Trends in Biotechnology

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Review

Engineering Synthetic Gene Circuits in Living Cells with CRISPR Technology

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One of the goals of synthetic biology is to build regulatory circuits that control cell behavior, for both basic research purposes and biomedical applications. The ability to build transcriptional regulatory devices depends on the availability of programmable, sequence-specific, and effective synthetic transcription factors (TFs). The prokaryotic clustered regularly interspaced short palindromic repeat (CRISPR) system, recently harnessed for transcriptional regulation in various heterologous host cells, offers unprecedented ease in designing synthetic TFs. We review how CRISPR can be used to build synthetic gene circuits and discuss recent advances in CRISPR-mediated gene regulation that offer the potential to build increasingly complex, programmable, and efficient gene circuits in the future.

Introduction: Synthetic Gene Circuits

Synthetic regulatory circuits in living cells are an area of intensive research in synthetic biology because of their potential to advance basic research as well as to enable practical applications in medicine and in the production of biofuels and commodity chemicals (reviewed in [1,2]). Gene circuits typically include three functional modules: sensors, processors, and actuators. Sensors detect environmental or cellular inputs, processors determine the appropriate response for the given inputs, and actuators transmit the signals that modify cellular function. In biological terms, a synthetic circuit may include transcriptional, post-transcriptional, and/or post-translational regulation, with components such as regulatory DNA sequences, noncoding RNAs, and proteins such as TFs. Combinations of such elements can generate circuits that execute user-defined functions such as logic gates [3–7], analog computing circuits [8], gene expression switches [9,10], oscillators [11], timers [12], counters and clocks [13,14], pattern detectors [15], band-pass filters [16], memory devices [17], sensors [18], and intercellular communication systems [19,20].

A fundamental challenge in building gene circuits has been the shortage of functional parts that can operate together in one cell without crosstalk – an important feature for building circuits that display predictable behavior. Most synthetic circuits built to date have used transcriptional regulation and hence our ability to construct complex circuits relies on the availability of synthetic TFs that are effective (capable of regulating their target gene at the desired level of expression) and programmable (able to act on any user-defined target sequence). Three major classes of programmable synthetic TFs are currently available (Box 1): zinc-finger proteins (ZFPs), transcription activator-like effectors (TALEs), and CRISPR, the latter being the focus of this review.

Trends

Regulatory cascades and logic gates have been built in bacteria and in mammalian cells using CRISPR–dCas9-based transcriptional regulatory systems.

Advances in CRISPR-mediated transcriptional regulation allow multiple genes to be modulated simultaneously, a vital feature for constructing complex regulatory circuits.

Modified guide RNAs (gRNAs) enable increased levels of transcriptional regulation by CRISPR as well as bidirectional regulation – the ability to activate some target genes and repress others within the same cell.

New methods for inducible circuit activation include expression of gRNAs from inducible promoters as well as activation of the dCas9 protein by light.

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Box 1. Programmable Synthetic TFs: ZFPs, TALEs, and CRISPR

ZFPs, TALEs, and CRISPR offer three approaches to building programmable, sequence-specific synthetic TFs.

ZFPs utilize tandem arrays of zinc-finger motifs (ZFs), small protein domains that occur in many eukaryotic TFs and that bind specific sequences of three DNA base pairs [74,75]. Likewise, TALEs, transcriptional regulatory proteins derived from *Xanthomonas* spp. plant pathogens, include tandem arrays of small protein domains, each binding a specific DNA base pair [76]. Both ZFPs and TALEs allow researchers to target specific loci by arranging small DNA-binding protein domains in the desired order. The resulting DNA-binding array can be tethered to a transcriptional activator or repressor such as VP64 or KRAB for regulating target gene expression [57,77–79] or to an endonuclease for site-specific genome editing [80–82]. A more recent alternative to ZFPs and TALEs is the CRISPR system, in which a small gRNA targets the associated Cas9 protein to its substrate DNA [24]. Active Cas9 endonuclease is used for genome editing [25,26] while dCas9 (a deactivated point-mutant protein) acts as a TF by recruiting transcriptional activators or repressors to its target gene [30–36].

ZFPs, TALEs, and CRISPR-based TFs offer distinct advantages and disadvantages relative to each other. ZFPs are smaller than TALEs or dCas9 and hence easier to introduce into host cells. An advantage of ZFPs and TALEs is that the number of tandem DNA-binding motifs in a protein can be adjusted by the user, with longer DNA-binding domains allowing higher sequence specificity. Moreover, the activity of ZFPs and TALEs can be controlled by expressing them from inducible promoters [77] or by making fusion proteins that become active when exposed to a ligand [83–85] or a specific wavelength of light [86,87]. However, one of the main strengths of CRISPR-based TFs lies in the ease of designing TFs against any locus of interest: dCas9 targeting depends entirely on the associated gRNA, which is much cheaper and easier to construct and engineer than a full-length protein. Recent studies showed that modified dCas9 variants can achieve strong transcriptional activation of mammalian genes with a single gRNA per gene [38–42], with the ability to regulate multiple genes simultaneously [40,42]. Their simplicity of design, combined with their ability to regulate target genes robustly, make CRISPR-based TFs well suited to building complex gene circuits that involve the regulation of multiple genes.

Mechanism and Applications of CRISPR

Naturally occurring CRISPR systems defend bacteria and archaea against invasive DNA elements such as viruses and plasmids [21,22]. A fragment of the foreign DNA is integrated into a tandem array at the host cell's CRISPR locus, from which it is later transcribed and processed into a CRISPR RNA (crRNA). In a Type II CRISPR system, the crRNA forms a complex with a transactivating CRISPR RNA (tracrRNA) and CRISPR-associated protein 9 (Cas9). The crRNA then guides the Cas9–RNA complex to its target DNA, where the Cas9 endonuclease catalyzes a double-stranded break, thus cleaving the invasive DNA and protecting the host [23,24]. *In vitro* analysis of Cas9 from *Streptococcus pyogenes* found that the crRNA and tracrRNA can be replaced by a single RNA molecule, the guide RNA (gRNA) [24].

The ability of the Cas9–gRNA complex to cleave specific DNA sequences has inspired its use as a genome-editing tool in heterologous host cells and organisms including bacteria, yeast, and mammalian cells [25–27]. CRISPR–Cas9-mediated mutagenesis is effective and conceptually simple, requiring only the Cas9 endonuclease and a gRNA against the locus of interest. The main design constraint is the need for a protospacer adjacent motif (PAM) immediately downstream of the gRNA target sequence in the genome. The PAM, a short sequence motif required for target recognition by Cas9–gRNA, varies depending on the Cas9 protein [28,29]; for *S. pyogenes* Cas9, the PAM is NGG [24]. The ability to direct Cas9 to virtually any target by changing only the base-pairing region of the gRNA makes CRISPR–Cas9 easier to use than ZFPs and TALEs, both of which require protein engineering for sequence specificity (Box 1).

