yield were increased by up to 2.15-fold and 41%, respectively [83].

The introduction of exogenous genes is also a common strategy for genetic engineering. The enzymes expressed by exogenous genes usually have higher activity than the original homologous enzymes, or may introduce new metabolic pathway. Some exogenous proteins have varying degrees of toxicity, therefore, the expression needs to be tightly controlled. Several systems based on CRISPRi have been designed to solve this problem. CRISPR- dCas9-Based Leaky- Expression Inhibition Module (CRISPR-Lim) (Fig. 2A) was designed to reduce the leakage of the T7 promoter. CRISPR-Lim consists of two parts, the aTc-induced CRISPRi system and the IPTG-induced T7 expression system. CRISPRi targets to the T7 promoter so that the T7 promoter is dually inhibited by the lacI repressor and dCas9 protein, increasing the stringency of the T7 expression system [84]. A CRiPi system (Fig. 2C) consisting of the protein interference system (PROTi) (Fig. 2B) and CRISPRi system able to efficient control the expression of target protein. In PROTi system an N-degrons degradation tag with a TEV site is added to the N-terminus of the target protein. Upon induction of the TEV protease, the exposure of N-degron leads to protein degradation by the N-end rule pathway. At the same time, CRISPRi can reduce the expression of the target protein at transcription level and accelerate the consumption of the target protein, achieving rapidly and dynamically regulating the expression of the specific protein [85]. Virginia Martínez *et al.* explored CRiPi' potential as a tool for creating antibiotic hypersensitive strains for use in antibiotic discovery. MurE, as same as MurA, is a central enzyme in peptidoglycan biosynthesis. Fosfomycin is an antibiotic that causes specific inhibition of the enzyme MurA. When CRiPi targets *murE*, the sensitivity to the antibiotic increased, depicted as complete growth inhibition at lower concentrations of the antibiotic, compared to the non-induced control. Then *murE* is demonstrated as a potential target for creating hypersensitive strains that can be used for screening compound libraries to identify agents with antibacterial activity [94].

5. CRISPRi as a Tool for Genomic Screening

With the development of DNA high throughput sequencing technology, genome-scale research has gradually attracted widespread attention. Prior to CRISPRi, the primary tool for genome-wide perturbation screening was RNAi [95-97]. However, RNAi has obvious limitations such as high sequence-specific off-target effect, different knockdown efficiencies, and inapplicability to prokaryotes [98-101]. Compared with RNAi, CRISPRi has a lower risk of off-target and has been successfully used in genome screening experiments [101]. CRISPRi-mediated genomic screening can be combined with bioinformatics to rapidly screen for genes with a specific function. First, gRNA library is obtained based on the special bioinformatics alignment to exclude the inefficient sites and avoid off-target effects. Then the sgRNA library is transferred to the target organism and activated the CRISPRi screening system. Finally, the corresponding phenotype can be enriched under the specific screening environment. To screen for unknown carboxylesterases in *Corynebacterium*, a candidate sequence highly homologous to the known lipase PvMekB was found by bioinformatics analysis and the candidate sequences were characterized for inhibition by the CRISPRi library. Finally, the *Cg0961* gene with natural esterase activity was successfully identified [86]. CRISPRi-mediated genomic screening can also randomly target the whole genome to interrogate the relationship between genotype and phenotype. 92,000 gRNAs were synthesized to targeted to the entire genome of *E. coli* randomly. The cultured gRNA abundance was tested to verify the necessity of 79% of the previously reported essential genes. Some phage host factors have also been identified, and colanic acid capsule synthesis is a shared resistance mechanism to phages λ, 186 and T4 [87]. Besides, Ana M. Mariscal *et al*. created a functional CRISPRi gene suppression system for *Mycoplasma pneumoniae* and *Mycoplasma mycoides* to unobstructed explore the function of essential genes [88].

6. The Limitation of CRISPRi and Future Perspectives

The widespread utilization of CRISPR gene editing technology promoting the development of biotechnology. CRISPRi derived from the CRISPR system is also important in its specific area, especially for regulation. The features of simple, economical, manageable, reversible, and the ability that does not completely block the expression of target gene confer CRISPRi an unparalleled advantage in regulating essential gene expression, interrogating unknown gene functions, and screening target genes on a genome scale.

Table 2. Limitations in CRISPRi/a system and the corresponding improvement strategies.