Shortly after the development of Cas9-mediated genome editing, a deactivated Cas9 (dCas9) was introduced with two point mutations (D10A, H841A) that abolish the protein's endonuclease activity while maintaining its ability to form a complex with gRNA and be recruited to specific DNA sequences [24,30]. When fused with effector domains, dCas9 can repress or activate transcription of target genes (Figure 1) [30–43]. Using dCas9 to inhibit or activate target genes is sometimes referred to as CRISPRi or CRISPRa, respectively [30,32,38]. The remainder of this review focuses on dCas9-mediated transcriptional regulation; for more information on genome

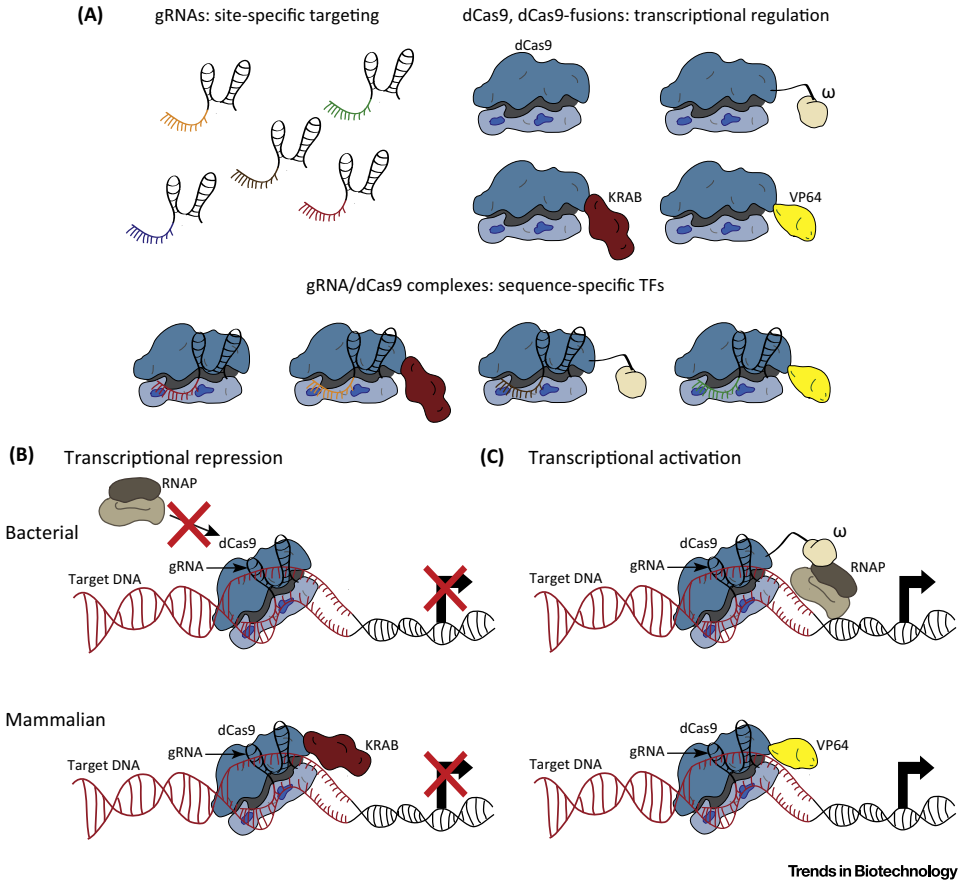


Figure 1. CRISPR–Based Regulation of Gene Expression. A CRISPR-based transcriptional regulator comprises two parts (A): a short guide RNA (gRNA) with a 5′ sequence specific for the target DNA and a deactivated Cas9 (dCas9) protein that may be fused to a transcription regulatory domain. The resulting dCas9–gRNA complex can repress (B) or activate (C) a gene of interest. In prokaryotes, dCas9–gRNA can repress transcription by sterically blocking transcription initiation (or elongation; not shown). A dCas9 fused to the omega (ω) subunit of the RNA polymerase (RNAP) can stabilize bacterial RNAP–promoter interactions and hence activate gene expression. In mammalian cells, dCas9 can be fused to the VP64 transcriptional activator or to the KRAB silencing domain (or other regulatory domains) to effect transcriptional activation or repression, respectively.

editing by CRISPR–Cas9, see [44,45]. Table 1 summarizes resources available for CRISPR-mediated gene regulation.

Transcriptional Repression (CRISPRi)

In *Escherichia coli*, up to 1000-fold transcriptional repression has been achieved by targeting dCas9 to the promoter or coding region of a gene of interest, where it prevents RNA polymerase (RNAP) recruitment or progression [30,31] (Figure 1B). In *Saccharomyces cerevisiae*, dCas9 efficiently represses transcription, both alone and when fused to the Mxi1 silencing domain [32].

By contrast, dCas9 alone is an inefficient repressor in mammalian cells [30]. Fusion of dCas9 with the repressive Krüppel-associated box (KRAB) domain [46] improves the repression capabilities of dCas9 [32] (Figure 1B). The degree of repression depends on where dCas9–KRAB binds within the gene of interest, with the strongest effect (up to 100-fold) observed when the gRNA was aligned 50–100 bp downstream of the transcription start site (TSS) [38]. Because KRAB recruits chromatin-modifying proteins [47], reversing dCas9–KRAB-mediated silencing