Limitations	Possible reasons	Improvement strategies
Toxicity of dcas9 to host cell [103,104]	The "bad-seed" effect [102]	Decreasing the expression or strictly regulating the expression at certain time point [102]
	Off-target effect [102]	Truncating the length of gRNA; predicting the off-target effect via bioinformatic tools; engineering the dCas9 protein to enhance the specificity of DNA binding [111-115]
Variable efficiency of different sgRNA [28]	The binding position and secondary structure of gRNA [39]	Targeting to the cis-acting element upstream of the gene sequence or the coding region close to TSS; predicting the advanced structure of gRNA via bioinformatic tools [39,116,117]
	The structure and kinetics of chromatin affect the binding of dCas9 protein [105,106]	Design multiple gRNAs targeting the same gene
Less efficiency of CRISPRa in prokaryotes [60]	dCas9 binding sites is limited by PAM sequence [60]	Engineering of dCas9 to recognize more PAM sequence [118,119]
	Few effective transcriptional activators [60]	Finding more potential transcriptional activators [60]
The exist of inapplicable sites for dCas9 [101]	Multiple genes transcribed via the same transcript [101]	
	Some two-way promoter regions are superimposed [101,107]	Targeting the coding region of genes
Complexity of multi genes regulatory systems	Activation of multiple gRNAs requires high concentrations of dCas9 protein [108-110]	Increasing the expression of dCas9[120]
	Instability of the repeat sequence	Using a variety of sequences and components [121]

Of course, CRISRRi/a also has some limitations (Table 2). Off-target binding can block the expression of essential or fitness genes with as little as 9 nt of identity in the seed sequence, and an unexplained sequence-specific toxicity "bad-seed" effect has been determined by the 5 PAM-proximal bases [102]. These lead to overexpression of dCas9 in many bacteria are often toxic [103,104]. Furthermore, the effects of various gRNAs targeting the same gene are great different, which influence the accuracy of high-throughput genomic screening based on CRISPRi [28]. Studies have shown that when gRNA was designed to targeting the cis-acting element upstream of the gene sequence or the coding region close to the transcription start site (TSS), the inhibitory effect of CRISPRi is remarkable [39]. Moreover, the structure and kinetics of chromatin also affect the binding efficiency of dCas9 complex, thereby impacting regulation effect of target genes [105,106]. In prokaryotes, CRISPRa has not been widely used due to the lack of suitable transcriptional activators. Besides, the optimal activation effect achieved by CRISPRa requires targeting a narrow region of about 90 bp upstream the TSS. This limits the application of CRISPRa in genes that have no identifiable PAM sites near the 90 bp upstream of TSS [60]. Furthermore, some special sites, such as where multiple genes transcribed via the same transcript or some two-way promoter regions are superimposed, CRISPRi is no longer the optimal choice [98,107]. Finally, construction of complex multi-gene regulatory systems remains difficult. Multiple sgRNAs compete for binding to a limited number of dCas9, leading to abnormity of activity [108-110]. Multi-gRNA containing complex repetitive sequences is unstable and tends to homologous recombination.

There have been several strategies proposed to improve these restrictions (Table 2). Decreasing the expression of dCas9 can significantly alleviate the growth inhibition caused by the "bad seed" effect [102]. At the same time, many methods to reduce off-target risk have been designed. One of the simplest measures is the use of a truncated gRNA, which is 17–18 rather than 20 nt in length. Critical to specific binding is 12 bp seed sequences, so the truncated gRNAs could reduce the binding energy to an extent that is just sufficient to bind a perfect target, but not targets containing mismatches [111-113]. More recently, improved versions of Cas9 have been developed, such as the enhanced specificity Cas9 (eSpCas9) and the high-fidelity variant SpCas9-HF1. Both of eSpCas9 and SpCas9-HF1 reduced the binding energy and making the Cas9/gRNA complex less tolerant of mismatches [114,115]. It is also necessary to use gRNA prediction software such as the ViennaRNA web services (http://rna.tbi.univie.ac.at/) and CRISPy-web (http://crispy.secondarymetabolites.org/) to assess the off-target risk and predict secondary structure during design of gRNA [116,117]. Studies have shown that gRNA can achieve a desired inhibitory effect when targeting a transcriptional start site (TSS) or an upstream cis-acting

element such as a promoter region. When targeting the coding strand of the coding region, the closer the gRNA binding site is to TTS, the more remarkable the repression effect. Conversely, it is more difficult to obtain the desired suppression effect when moving away from the TTS. It should be noted that there is no inhibitory effect when the gRNA targets the template strand. Therefore, gRNA should be designed to target upstream cis-acting elements or near the TSS [39]. For complex chromatin regions, multiple gRNAs targeting the same gene should be designed to guarantee the regulation effect. xCas9 and SpCas9-NG reduce the Cas9 protein's requirement for the third base of the PAM site, making the recognition of PAM site from NGG to NG [118,119]. More transcriptional activators have been tested for prokaryotic CRISPRa systems, and it is believed that stronger, more flexible, and more versatile prokaryotic CRISPRa systems will be developed [60]. For construction of complex multi-gene regulatory systems, the dCas9*_PhlF, with mutation (R1335K) impaired the ability of recognizing PAM and fused a PhlF repressor to recovery specific regulation, significantly reduced dCas9 protein toxicity, resulting in increased the concentration of dCas9 [120]. Increasing the sequences diversity can reduce the occurrence of recombination and improve the CRISPRi/a stability. At the same time, the introduction of logic gates and oscillators makes the dynamic and reasonable expression of the control system, which can reduce the loss of resources and weaken the interference to the normal life of the host [121].

7. Conclusion

In short, the CRISPRi system is a powerful genome-wide transcriptional regulation system whose potential is far from fully developed. With the discovery of other orthogonal Cas proteins, more candidate repressors and activators tested and screened, innovative applications by combining other biology components, CRISPRi will play a pivotal role in improving production of the product, dynamically analyzing the specific metabolic process, designing new synthetic pathways, and creating chassis microbial cells with excellent performance.

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References

1. Curran, K. A. and H. S. Alper (2012) Expanding the chemical palate of cells by combining systems biology and metabolic engineering. *Metab. Eng.* 14: 289-297.
2. Ye, V. M. and S. K. Bhatia (2012) Metabolic engineering for the production of clinically important molecules: Omega-3 fatty acids, artemisinin, and taxol. *Biotechnol. J*. 7: 20-33.
3. Jullesson, D., F. David, B. Pfleger, and J. Nielsen (2015) Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals. *Biotechnol. Adv.* 33: 1395-1402.
4. Nielsen, J. and J. D. Keasling (2016) Engineering cellular metabolism. *Cell*. 164: 1185-1197.
5. Wendisch, V. F., J. M. P. Jorge, F. Perez-Garcia, and E. Sgobba (2016) Updates on industrial production of amino acids using *Corynebacterium glutamicum*. *World J. Microbiol. Biotechnol.* 32: 105.
6. Baritugo, K. A., H. T. Kim, Y. David, J. I. Choi, S. H. Hong, K. J. Jeong, J. H. Choi, J. C. Joo, and S. J. Park (2018) Metabolic engineering of *Corynebacterium glutamicum* for fermentative production of chemicals in biorefinery. *Appl. Microbiol. Biotechnol.* 102: 3915-3937.
7. Mota, L. J., L. M. Sarmento, and I. de Sa-Nogueira (2001) Control of the arabinose regulon in *Bacillus subtilis* by AraR *in vivo*: crucial roles of operators, cooperativity, and DNA looping. *J. Bacteriol.* 183: 4190-4201.
8. Lewis, M. (2005) The lac repressor. *C. R. Biol.* 328: 521-548.
9. Ramos, J. L., M. Martinez-Bueno, A. J. Molina-Henares, W. Teran, K. Watanabe, X. Zhang, M. T. Gallegos, R. Brennan, and R. Tobes (2005) The TetR family of transcriptional repressors. *Microbiol. Mol. Biol. Rev.* 69: 326-356.
10. Martinez-Antonio, A. and J. Collado-Vides (2003) Identifying global regulators in transcriptional regulatory networks in bacteria. *Curr. Opin. Microbiol.* 6: 482-489.
11. Savery, N., V. Rhodius, and S. Busby (1996) Protein-protein interactions during transcription activation: the case of the *Escherichia coli* cyclic AMP receptor protein. *Philos. Trans. R. Soc. Lond. B Biol. Sci*. 351: 543-550.
12. Harman, J. G. (2001) Allosteric regulation of the cAMP receptor protein. *Biochim. Biophys. Acta*. 1547: 1-17.
13. Nakashima, N. and K. Miyazaki (2014) Bacterial cellular engineering by genome editing and gene silencing. *Int. J. Mol. Sci*. 15: 2773-2793.
14. McNerney, M. P., D. M. Watstein, and M. P. Styczynski (2015) Precision metabolic engineering: The design of responsive, selective, and controllable metabolic systems. *Metab. Eng.* 31: 123-131.
15. Didovyk, A., B. Borek, L. Tsimring, and J. Hasty (2016) Transcriptional regulation with CRISPR-Cas9: principles, advances, and applications. *Curr. Opin. Biotechnol.* 40: 177-184.
16. Engstrom, M. D. and B. F. Pfleger (2017) Transcription control engineering and applications in synthetic biology. *Synth. Syst. Biotechnol*. 2: 176-191.
17. Manan, S., B. Chen, G. She, X. Wan, and J. Zhao (2017) Transport and transcriptional regulation of oil production in plants. *Crit. Rev. Biotechnol.* 37: 641-655.
18. Stensjo, K., K. Vavitsas, and T. Tyystjarvi (2018) Harnessing transcription for bioproduction in cyanobacteria. *Physiol. Plant.* 162: 148-155.
19. Uguru, G. C., M. Mondhe, S. Goh, A. Hesketh, M. J. Bibb, L. Good, and J. E. Stach (2013) Synthetic RNA Silencing of Actinorhodin Biosynthesis in *Streptomyces coelicolor* A3(2). *PLoS One*. 8: e67509.
20. Yang, Y., Y. Lin, L. Li, R. J. Linhardt, and Y. Yan (2015) Regulating malonyl-CoA metabolism via synthetic antisense