Table 1. CRISPR–dCas9-Based Platforms for Gene Regulation

Function	dCas9 Fusion	Host Cell	gRNAs	Maximum Fold Change	Addgene Vector #	Refs
Rep	– (dCas9 alone)	*Escherichia coli*; HEK293	1, 2/locus	1000-fold in *E. coli*	44246, 44249	[30]
Rep	– (dCas9 alone)	*E. coli*; *Streptococcus pneumoniae*	1/locus	>100-fold in *E. coli*	46569	[31]
Act/Rep	RNAP ω subunit	*E. coli*	1/locus	23-fold act	46570	[31]
Rep	BFP-KRAB	HEK293, HeLa, K562	1/locus	100-fold	46911	[32,38]
Act	VP64-BFP	HEK293	1/locus; 3 tandem sites	25-fold	46912	[32]
Act	P65AD-BFP	HEK293	1/locus; 3 tandem sites	12-fold	46913	[32]
Rep	– (dCas9 alone)	*Saccharomyces cerevisiae*	1/locus	18-fold	46920	[32]
Rep	Mxi	*S. cerevisiae*	1/locus	53-fold	46921	[32]
Act	VP64	HEK293T; MEF	1, 4/locus	2675-fold	47107	[33]
Act	VP64	HEK293	1, 3, 4/locus	>2000-fold	47754	[34]
Act	VP64	HEK293T	1, 5, 10/locus	>30-fold	47319	[35]
Act/Rep	VP64	*S. cerevisiae*; HEK293T	1, 2/locus; up to 12 tandem sites	7-fold rep, 70-fold act in yeast	49013, 49015	[36]
Act	VP160	HeLa, HEK293T; mESC, mouse embryos	Up to 10/locus	11-fold	48225, 48240	[37]
Act	VP64	HEK293T	4/locus; RNAP II expressed	1539-fold	55195, 55196, 55200-2	[49]
Act	$SunTag_{10x}$ + scFv–sfGFP-VP64	K562	1/locus	50-fold	60903, 60904	[38,39]
Act	VPR	HEK293T, hiPSC; *S. cerevisiae*; *Drosophila melanogaster* S2R+ cells; mouse Neuro-2A cells	3, 4/locus	20 000-fold	63798, 63801, 63802	[40]
Act	+ MCP–VP64 or PCP–VP64	*S. cerevisiae*; HEK293T	Mod gRNA, 1/locus	>140-fold in HEK293T	62282, 62313-9, 62331, 66564	[41]
Rep	+ Com–KRAB	HEK293T	Mod gRNA, 1/locus	5-fold	62338-9, 62342	[41]
Act	VP64 + MS2–P65–HSF1	HEK293FT, A375 melanoma cells	Mod gRNA, 1/locus; 7 tandem sites	>10 000-fold	61422-7	[42]
Act	p300Core	HEK293T	1, 4/locus	9920-fold	61357	[43]
Act	+ PP7–VP64 or MS2–VP64	HEK293FT	Mod gRNA, 4/locus	4000-fold	N/A	[61]
Ind Act			1, 4/locus			[62]

Table 1. (continued)

Function	dCas9 Fusion	Host Cell	gRNAs	Maximum Fold Change	Addgene Vector #	Refs
	trCIB1 + CRY2PHR–P65	HEK293, HeLa, COS-7		51-fold with blue light	64119, 64124	
Ind Act	CIBN + CRY2–VP64	HEK293T	4/locus	>1000-fold with blue light	60553-4	[63]

Abbreviations: Act, activation; Rep, repression; Ind Act, inducible activation; Act/Rep, the same dCas9 protein can activate or repress its target depending on where in/near the target gene it binds. Some dCas9 proteins require separate accessory proteins, indicated by a '+' sign; for example, 'CIBN + CRY2–VP64' means 'dCas9–CIBN coexpressed with CRY2–VP64'. The gRNA column indicates whether a modified (mod) gRNA is required and how many gRNAs were targeted to each locus. Maximum fold change indicates the largest change in gene expression observed with each system; note that results vary by orders of magnitude between loci and between different gRNAs at the same locus. CIBN, N-terminal domain of *Arabidopsis thaliana* CIB1 protein; CRY2PHR, photolyase homology region of *A. thaliana* CRY2 protein; HEK293, human embryonic kidney cells; hiPSC, human induced pluripotent stem cells; HSF1, HSF1 activation domain; K562, human myeloid leukemia cell line; MEF, mouse embryonic fibroblasts; mESC, mouse embryonic stem cells; P65AD, p65 activation domain; scFv, single-chain variable fragment antibody; sfGFP, superfolder GFP; $SunTag_{10x}$, ten copies of SunTag epitope; trCIB1, truncated CIB1; VP160, ten tandem copies of herpes simplex viral protein VP16; VP64, four copies of VP16; VPR, VP64–P65–Rta fusion.

can take several days after dCas9–KRAB expression ceases [38], an important consideration when designing dynamic regulatory circuits.

Transcriptional Activation (CRISPRa)

In bacteria, dCas9 fused with an omega (ω) protein enhances transcription by stabilizing interactions between the target promoter and RNAP [31] (Figure 1C). The strongest activation, observed for genes with low levels of expression at steady state, was ~23-fold in one study; genes controlled by stronger promoters are typically upregulated by less than tenfold [31].

In mammalian cells, early studies demonstrated transcriptional activation by fusing dCas9 to VP64 (four tandem repeats of the viral VP16 transcriptional activator) or the P65 subunit of nuclear factor kappa B (NF-κB) [32–36] (Figure 1C); dCas9–VP64 can also activate gene expression approximately threefold in *S. cerevisiae* [36]. VP64 enhances gene expression by recruiting elements of the basal transcription machinery to the target promoter [48]. However, dCas9–VP64 is a weak transcriptional activator in mammalian cells compared with ZFPs or TALEs. In most cases, multiple copies of dCas9–VP64 (or dCas9–VP160, which has ten tandem VP16 domains) must be targeted to a promoter to achieve effective activation [33–35,37,38,49]. For synthetic promoters, this problem can be solved by incorporating multiple binding sites for the same gRNA into the promoter [36]. However, strong activation of native mammalian genes typically requires three or more gRNAs per promoter [33,37]. Although multiple gRNAs can be introduced into one cell using a single vector [50], the need to target multiple gRNAs to a single promoter limits the ability to regulate many genes in parallel – a vital consideration for building complex circuits.

Recently, several groups modified CRISPR–dCas9 to achieve significantly stronger target gene activation in eukaryotes by making a single gRNA recruit multiple transcriptional activator domains [39–42] (Figure 2). Tanenbaum *et al.* fused dCas9 with multiple copies of a peptide named SunTag [39]. The resulting protein, sunCas9, can recruit multiple copies of a VP64 fusion protein and activate gene expression very efficiently with a single gRNA [38,39]. In an alternative approach, Chavez *et al.* fused dCas9 with a tripartite transcriptional activator comprising VP64, P65, and Rta [40]. This fusion protein, dCas9–VPR, achieves significantly higher induction of native mammalian genes than the original dCas9–VP64 [40]. The authors hypothesize that the three transactivators recruit distinct subsets of regulatory proteins, leading to synergistic activation [40]. Two other groups recruited transcriptional activators to the target gene via protein–RNA interactions [41,42]. Konermann *et al.* [42] modified gRNAs with RNA aptamers,

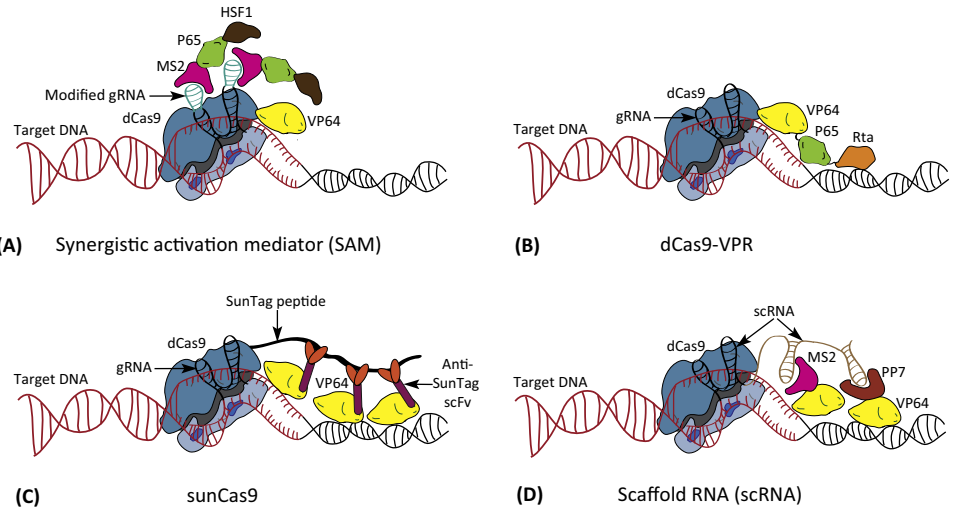


Figure 2. Improved Transcriptional Activation by CRISPR–Deactivated Cas9 (dCas9) in Eukaryotic Cells. Four novel methods offer improved transcriptional activation by recruiting multiple copies or multiple types of transcriptional activator domains to the target gene with a single guide RNA (gRNA). (A) The synergistic activation mediator (SAM) combines a dCas9–VP64 fusion with a modified gRNA that interacts with the RNA-binding MS2 protein. The MS2 is in turn fused to the transcriptional activators P65 and HSF1, resulting in three types of activator domain [VP64, P65, and heat-shock factor 1 (HSF1)] at the target locus. (B) dCas9–VPR features three activator domains – VP64, P65, and Rta – fused in tandem to the C terminus of dCas9. (C) In sunCas9, dCas9 is fused to a SunTag peptide, which provides multiple binding sites for an antibody fragment fused to the VP64 activator (for simplicity, not all copies of the VP64 fusion are shown). (D) A scaffold RNA (scRNA) is a gRNA with a 3′ extension that recruits the DNA-binding proteins MS2 and PP7, which are in turn fused to VP64 proteins. As a result, each scRNA recruits two copies of VP64 for improved transcriptional activation. A scRNA can also recruit KRAB domains for transcriptional repression (not shown). (A) Modified from [42], (B) from [40], (C) from [39], and (D) from [41].

which recruit the RNA-binding protein MS2 fused to two transcriptional activator domains, P65 and human heat-shock factor 1 (HSF1). Combined with dCas9–VP64, the RNA aptamer recruits three distinct transcriptional activators to the target gene: VP64, P65, and HSF1. The system, named synergistic activation mediator (SAM), is as effective as multiple copies of VP64 at the same promoter [42]. Finally, Zalatan *et al.* [41] replaced the standard gRNA with a scaffold RNA (scRNA), which recruits both dCas9 and an RNA aptamer-binding protein fused to a transcriptional activator or repressor. Recruiting two copies of a VP64 fusion protein to a single scRNA resulted in improved transcriptional activation [41].

The four platforms described above were analyzed using different cell lines and gRNAs, making direct comparisons among them difficult. Nonetheless, SAM, dCas9–VPR, and sunCas9 all function robustly in mammalian cells: each can activate its target gene more than tenfold higher than dCas9–VP64 with the same gRNA, although induction efficiency varies widely among target genes [38–40,42]. For scRNA, only one mammalian gene was tested, yielding approximately threefold higher activation relative to dCas9–VP64; scRNA was characterized mostly in yeast [41]. Together, these new methods provide a toolkit for potent engineering of gene expression, enabling exciting applications such as stem cell reprogramming [40], regulating metabolite synthesis in *S. cerevisiae* [41], and genome-wide gain-of-function screens in mammalian cells [38,42].

The above methods use multiple copies of VP64 or combinations of VP64 with other activator domains. Alternatively, high-level transcriptional activation can be achieved with a single gRNA per gene by fusing dCas9 with the core domain of the human p300 histone acetyltransferase

[43]. The resulting protein, dCas9–p300Core, showed significantly higher activation of three mammalian target genes compared with dCas–VP64, with maximum activation of over 4000-fold above background when used with a single gRNA. When used with four gRNAs per gene, dCas9-p300Core can activate its target over 9000-fold (Table 1) [43].

Use of CRISPR in Synthetic Gene Circuits

Two early examples of CRISPR-based circuits demonstrated layering, whereby the output of an upstream component of the circuit becomes the input to a downstream component – a key property for building complex regulatory circuits. In these two circuits, the upstream gRNA together with a dCas9 protein regulated the expression of a downstream gRNA that in turn controlled the expression of a fluorescent protein in mammalian cells [49,51]. One of these studies combined CRISPR–dCas9 and RNAi in one circuit, highlighting the potential to integrate CRISPR with other types of synthetic regulatory devices [49].

The two proof-of-principle CRISPR circuits described above used fluorescent protein expression as an easily observable output. Another recent study used a CRISPR-based logic gate for a therapeutic application, selective targeting of bladder cancer cells [52]. This circuit comprised a cancer cell-specific promoter (*hTERT*) driving dCas9 and a bladder cell-specific promoter (*hUPII*) driving a gRNA against a *lacI* transgene, which encoded a transcriptional repressor (Figure 3A). The dCas9–gRNA complex repressed *lacI*, thus relieving LacI-mediated repression of the output transgene. As a result, the output transgene was expressed only in cells where *hTERT* and *hUPII* was active; that is, bladder cancer cells. This circuit was used express three therapeutically relevant transgenes: *hBax* (proapoptotic), *p21* (antiproliferation), and *E-cadherin* (cell migration inhibitor) [52].