RNAs for enhanced biosynthesis of natural products. *Metab. Eng.* 29: 217-226.
21. Leistra, A. N., N. C. Curtis, and L. M. Contreras (2019) Regulatory non-coding sRNAs in bacterial metabolic pathway engineering. *Metab. Eng.* 52: 190-214.
22. Dambach, M., I. Irnov, and W. C. Winkler (2013) Association of RNAs with *Bacillus subtilis* Hfq. *PLoS One*. 8: e55156.
23. Miyakoshi, M., G. Matera, K. Maki, Y. Sone, and J. Vogel (2019) Functional expansion of a TCA cycle operon mRNA by a 3' end-derived small RNA. *Nucleic Acids Res.* 47: 2075-2088.
24. Mercer, A. C., T. Gaj, S. J. Sirk, B. M. Lamb, and C. F. Barbas, 3rd (2014) Regulation of endogenous human gene expression by ligand-inducible TALE transcription factors. *ACS Synth. Biol*. 3: 723-730.
25. Copeland, M. F., M. C. Politz, C. B. Johnson, A. L. Markley, and B. F. Pfleger (2016) A transcription activator-like effector (TALE) induction system mediated by proteolysis. *Nat. Chem. Biol.* 12: 254-260.
26. Kim, D. and J. Rossi (2008) RNAi mechanisms and applications. *BioTechniques*. 44: 613-616.
27. Klug, A. (2010) The discovery of zinc fingers and their development for practical applications in gene regulation and genome manipulation. *Q. Rev. Biophys.* 43: 1-21.
28. Gilbert, L. A., M. A. Horlbeck, B. Adamson, J. E. Villalta, Y. Chen, E. H. Whitehead, C. Guimaraes, B. Panning, H. L. Ploegh, M. C. Bassik, L. S. Qi, M. Kampmann, and J. S. Weissman (2014) Genome-scale CRISPR-mediated control of gene repression and activation. *Cell*. 159: 647-661.
29. Czarnek, M. and J. Bereta (2016) The CRISPR-Cas system - from bacterial immunity to genome engineering. *Postepy Hig Med Dosw (Online)*. 70: 901-916.
30. Peterson, A. (2017) CRISPR: express delivery to any DNA address. *Oral Dis*. 23: 5-11.
31. Wiedenheft, B., S. H. Sternberg, and J. A. Doudna (2012) RNA-guided genetic silencing systems in bacteria and archaea. *Nature*. 482: 331-338.
32. Gasiunas, G., R. Barrangou, P. Horvath, and V. Siksnys (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. USA*. 109: E2579-2586.
33. Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 337: 816-821.
34. Chhotaray, C., Y. Tan, J. Mugweru, M. M. Islam, H. M. Adnan Hameed, S. Wang, Z. Lu, C. Wang, X. Li, S. Tan, J. Liu, and T. Zhang (2018) Advances in the development of molecular genetic tools for *Mycobacterium tuberculosis*. *J. Genet. Genomics*. 45: 281-297.
35. Tarasava, K., E. J. Oh, C. A. Eckert, and R. T. Gill (2018) CRISPR-enabled tools for engineering microbial genomes and phenotypes. *Biotechnol. J.* 13: e1700586.
36. Xu, X. and L. S. Qi (2019) A CRISPR-dCas toolbox for genetic engineering and synthetic biology. *J. Mol. Biol.* 431: 34-47.
37. Sapranauskas, R., G. Gasiunas, C. Fremaux, R. Barrangou, P. Horvath, and V. Siksnys (2011) The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res.* 39: 9275-9282.
38. Taylor, G. K., D. F. Heiter, S. Pietrokovski, and B. L. Stoddard (2011) Activity, specificity and structure of I-Bth0305I: a representative of a new homing endonuclease family. *Nucleic Acids Res.* 39: 9705-9719.
39. Qi, L. S., M. H. Larson, L. A. Gilbert, J. A. Doudna, J. S. Weissman, A. P. Arkin, and W. A. Lim (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*. 152: 1173-1183.
40. Bikard, D., W. Jiang, P. Samai, A. Hochschild, F. Zhang, and L. A. Marraffini (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res.* 41: 7429-7437.
41. Gagarinova, A. and A. Emili (2012) Genome-scale genetic manipulation methods for exploring bacterial molecular biology. *Mol. Biosyst*. 8: 1626-1638.
42. Li, Q., J. Chen, N. P. Minton, Y. Zhang, Z. Wen, J. Liu, H. Yang, Z. Zeng, X. Ren, J. Yang, Y. Gu, W. Jiang, Y. Jiang, and S. Yang (2016) CRISPR-based genome editing and expression control systems in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. *Biotechnol. J.* 11: 961-972.
43. Nakashima, N., T. Tamura, and L. Good (2006) Paired termini stabilize antisense RNAs and enhance conditional gene silencing in *Escherichia coli*. *Nucleic Acids Res.* 34: e138.
44. Peters, J. M., A. Colavin, H. Shi, T. L. Czarny, M. H. Larson, S. Wong, J. S. Hawkins, C. H. S. Lu, B. M. Koo, E. Marta, A. L. Shiver, E. H. Whitehead, J. S. Weissman, E. D. Brown, L. S. Qi, K. C. Huang, and C. A. Gross (2016) A Comprehensive, CRISPR-based functional analysis of essential genes in bacteria. *Cell*. 165: 1493-1506.
45. Margolin, J. F., J. R. Friedman, W. K. Meyer, H. Vissing, H. J. Thiesen, and F. J. Rauscher, 3rd (1994) Kruppel-associated boxes are potent transcriptional repression domains. *Proc. Natl. Acad. Sci. USA*. 91: 4509-4513.
46. Schreiber-Agus, N., L. Chin, K. Chen, R. Torres, G. Rao, P. Guida, A. I. Skoultchi, and R. A. DePinho (1995) An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. *Cell*. 80: 777-786.
47. Fisher, A. L., S. Ohsako, and M. Caudy (1996) The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol. Cell. Biol.* 16: 2670-2677.
48. Hathaway, N. A., O. Bell, C. Hodges, E. L. Miller, D. S. Neel, and G. R. Crabtree (2012) Dynamics and memory of heterochromatin in living cells. *Cell*. 149: 1447-1460.
49. Gilbert, L. A., M. H. Larson, L. Morsut, Z. Liu, G. A. Brar, S. E. Torres, N. Stern-Ginossar, O. Brandman, E. H. Whitehead, J. A. Doudna, W. A. Lim, J. S. Weissman, and L. S. Qi (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*. 154: 442-451.
50. Konermann, S., M. D. Brigham, A. Trevino, P. D. Hsu, M. Heidenreich, L. Cong, R. J. Platt, D. A. Scott, G. M. Church, and F. Zhang (2013) Optical control of mammalian endogenous transcription and epigenetic states. *Nature*. 500: 472-476.
51. Lundh, M., K. Plucinska, M. S. Isidor, P. S. S. Petersen, and B. Emanuelli (2017) Bidirectional manipulation of gene expression in adipocytes using CRISPRa and siRNA. *Mol. Metab*. 6: 1313-1320.
52. Xiong, K., Y. Zhou, K. A. Blichfeld, P. Hyttel, L. Bolund, K. K. Freude, and Y. Luo (2017) RNA-guided activation of pluripotency genes in human fibroblasts. *Cell Reprogram*. 19: 189-198.
53. Putri, R. R. and L. Chen (2018) Spatiotemporal control of zebrafish (*Danio rerio*) gene expression using a light-activated CRISPR activation system. *Gene*. 677: 273-279.
54. Wang, X. G., S. Y. Ma, J. S. Chang, R. Shi, R. L. Wang, P. Zhao, and Q. Y. Xia (2019) Programmable activation of *Bombyx* gene expression using CRISPR/dCas9 fusion systems. *Insect. Sci.* 26: 983-990.
55. Tanenbaum, M. E., L. A. Gilbert, L. S. Qi, J. S. Weissman, and R. D. Vale (2014) A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell*. 159: 635-646.