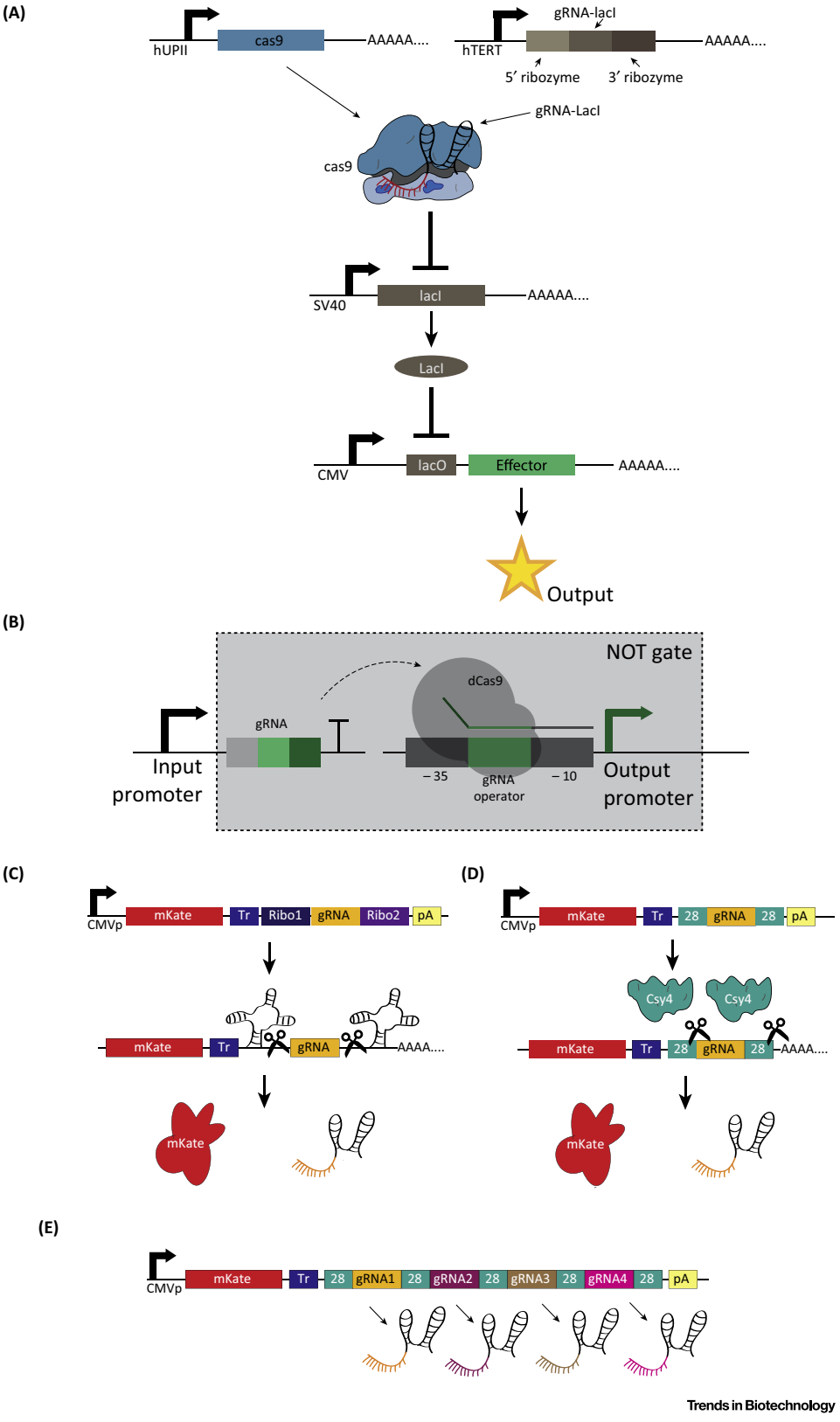
Another recent study built CRISPR-based logic gates to regulate gene expression in *E. coli* [53] (Figure 3B). An inducible promoter drove gRNA expression in response to a small-molecule input. The gRNA, when present, recruited dCas9 to the constitutive promoter of an output gene, inhibiting RNAP binding to the output promoter. As a result, the output gene was transcribed only in the absence of the input (a NOT gate). Expressing the gRNA from two independent promoters, each responsive to a different small-molecule inducer, resulted in a two-input NOR gate, where the output promoter was active only when neither input was present. Importantly, the NOT and NOR gates could be layered to build OR and AND gates, thus expanding the number of logical operations that the cell could perform. The output of these circuits can be a protein-coding transgene or a gRNA against an endogenous *E. coli* gene. Such logic circuits could control various bacterial phenotypes, such as biofilm formation, metabolism, and antimicrobial resistance [53]. They could also be used to regulate synthetic gene expression in commensal gut microbes [54].

Considerations for Building CRISPR-Based Synthetic Circuits

Orthogonality and Inducibility

In a synthetic gene circuit comprising more than one TF–promoter pair, it is important to avoid crosstalk between TFs. The ability of regulatory elements to function in the same cell without crosstalk is known as orthogonality [55–57]. The dCas9–gRNA complex can target any DNA sequence adjacent to NGG, the PAM for *S. pyogenes* Cas9, and specificity is achieved simply by changing the base-pairing region of the gRNA, making dCas9 attractive for designing orthogonal synthetic TFs. In addition, orthogonal regulation with CRISPR could be achieved with Cas9 orthologs from different species that recognize different PAM sequences and thus avoid crosstalk with each other. Inducible expression of distinct Cas9 orthologs in the same cell would then allow independent regulation of genes recognized by each Cas9 variant [58].

In addition to avoiding crosstalk, the components of a gene circuit often need to be inducible; that is, different genes in the circuit may need to be activated or repressed at different times to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achieve the desired behavior. In bacteria, this can be done by coupling the expression of different gRNAs to distinct molecular inputs [53]. By contrast, gRNA expression in eukaryotic cells is complicated by the need to retain the gRNA in the nucleus where it can form a complex with dCas9. Hence, gRNAs in most mammalian applications have been produced from Pol III promoters [32,34,36], which favor nuclear retention of the RNA but usually result in constitutive expression [59]. Since dCas9 is the shared effector for all gRNAs, all targets in such systems will be acted on simultaneously. This is an important drawback for designing gene circuits in mammalian cells, particularly compared with TALEs or ZFPs, which can be regulated by inducible Pol II promoters (Box 1).

One way to achieve inducible CRISPR-based regulation in eukaryotic cells is to express gRNAs from inducible Pol II promoters and then release the gRNA from the resulting transcript in the nucleus [49,51,60]. Two recent studies demonstrated this by embedding gRNA sequences in introns of protein-coding transgenes and flanking them with intron-splicing sites or self-cleaving ribozymes (Figure 3C) [49,51]. Nissim *et al.* presented an additional strategy by flanking the gRNA sequence with recognition motifs for the bacterial Csy4 endonuclease [49] (Figure 3D). Csy4 acts on the RNA and releases functional gRNAs from transcripts expressed from RNA Pol II promoters. Since Csy4 does not occur naturally in mammalian cells, this system presents an additional layer of regulation at the RNA level, as demonstrated in circuits that produce different outputs in the presence versus absence of the Csy4-coding transgene [49]. Alternatively, certain Pol II expression systems may be used that allow nuclear retention of the transcript. The combination of a Pol II promoter with the 3′ box terminator from the U1 gene results in nuclear retention of the gRNA transcript and the formation of a functional dCas9–gRNA complex [61]. Such expression systems could be coupled with inducible synthetic enhancers to control gRNA synthesis.

Rather than regulating the synthesis of dCas9 or gRNAs, yet another option is to control CRISPR activity post-translationally. Two recent studies built a light-inducible CRISPR–dCas9 system using the CRY2 and CIB1 proteins from *Arabidopsis thaliana* that form a heterodimer when illuminated with blue light [62,63]. Illumination with blue light brings together dCas9–CIB1 and CRY2 fused to a transcriptional activator, triggering target gene expression [62,63]. A drawback of this system is that all target genes become activated by blue light. Future studies could fuse different dCas9 orthologs to protein domains that respond to different wavelengths of light, allowing control of distinct subsets of genes in the same cell.

Bidirectional Regulation

The ability to repress and activate different genes simultaneously in the same cell should enable the construction of sophisticated circuits that more closely resemble endogenous gene regulatory networks. In bacteria and yeast, the same dCas9 fusion protein can activate or repress

Figure 3. CRISPR-Based Synthetic Regulatory Circuits. (A) A logic circuit activates the expression of an effector gene only in bladder cancer cells, identified as cells that simultaneously activate *hUPII* and *hTERT* promoters, which drive the expression of deactivated Cas9 (dCas9) and guide RNA (gRNA)–lacI, respectively. The dCas9–gRNA complex represses the *lacI* transgene, which in turn encodes a transcription factor (TF) that represses the effector transgene by binding to the *lacO* sequence 5′ of the effector transgene. (B) A NOT logic gate in *Escherichia coli* expresses a gRNA in response to a small-molecule input detected by the input promoter. The gRNA recruits dCas9 to a gRNA operator sequence in the output gene's promoter, blocking RNA polymerase binding and hence preventing transcription of the output gene. (C–E) Coexpression of a protein-coding region and gRNA from a single promoter in mammalian cells. A strong mammalian promoter, CMVp, drives the expression of a transgene encoding an RFP, mKate, followed by a gRNA flanked by sequences that allow the gRNA to be excised from the transcript and retained in the cell nucleus. In (C), two ribozymes (Ribo1 and Ribo2) flanking the gRNA cleave the transcript and release the gRNA. In (D), the gRNA is flanked by 28-nt sequences recognized by the bacterial Csy4 endonuclease (supplied as a separate transgene). The Csy4-based system has been used to express up to four gRNAs and a protein-coding region from a single promoter (E). In (C–E), a triplex sequence (Tr) downstream of the mKate-coding region stabilizes the protein-coding transcript after the 3′ portion is cleaved off. CMVp, cytomegalovirus promoter; pA, polyadenylation sequence. (A) Modified from [52], (B) from [53], and (C–E) from [49].

transcription depending on where in the target gene it binds [31,36]. In *S. cerevisiae*, dCas9–VP64 can activate its target when recruited upstream of the TSS and repress its target when bound to or downstream of the TSS, presumably by interfering with transcription initiation or elongation [36]. A qualitatively similar effect is observed with dCas9–VP64 in mammalian cells [36], although dCas9 tends not to repress native mammalian genes effectively in the absence of the KRAB domain [32].

An improved method for bidirectional regulation in mammalian cells is to modify the gRNA with a motif that can recruit a transcriptional activator or repressor via protein–RNA interactions. In the scRNA platform described above [41], each scRNA encodes information about both its target locus and its interacting protein. Thus, a scRNA with one type of aptamer recruits the VP64 transactivator to one gene while a scRNA with a different aptamer recruits the KRAB repressor to a different gene; the dCas9 protein is identical in the two cases [41]. A method called CRISPR Display (CRISP-Disp) also uses gRNAs modified with various protein-binding RNA domains, enabling the cell to perform different functions at different gRNA target loci [61]. Shechner *et al.* used CRISP-Disp to simultaneously activate one locus and image another, by coexpressing gRNAs that interacted with VP64 and with a fluorescent protein, respectively [61]. Future applications of CRISP-Disp may include simultaneously targeting activators and repressors to distinct loci. Future work might also modify the sunCas9 and SAM systems described above to allow bidirectional regulation [39,42].

System Specificity

The need to avoid off-target gene regulation by CRISPR–dCas9 is a key consideration in building genetic circuits, especially for mammalian cells, which have large genomes with many potential off-target sites. In contrast to off-target cleavage of DNA by the Cas9 nuclease [64,65], transcriptional regulation by CRISPR-based TFs appears to have reduced off-target effects, based on genome-wide sequencing analyses of cells expressing CRISPR-based TFs [32,42,43]. One reason for this high level of specificity may be the requirement for the gRNA to bind within a narrow window relative to the target gene's TSS: gRNAs that bind <400 bp upstream of the TSS produce the strongest transcriptional activation in mammalian cells [38,42], while binding 50–100 bp downstream of the TSS results in the strongest silencing by dCas9–KRAB [38]. These findings may help in the design of gRNAs that provide strong on-target regulation while minimizing off-target regulation, by screening out gRNAs predicted to bind near the TSS of annotated off-target genes.

Multiplexing and Scalability

Multiplexing – the ability to control multiple targets simultaneously – is vital for building complex gene circuits and for mimicking naturally occurring gene networks, which often regulate multiple genes. Compared with ZFPs and TALEs, dCas9 is uniquely well suited for multiplexing because of its modular nature: the same dCas9 effector can target different genes simply by changing the associated gRNA. Recent advances in CRISPR-based transcriptional activation that allow a gene to be activated strongly with a single gRNA [39,41–43] will enable the construction of complex devices that regulate multiple genes. For example, a recent study demonstrated simultaneous activation of up to ten genes in a cell population cotransfected with 10 gRNAs, although activation of any single gene was weaker in cells with multiple gRNAs than in cells with a single gRNA [42].

The scale of a genetic circuit depends not only on the ability to regulate multiple genes in parallel but also on the ability to express multiple gRNAs in the same cell. Most CRISPR-based regulatory devices in mammalian cells published to date simply used transient cotransfection of multiple plasmids, each encoding one gRNA [40,42]. However, a recent study showed that up to four gRNAs can be expressed from a single transcript using a site-specific endonuclease that

cleaves the transcript between the gRNA sequences (Figure 3E) [49]. The combination of such innovative gRNA expression systems with potent CRISPR-based TFs should enable researchers to build scalable synthetic circuits with multiple regulatory units.

Concluding Remarks

Synthetic biology has the potential for many practical applications [66,67], including the synthesis of biofuels and commodity chemicals, environmental sensing and bioremediation, and sophisticated medical therapies [68,69]. However, despite some successes the engineering of synthetic gene circuits remains slow and limited in many settings. One limitation of synthetic biology is the difficulty in designing synthetic TFs that can target user-defined sequences with high specificity and regulate target genes effectively. CRISPR-based TFs provide an exciting new option for synthetic TF design due to their ease of use combined with recent advances in efficiently regulating multiple genes in parallel. By circumventing the significant amount of time and effort that was previously required to engineer libraries of protein-based regulators for scalable gene circuits, CRISPR-based regulators should enable an accelerated design–build–test cycle for synthetic biology and a greater focus on circuit-level design rather than part-level design. Future studies will expand the repertoire of functions available with CRISPR-based regulators; for example, the recently described CRISP-Disp platform can recruit long noncoding RNAs to specific genomic loci, potentially enabling a novel layer of regulation in synthetic circuits [61]. Another recent study showed that CRISPR–Cas9 can target RNA [70], suggesting the possibility of future CRISPR-based circuits that combine transcriptional and post-transcriptional regulation.