56. Chavez, A., J. Scheiman, S. Vora, B. W. Pruitt, M. Tuttle, E. P. R. Iyer, S. Kiani, C. D. Guzman, D. J. Wiegand, D. Ter-Ovanesyan, J. L. Braff, N. Davidsohn, B. E. Housden, N. Perrimon, R. Weiss, J. Aach, J. J. Collins, and G. M. Church (2015) Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods*. 12: 326-328.
57. Konermann, S., M. D. Brigham, A. E. Trevino, J. Joung, O. O. Abudayyeh, C. Barcena, P. D. Hsu, N. Habib, J. S. Gootenberg, H. Nishimasu, O. Nureki, and F. Zhang (2015) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*. 517: 583-588.
58. Chavez, A., M. Tuttle, B. W. Pruitt, B. Ewen-Campen, R. Chari, D. Ter-Ovanesyan, S. J. Haque, R. J. Cecchi, E. J. K. Kowal, J. Buchthal, B. E. Housden, N. Perrimon, J. J. Collins, and G. Church (2016) Comparison of Cas9 activators in multiple species. *Nat. Methods*. 13: 563-567.
59. Zhou, H., J. Liu, C. Zhou, N. Gao, Z. Rao, H. Li, X. Hu, C. Li, X. Yao, X. Shen, Y. Sun, Y. Wei, F. Liu, W. Ying, J. Zhang, C. Tang, X. Zhang, H. Xu, L. Shi, L. Cheng, P. Huang, and H. Yang (2018) *In vivo* simultaneous transcriptional activation of multiple genes in the brain using CRISPR-dCas9-activator transgenic mice. *Nat. Neurosci.* 21: 440-446.
60. Dong, C., J. Fontana, A. Patel, J. M. Carothers, and J. G. Zalatan (2018) Synthetic CRISPR-Cas gene activators for transcriptional reprogramming in bacteria. *Nat. Commun*. 9: 2489.
61. Peters, J. M., M. R. Silvis, D. Zhao, J. S. Hawkins, C. A. Gross, and L. S. Qi (2015) Bacterial CRISPR: accomplishments and prospects. *Curr. Opin. Microbiol.* 27: 121-126.
62. Choi, K. R. and S. Y. Lee (2016) CRISPR technologies for bacterial systems: Current achievements and future directions. *Biotechnol. Adv.* 34: 1180-1209.
63. Otoupal, P. B., K. E. Erickson, A. Escalas-Bordoy, and A. Chatterjee (2017) CRISPR perturbation of gene expression alters bacterial fitness under stress and reveals underlying epistatic constraints. *ACS Synth. Biol*. 6: 94-107.
64. Peng, R., Y. Wang, W. W. Feng, X. J. Yue, J. H. Chen, X. Z. Hu, Z. F. Li, D. H. Sheng, Y. M. Zhang, and Y. Z. Li (2018) CRISPR/dCas9-mediated transcriptional improvement of the biosynthetic gene cluster for the epothilone production in *Myxococcus xanthus*. *Microb. Cell Fact*. 17: 15.
65. Sung, L. Y., M. Y. Wu, M. W. Lin, M. N. Hsu, V. A. Truong, C. C. Shen, Y. Tu, K. Y. Hwang, A. P. Tu, Y. H. Chang, and Y. C. Hu (2019) Combining orthogonal CRISPR and CRISPRi systems for genome engineering and metabolic pathway modulation in *E. coli*. *Biotechnol. Bioeng.* 116: 1066-1079.
66. Ferreira, R., C. Skrekas, J. Nielsen, and F. David (2018) Multiplexed CRISPR/Cas9 genome editing and gene regulation using Csy4 in *Saccharomyces cerevisiae*. *ACS Synth. Biol*. 7: 10-15.
67. Zetsche, B., J. S. Gootenberg, O. O. Abudayyeh, I. M. Slaymaker, K. S. Makarova, P. Essletzbichler, S. E. Volz, J. Joung, J. van der Oost, A. Regev, E. V. Koonin, and F. Zhang (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell.* 163: 759-771.
68. Zhang, X., J. Wang, Q. Cheng, X. Zheng, G. Zhao, and J. Wang (2017) Multiplex gene regulation by CRISPR-ddCpf1. *Cell Discov.* 3: 17018.
69. Zhang, J. L., Y. Z. Peng, D. Liu, H. Liu, Y. X. Cao, B. Z. Li, C. Li, and Y. J. Yuan (2018) Gene repression via multiplex gRNA strategy in *Y. lipolytica*. *Microb. Cell Fact*. 17: 62.
70. Li, L., K. Wei, G. Zheng, X. Liu, S. Chen, W. Jiang, and Y. Lu (2018) CRISPR-Cpf1-Assisted multiplex genome editing and transcriptional repression in *streptomyces*. *Appl. Environ. Microbiol.* 84: e00827-18.
71. Park, J., H. Shin, S. M. Lee, Y. Um, and H. M. Woo (2018) RNA-guided single/double gene repressions in *Corynebacterium glutamicum* using an efficient CRISPR interference and its application to industrial strain. *Microb. Cell Fact*. 17: 4.
72. Shabestary, K., J. Anfelt, E. Ljungqvist, M. Jahn, L. Yao, and E. P. Hudson (2018) Targeted repression of essential genes to arrest growth and increase carbon partitioning and biofuel titers in Cyanobacteria. *ACS Synth. Biol*. 7: 1669-1675.
73. Kaczmarzyk, D., I. Cengic, L. Yao, and E. P. Hudson (2018) Diversion of the long-chain acyl-ACP pool in *Synechocystis* to fatty alcohols through CRISPRi repression of the essential phosphate acyltransferase PlsX. *Metab. Eng.* 45: 59-66.
74. Schwartz, C., N. Curtis, A. K. Lobs, and I. Wheeldon (2018) Multiplexed CRISPR activation of cryptic sugar metabolism enables *Yarrowia Lipolytica* growth on cellobiose. *Biotechnol. J.* 13: e1700584.
75. Wu, Y., T. Chen, Y. Liu, X. Lv, J. Li, G. Du, R. Ledesma-Amaro, and L. Liu (2018) CRISPRi allows optimal temporal control of N-acetylglucosamine bioproduction by a dynamic coordination of glucose and xylose metabolism in *Bacillus subtilis*. *Metab. Eng.* 49: 232-241.
76. Gao, C., S. Wang, G. Hu, L. Guo, X. Chen, P. Xu, and L. Liu (2018) Engineering *Escherichia coli* for malate production by integrating modular pathway characterization with CRISPRi-guided multiplexed metabolic tuning. *Biotechnol. Bioeng.* 115: 661-672.
77. Moser, F., A. Espah Borujeni, A. N. Ghodasara, E. Cameron, Y. Park, and C. A. Voigt (2018) Dynamic control of endogenous metabolism with combinatorial logic circuits. *Mol. Syst. Biol.* 14: e8605.
78. Higo, A., A. Isu, Y. Fukaya, S. Ehira, and T. Hisabori (2018) Application of CRISPR interference for metabolic engineering of the heterocyst-forming multicellular *Cyanobacterium Anabaena* sp. PCC 7120. *Plant Cell Physiol.* 59: 119-127.
79. Woolston, B. M., D. F. Emerson, D. H. Currie, and G. Stephanopoulos (2018) Rediverting carbon flux in *Clostridium ljungdahlii* using CRISPR interference (CRISPRi). *Metab. Eng.* 48: 243-253.
80. Lobs, A. K., C. Schwartz, S. Thorwall, and I. Wheeldon (2018) Highly multiplexed CRISPRi repression of respiratory functions enhances mitochondrial localized ethyl acetate biosynthesis in *Kluyveromyces marxianus*. *ACS Synth. Biol*. 7: 2647-2655.
81. Zhang, X. C., Y. Guo, X. Liu, X. G. Chen, Q. Wu, and G. Q. Chen (2018) Engineering cell wall synthesis mechanism for enhanced PHB accumulation in *E. coli*. *Metab. Eng.* 45: 32-42.
82. Zhang, J. and C. L. Poh (2018) Regulating exopolysaccharide gene *wcaF* allows control of *Escherichia coli* biofilm formation. *Sci. Rep*. 8: 13127.
83. Li, S., C. B. Jendresen, A. Grunberger, C. Ronda, S. I. Jensen, S. Noack, and A. T. Nielsen (2016) Enhanced protein and biochemical production using CRISPRi-based growth switches. *Metab. Eng.* 38: 274-284.
84. McCutcheon, S. R., K. L. Chiu, D. D. Lewis, and C. Tan (2018) CRISPR-Cas expands dynamic range of gene expression from T7RNAP promoters. *Biotechnol. J.* 13: e1700167.
85. Lauritsen, I., V. Martinez, C. Ronda, A. T. Nielsen, and M. H. H. Norholm (2018) Bacterial genome editing strategy for control of transcription and protein stability. *Methods Mol. Biol*. 1671: 27-37.
86. Lee, S. S., H. Shin, S. Jo, S. M. Lee, Y. Um, and H. M. Woo (2018) Rapid identification of unknown carboxyl esterase activity in *Corynebacterium glutamicum* using RNA-guided CRISPR interference. *Enzyme Microb. Technol.* 114: 63-68.
87. Rousset, F., L. Cui, E. Siouve, C. Becavin, F. Depardieu, and D. Bikard (2018) Genome-wide CRISPR-dCas9 screens in *E. coli* identify essential genes and phage host factors. *PLoS Genet.* 14: e1007749.
88. Mariscal, A. M., S. Kakizawa, J. Y. Hsu, K. Tanaka, L.