We expect that future applications will take advantage of new and improved CRISPR regulators (see Outstanding Questions). For example, one could express multiple gRNAs from a single transcript, as previously described [49], and use them to regulate multiple endogenous genes to control a complex phenotype such as cell fate [40]. The metabolic engineering of microbes may be accelerated by the facile design of gRNAs to activate or repress multiple genes in biosynthetic pathways, to test hypotheses and optimize productivity. Although Cas9 nuclease and associated gRNAs have been delivered via bacteriophages as sequence-specific antimicrobials [71], one could envision the use of dCas9-based regulation to repress virulence or antibiotic-resistance genes in pathogenic bacteria instead [31]. In addition, instead of gene therapies that target only a single gene for well-defined genetic diseases, one could envision 'gene circuit therapies' that simultaneously modulate multiple targets using CRISPR-based regulation to treat complex multigenic human diseases. By combining CRISPR-based gene regulation with strategies for massively parallel combinatorial genetic perturbation [72,73], we should be able to map in a high-throughput manner how genetic networks regulate medically relevant biological phenotypes. By titrating the levels of gene repression and activation with CRISPR activators or repressors, one could further understand how graded changes in gene activity, rather than complete knockout or high overexpression, impact biological function. In summary, despite limitations that remain to be overcome, CRISPR-based gene regulation provides new opportunities for constructing scalable regulatory circuits in living cells with broad applications in synthetic biology.

Outstanding Questions

What is the limit to the number of genes that can be regulated simultaneously in one cell by CRISPR–dCas9?

How can bidirectional CRISPR regulatory systems be improved to enable high-level activation and repression at distinct loci in one cell?

What parameters, besides distance to the TSS, determine the strength of target gene activation or repression by a given gRNA?

How can transcriptional and post-transcriptional regulation (e.g., CRISPR-based RNA targeting) be combined to enable construction of more complex circuit behaviors?

What additional methods could be used for inducible activation of CRISPR-based circuits?

References

1. Brophy, J.A. and Voigt, C.A. (2014) Principles of genetic circuit design. *Nat. Methods* 11, 508–520
2. Lienert, F. *et al.* (2014) Synthetic biology in mammalian cells: next generation research tools and therapeutics. *Nat. Rev. Mol. Cell Biol.* 15, 95–107
3. Liu, C.C. *et al.* (2012) An adaptor from translational to transcriptional control enables predictable assembly of complex regulation. *Nat. Methods* 9, 1088–1094
4. Lohmueller, J.J. *et al.* (2012) A tunable zinc finger-based framework for Boolean logic computation in mammalian cells. *Nucleic Acids Res.* 40, 5180–5187
5. Moon, T.S. *et al.* (2012) Genetic programs constructed from layered logic gates in single cells. *Nature* 491, 249–253
6. Siuti, P. *et al.* (2013) Synthetic circuits integrating logic and memory in living cells. *Nat. Biotechnol.* 31, 448–452
7. Auslander, S. *et al.* (2012) Programmable single-cell mammalian biocomputers. *Nature* 487, 123–127
8. Daniel, R. *et al.* (2013) Synthetic analog computation in living cells. *Nature* 497, 619–623
9. Gardner, T.S. *et al.* (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 403, 339–342

10. Deans, T.L. *et al.* (2007) A tunable genetic switch based on RNAi and repressor proteins for regulating gene expression in mammalian cells. *Cell* 130, 363–372
11. Stricker, J. *et al.* (2008) A fast, robust and tunable synthetic gene oscillator. *Nature* 456, 516–519
12. Ellis, T. *et al.* (2009) Diversity-based, model-guided construction of synthetic gene networks with predicted functions. *Nat. Biotechnol.* 27, 465–471
13. Friedland, A.E. *et al.* (2009) Synthetic gene networks that count. *Science* 324, 1199–1202
14. Danino, T. *et al.* (2010) A synchronized quorum of genetic clocks. *Nature* 463, 326–330
15. Tabor, J.J. *et al.* (2009) A synthetic genetic edge detection program. *Cell* 137, 1272–1281
16. Basu, S. *et al.* (2005) A synthetic multicellular system for programmed pattern formation. *Nature* 434, 1130–1134
17. Farzadfard, F. and Lu, T.K. (2014) Synthetic biology Genomically encoded analog memory with precise in vivo DNA writing in living cell populations. *Science* 346, 1256272
18. Auslander, D. *et al.* (2014) A designer cell-based histamine-specific human allergy profiler. *Nat. Commun.* 5, 4408
19. You, L. *et al.* (2004) Programmed population control by cell–cell communication and regulated killing. *Nature* 428, 868–871
20. Bacchus, W. *et al.* (2012) Synthetic two-way communication between mammalian cells. *Nat. Biotechnol.* 30, 991–996
21. Barrangou, R. *et al.* (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712
22. Marraffini, L.A. and Sontheimer, E.J. (2008) CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322, 1843–1845
23. Deltcheva, E. *et al.* (2011) CRISPR RNA maturation by *trans*-encoded small RNA and host factor RNase III. *Nature* 471, 602–607
24. Jinek, M. *et al.* (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821
25. Mali, P. *et al.* (2013) RNA-guided human genome engineering via Cas9. *Science* 339, 823–826
26. Jiang, W. *et al.* (2013) RNA-guided editing of bacterial genomes using CRISPR–Cas systems. *Nat. Biotechnol.* 31, 233–239
27. DiCarlo, J.E. *et al.* (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR–Cas systems. *Nucleic Acids Res.* 41, 4336–4343
28. Mojica, F.J. *et al.* (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 155, 733–740
29. Shah, S.A. *et al.* (2013) Protospacer recognition motifs: mixed identities and functional diversity. *RNA Biol.* 10, 891–899
30. Qi, L.S. *et al.* (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152, 1173–1183
31. Bikard, D. *et al.* (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR–Cas system. *Nucleic Acids Res.* 41, 7429–7437
32. Gilbert, L.A. *et al.* (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154, 442–451
33. Perez-Pinera, P. *et al.* (2013) RNA-guided gene activation by CRISPR–Cas9-based transcription factors. *Nat. Methods* 10, 973–976
34. Maeder, M.L. *et al.* (2013) CRISPR RNA-guided activation of endogenous human genes. *Nat. Methods* 10, 977–979
35. Mali, P. *et al.* (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.* 31, 833–838
36. Farzadfard, F. *et al.* (2013) Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. *ACS Synth. Biol.* 2, 604–613
37. Cheng, A.W. *et al.* (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res.* 23, 1163–1171
38. Gilbert, L.A. *et al.* (2014) Genome-scale CRISPR-mediated control of gene repression and activation. *Cell* 159, 647–661
39. Tanenbaum, M.E. *et al.* (2014) A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell* 159, 635–646
40. Chavez, A. *et al.* (2015) Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods* 12, 326–328
41. Zalatan, J.G. *et al.* (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell* 160, 339–350
42. Konermann, S. *et al.* (2015) Genome-scale transcriptional activation by an engineered CRISPR–Cas9 complex. *Nature* 517, 583–588
43. Hilton, I.B. *et al.* (2015) Epigenome editing by a CRISPR–Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat. Biotechnol.* 33, 510–517
44. Sander, J.D. and Joung, J.K. (2014) CRISPR–Cas systems for editing, regulating and targeting genomes. *Nat. Biotechnol.* 32, 347–355
45. Hsu, P.D. *et al.* (2014) Development and applications of CRISPR–Cas9 for genome engineering. *Cell* 157, 1262–1278
46. Margolin, J.F. *et al.* (1994) Krüppel-associated boxes are potent transcriptional repression domains. *Proc. Natl. Acad. Sci. U.S.A.* 91, 4509–4513
47. Urrutia, R. (2003) KRAB-containing zinc-finger repressor proteins. *Genome Biol.* 4, 231
48. Mittler, G. *et al.* (2003) A novel docking site on Mediator is critical for activation by VP16 in mammalian cells. *EMBO J.* 22, 6494–6504
49. Nissim, L. *et al.* (2014) Multiplexed and programmable regulation of gene networks with an integrated RNA and CRISPR/Cas toolkit in human cells. *Mol. Cell* 54, 698–710
50. Kabadi, A.M. *et al.* (2014) Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. *Nucleic Acids Res.* 42, e147
51. Kiani, S. *et al.* (2014) CRISPR transcriptional repression devices and layered circuits in mammalian cells. *Nat. Methods* 11, 723–726
52. Liu, Y. *et al.* (2014) Synthesizing AND gate genetic circuits based on CRISPR–Cas9 for identification of bladder cancer cells. *Nat. Commun.* 5, 5393
53. Nielsen, A.A. and Voigt, C.A. (2014) Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks. *Mol. Syst. Biol.* 10, 763
54. Mimee, M. *et al.* (2015) Programming a human commensal bacterium Bacteroides thetaiotaomicron, to sense and respond to stimuli in the murine gut microbiota. *Cell Syst.* 1, 62–71
55. Wang, B. *et al.* (2011) Engineering modular and orthogonal genetic logic gates for robust digital-like synthetic biology. *Nat. Commun.* 2, 508
56. Blount, B.A. (2012) Rational diversification of a promoter providing fine-tuned expression and orthogonal regulation for synthetic biology. *PLoS ONE* 7, e33279
57. Khalil, A.S. *et al.* (2012) A synthetic biology framework for programming eukaryotic transcription functions. *Cell* 150, 647–658
58. Esvelt, K.M. *et al.* (2013) Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat. Methods* 10, 1116–1121
59. Orioli, A. *et al.* (2012) RNA polymerase III transcription control elements: themes and variations. *Gene* 493, 185–194
60. Gao, Y. and Zhao, Y. (2014) Self-processing of ribozyme-flanked RNAs into guide RNAs *in vitro* and *in vivo* for CRISPR-mediated genome editing. *J. Integr. Plant Biol.* 56, 343–349
61. Shechner, D.M. *et al.* (2015) Multiplexable, locus-specific targeting of long RNAs with CRISPR-Display. *Nat. Methods* 12, 664–670
62. Nihongaki, Y. *et al.* (2015) CRISPR–Cas9-based photoactivatable transcription system. *Chem. Biol.* 22, 169–174
63. Polstein, L.R. and Gersbach, C.A. (2015) A light-inducible CRISPR–Cas9 system for control of endogenous gene activation. *Nat. Chem. Biol.* 11, 198–200
64. Fu, Y. *et al.* (2013) High-frequency off-target mutagenesis induced by CRISPR–Cas nucleases in human cells. *Nat. Biotechnol.* 31, 822–826
65. Pattanayak, V. *et al.* (2013) High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* 31, 839–843

66. Khalil, A.S. and Collins, J.J. (2010) Synthetic biology: applications come of age. *Nat. Rev. Genet.* 11, 367–379
67. Cheng, A.A. and Lu, T.K. (2012) Synthetic biology: an emerging engineering discipline. *Annu. Rev. Biomed. Eng.* 14, 155–178
68. Boyle, P.M. and Silver, P.A. (2012) Parts plus pipes: synthetic biology approaches to metabolic engineering. *Metab. Eng.* 14, 223–232
69. Ye, H. *et al.* (2013) Synthetic mammalian gene circuits for biomedical applications. *Curr. Opin. Chem. Biol.* 17, 910–917
70. O'Connell, M.R. *et al.* (2014) Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* 516, 263–266
71. Citorik, R.J. *et al.* (2014) Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nat. Biotechnol.* 32, 1141–1145
72. Cheng, A.A. *et al.* (2014) Enhanced killing of antibiotic-resistant bacteria enabled by massively parallel combinatorial genetics. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12462–12467
73. Wong, A.S. *et al.* (2015) Massively parallel high-order combinatorial genetics in human cells. *Nat. Biotechnol.* 33, 952–961
74. Beerli, R.R. and Barbas, C.F., III (2002) Engineering polydactyl zinc-finger transcription factors. *Nat. Biotechnol.* 20, 135–141
75. Liu, Q. *et al.* (1997) Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5525–5530
76. Bogdanove, A.J. *et al.* (2010) TAL effectors: finding plant genes for disease and defense. *Curr. Opin. Plant Biol.* 13, 394–401
77. Beerli, R.R. *et al.* (2000) Positive and negative regulation of endogenous genes by designed transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1495–1500
78. Zhang, F. *et al.* (2011) Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nat. Biotechnol.* 29, 149–153
79. Zhang, Z. *et al.* (2013) Dissecting the roles of miR-302/367 cluster in cellular reprogramming using TALE-based repressor and TALEN. *Stem Cell Rep.* 1, 218–225
80. Urnov, F.D. *et al.* (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435, 646–651
81. Christian, M. *et al.* (2010) Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186, 757–761
82. Miller, J.C. *et al.* (2011) A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* 29, 143–148
83. Beerli, R.R. *et al.* (2000) Chemically regulated zinc finger transcription factors. *J. Biol. Chem.* 275, 32617–32627
84. Magnenat, L. *et al.* (2008) Drug-inducible and simultaneous regulation of endogenous genes by single-chain nuclear receptor-based zinc-finger transcription factor gene switches. *Gene Ther.* 15, 1223–1232
85. Mercer, A.C. *et al.* (2013) Regulation of endogenous human gene expression by ligand-inducible TALE transcription factors. *ACS Synth. Biol.* 3, 723–730
86. Polstein, L.R. and Gersbach, C.A. (2012) Light-inducible spatiotemporal control of gene activation by customizable zinc finger transcription factors. *J. Am. Chem. Soc.* 134, 16480–16483
87. Konermann, S. *et al.* (2013) Optical control of mammalian endogenous transcription and epigenetic states. *Nature* 500, 472–476