Gonzalez-Gonzalez, A. Broto, E. Querol, M. Lluch-Senar, C. Pinero-Lambea, L. Sun, P. D. Weyman, K. S. Wise, C. Merryman, G. Tse, A. J. Moore, C. A. Hutchison, 3rd, H. O. Smith, M. Tomita, J. C. Venter, J. I. Glass, J. Pinol, and Y. Suzuki (2018) Tuning gene activity by inducible and targeted regulation of gene expression in minimal bacterial cells. *ACS Synth. Biol*. 7: 1538-1552.

89. Cho, S., J. Shin, and B. K. Cho (2018) Applications of CRISPR/Cas system to bacterial metabolic engineering. *Int. J. Mol. Sci*. 19: 1089.
90. Mengin-Lecreulx, D., C. Parquet, L. R. Desviat, J. Pla, B. Flouret, J. A. Ayala, and J. van Heijenoort (1989) Organization of the *murE-murG* region of *Escherichia coli*: identification of the *murD* gene encoding the D-glutamic-acid-adding enzyme. *J. Bacteriol.* 171: 6126-6134.
91. Liu, Y., J. P. Rodrigues, A. M. Bonvin, E. A. Zaal, C. R. Berkers, M. Heger, K. Gawarecka, E. Swiezewska, E. Breukink, and M. R. Egmond (2016) New insight into the catalytic mechanism of bacterial MraY from enzyme kinetics and docking studies. *J. Biol. Chem.* 291: 15057-15068.
92. Mohammadi, T., R. Sijbrandi, M. Lutters, J. Verheul, N. I. Martin, T. den Blaauwen, B. de Kruijff, and E. Breukink (2014) Specificity of the transport of lipid II by FtsW in *Escherichia coli*. *J. Biol. Chem.* 289: 14707-14718.
93. Stevenson, G., K. Andrianopoulos, M. Hobbs, and P. R. Reeves (1996) Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *J. Bacteriol.* 178: 4885-4893.
94. Martinez, V., I. Lauritsen, T. Hobel, S. Li, A. T. Nielsen, and M. H. H. Norholm (2017) CRISPR/Cas9-based genome editing for simultaneous interference with gene expression and protein stability. *Nucleic Acids Res.* 45: e171.
95. Boettcher, M. and M. T. McManus (2015) Choosing the right tool for the job: RNAi, TALEN, or CRISPR. *Mol. Cell*. 58: 575-585.
96. Torres-Martinez, S. and R. M. Ruiz-Vazquez (2016) RNAi pathways in *Mucor*: A tale of proteins, small RNAs and functional diversity. *Fungal Genet. Biol.* 90: 44-52.
97. Torres-Martinez, S. and R. M. Ruiz-Vazquez (2017) The RNAi universe in Fungi: A varied landscape of small RNAs and biological functions. *Annu. Rev. Microbiol.* 71: 371-391.
98. Jackson, A. L., S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet, and P. S. Linsley (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21: 635-637.
99. Dominguez, A. A., W. A. Lim, and L. S. Qi (2016) Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nat. Rev. Mol. Cell Biol.* 17: 5-15.
100. Quebatte, M. and C. Dehio (2017) Systems-level interference strategies to decipher host factors involved in bacterial pathogen interaction: from RNAi to CRISPRi. *Curr. Opin. Microbiol.* 39: 34-41.
101. Schuster, A., H. Erasimus, S. Fritah, P. V. Nazarov, E. van Dyck, S. P. Niclou, and A. Golebiewska (2019) RNAi/CRISPR screens: from a pool to a valid hit. *Trends Biotechnol.* 37: 38-55.
102. Cui, L., A. Vigouroux, F. Rousset, H. Varet, V. Khanna, and D. Bikard (2018) A CRISPRi screen in *E. coli* reveals sequence-specific toxicity of dCas9. *Nat. Commun*. 9: 1912.
103. Rock, J. M., F. F. Hopkins, A. Chavez, M. Diallo, M. R. Chase, E. R. Gerrick, J. R. Pritchard, G. M. Church, E. J. Rubin, C. M. Sassetti, D. Schnappinger, and S. M. Fortune (2017) Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nat. Microbiol*. 2: 16274.
104. Cho, S., D. Choe, E. Lee, S. C. Kim, B. Palsson, and B. K. Cho (2018) High-level dCas9 expression induces abnormal cell morphology in *Escherichia coli*. *ACS Synth. Biol*. 7: 1085-1094.
105. Wilson, R. C. and J. A. Doudna (2013) Molecular mechanisms of RNA interference. *Annu. Rev. Biophys*. 42: 217-239.
106. Kuscu, C., S. Arslan, R. Singh, J. Thorpe, and M. Adli (2014) Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. *Nat. Biotechnol.* 32: 677-683.
107. Rosenbluh, J., H. Xu, W. Harrington, S. Gill, X. Wang, F. Vazquez, D. E. Root, A. Tsherniak, and W. C. Hahn (2017) Complementary information derived from CRISPR Cas9 mediated gene deletion and suppression. *Nat. Commun*. 8: 15403.
108. Del Vecchio, D., A. J. Ninfa, and E. D. Sontag (2008) Modular cell biology: retroactivity and insulation. *Mol. Syst. Biol.* 4: 161.
109. Brewster, R. C., F. M. Weinert, H. G. Garcia, D. Song, M. Rydenfelt, and R. Phillips (2014) The transcription factor titration effect dictates level of gene expression. *Cell*. 156: 1312-1323.
110. Qian, Y., H. H. Huang, J. I. Jimenez, and D. Del Vecchio (2017) Resource competition shapes the response of genetic circuits. *ACS Synth. Biol*. 6: 1263-1272.
111. Fu, Y., J. A. Foden, C. Khayter, M. L. Maeder, D. Reyon, J. K. Joung, and J. D. Sander (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* 31: 822-826.
112. Fu, Y., D. Reyon, and J. K. Joung (2014) Targeted genome editing in human cells using CRISPR/Cas nucleases and truncated guide RNAs. *Methods Enzymol.* 546: 21-45.
113. Fu, Y., J. D. Sander, D. Reyon, V. M. Cascio, and J. K. Joung (2014) Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* 32: 279-284.
114. Kleinstiver, B. P., V. Pattanayak, M. S. Prew, S. Q. Tsai, N. T. Nguyen, Z. Zheng, and J. K. Joung (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*. 529: 490-495.
115. Slaymaker, I. M., L. Gao, B. Zetsche, D. A. Scott, W. X. Yan, and F. Zhang (2016) Rationally engineered Cas9 nucleases with improved specificity. *Science*. 351: 84-88.
116. Gruber, A. R., S. H. Bernhart, and R. Lorenz (2015) The ViennaRNA web services. *Methods Mol. Biol*. 1269: 307-326.
117. Blin, K., L. E. Pedersen, T. Weber, and S. Y. Lee (2016) CRISPy-web: An online resource to design sgRNAs for CRISPR applications. *Synth. Syst. Biotechnol*. 1: 118-121.
118. Hu, J. H., S. M. Miller, M. H. Geurts, W. Tang, L. Chen, N. Sun, C. M. Zeina, X. Gao, H. A. Rees, Z. Lin, and D. R. Liu (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature*. 556: 57-63.
119. Nishimasu, H., X. Shi, S. Ishiguro, L. Gao, S. Hirano, S. Okazaki, T. Noda, O. O. Abudayyeh, J. S. Gootenberg, H. Mori, S. Oura, B. Holmes, M. Tanaka, M. Seki, H. Hirano, H. Aburatani, R. Ishitani, M. Ikawa, N. Yachie, F. Zhang, and O. Nureki (2018) Engineered CRISPR-Cas9 nuclease with expanded targeting space. *Science*. 361: 1259-1262.
120. Zhang, S. and C. A. Voigt (2018) Engineered dCas9 with reduced toxicity in bacteria: implications for genetic circuit design. *Nucleic Acids Res.* 46: 11115-11125.
121. Li, Z. and Q. Yang (2018) Systems and synthetic biology approaches in understanding biological oscillators. *Quant Biol*. 6: 1-14.