System-level perturbations of cell metabolism using CRISPR/Cas9

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Abstract

CRISPR/Cas9 (clustered regularly interspaced palindromic repeats and the associated protein Cas9) techniques have made genome engineering and transcriptional reprogramming studies more advanced and cost-effective. For metabolic engineering purposes, the CRISPR-based tools have been applied to single and multiplex pathway modifications and transcriptional regulations. The effectiveness of these tools allows researchers to implement genome-wide perturbations, test model-guided genome editing strategies, and perform transcriptional reprogramming perturbations in a more advanced manner than previously possible. In this mini-review we highlight recent studies adopting CRISPR/Cas9 for systems-level perturbations and model-guided metabolic engineering.

Introduction

CRISPR/Cas9 is revolutionizing biotechnology due to the speed and precision by which genomes and transcript abundances can be modified [1,2]. Genome edits and transcriptional reprogramming which used to require years, non-natural selection pressure, and sequential transformation procedures, can now be completed in multiplex, and without the introduction of markers, within a week [3,4]. In brief, CRISPR/Cas9, which originally was described as an adaptive immune system in bacteria and archaea [5–7], relies on the flexible guiding of the Cas9 nuclease by a short guide RNA (gRNA) to the genomic site of

interest [8,9]. The guiding of Cas9, or nuclease deficient versions thereof (dCas9), has allowed the development of a variety of tools for integration of genes into chromosomes [3,10–12], gene knock-outs [13*, 14*–16], directed evolution experiments [17*], epigenetic perturbations [18], and transcriptional regulation [4**,19**,20*,21*]. Indeed, CRISPR/Cas9 has been adapted and used in many different organisms for a variety of purposes [22] (Table 1 [16,23–37]), and most importantly, tools based on CRISPR have enabled inexpensive genetic manipulations in organisms which were previously troublesome, or even impossible, to engineer [27,31,32,34,37].

In relation to the design of cell factories for biobased production of chemicals, the CRISPR/Cas9 technologies enable researchers to perturb cellular metabolism more cost-effectively. This includes facile integration of biosynthetic genes or whole pathways, limiting flux through competing pathways, (re-)direction of pathway intermediates and co-factors to the desired product, and engineering of enzymes with increased catalytic activity [1*]. With such efficient tools at hand, metabolic engineers are confronted with an immense genome-wide solution space for improving cell factory performance. To assist genome-engineering decision-making, metabolic engineers are now starting to adopt i) systems-level perturbations coupled with selection, and ii) metabolic models to guide the genomic edits and flux perturbations required for improving the production of a desired chemicals or pharmaceuticals. Both routes are mutually important, in order to rationalize the design of best-performing cell factories.

In this mini-review we first present the emerging applications where CRISPR/Cas9 technologies have been used for systems-level genome and metabolic engineering. Secondly, we present how dCas9 has been used for balanced and model-guided transcriptional reprogramming of microbes, enabling metabolic models to leverage the CRISPR/Cas9 technology for experimental validation of model-guided designs. Lastly we put forward future perspectives on how CRISPR/Cas9 technology can further support the mapping of phenotypes onto genotypes, and thereby further support the refinement of metabolic models.

CRISPR/Cas9 for systems-level genome and metabolic engineering

To optimally design and engineer cell factories for increased production of chemicals, fuels and pharmaceuticals, data on the overall cellular states, chemical-genetic interactions, and metabolism should be well understood at the systems level. Nevertheless, even with extensive usage of a variety of omics approaches, many protein-coding gene functions, and

how they are regulated, are still poorly understood. To overcome these issues and better understand them, genome-wide studies and large library screens are sought after (Figure 1). Perturbation of gene expression is an often sought after means to experimentally determine gene function. For this purpose, several studies have employed CRISPR/Cas9 for single gene repression/activation or knock-out [38–40,41,42]. Moreover, in order to infer conditional functionality, a recent study explored dCas9-mediated CRISPR interference to identify chemical-genetic interactions in yeast (Figure 2a) [43]. Here, the authors tested a library of 989 unique gRNAs in the presence of various compounds known to inhibit growth, and from this determined both useful design rules for gRNAs and important genetic-chemical interactions [43]. In another study, Cress and colleagues interrogated dCas9 repressive capabilities on a mutagenesis library of T7-lac promoter [44]. This approach allowed generation of a set of inducible and variable-strength promoters, which could be regulated by both LacI and dCas9. Further, the authors designed and implemented selected promoters for throttling and selectively driving the violacein biosynthetic pathway in *E. coli* for targeted redirection of carbon flux. Using this synthetic promoter approach authors were able to increase production of proviolacein and deoxyviolacein more than ten-fold compared to a reference design.

Beyond systematic single-gene targeting, mapping complex traits onto genotypes, requires systems-level methodologies to target genome-wide interactions between multiple genes. To tackle this, Perturb-Seq and CRISP-seq methods were recently outlined [45,46]. These methods basically consists of combining the modularity of CRISPR/Cas9 to perform multi-locus (*ie.* delivery of multiple gRNAs in combination) gene perturbation with massively parallel single cell RNA sequencing as a genome-wide functional readout [45,46]. From these methods it is possible to screen the transcriptional effects in several hundred thousands of single cell combinatorial gene knock-out events. In both cases, the methods allowed mapping phenotypes onto the identity of the perturbation, based on an expressed guide barcode. Though these studies were performed in mammalian cells and largely focused on developmental traits, the strategy of pooled gRNA screening and single-cell sequencing should be applicable to diverse cell types with a sequenced genome and amenable to genetic transformation. Likewise, Perturb-seq, but presumably also CRISP-seq, is useful for dCas9-mediated genome-wide expression perturbations [47].

In another systems-level study of relevance to metabolic engineering, Garst *et al.*, applied CRISPR-enabled trackable genome engineering (CREATE) to reverse engineer all 645 nonsynonymous mutations identified in an evolved thermotolerant *E. coli* strain [48,49]

(Figure 2b). Not only does this method demonstrate multi-loci editing at the genome scale, but since CREATE libraries mapping tens of thousands genome-wide mutations can be built, transformed and selected for by a single researcher in just 1–2 weeks, this method also significantly reduces the turnaround time of design-build-test cycles compared to month- and year-long adaptive laboratory evolution experiments.

Summing up, through cost-effective DNA synthesis and efficient multiplex CRISPR/Cas9 technologies, more and more gRNAs are experimentally tested and higher-order genetic interactions are being uncovered. This improves the predictive power for designing functional gRNAs, and should furthermore allow genome-wide screens to uncover optimal genotypes for desired metabolic states and phenotypic traits.

Balanced metabolic engineering by CRISPR/dCas9

As evidenced in the examples above, balancing expression of both heterologous and endogenous genes or pathways is of paramount importance, and dCas9-mediated transcriptional reprogramming is becoming a widely used technique in the fields of synthetic and systems biology (Figure 1). In this section we will review examples of dCas9 applications for multiplex, systems-level and model-guided repression of gene expression in relation to metabolic engineering purposes.

Firstly, a recent study explored dCas9 for balancing expression in the mevalonate biosynthetic pathway in *E. coli* for production of terpenoids [19^{**}]. Here, the expression of target genes was tuned to minimize the production load, by applying L-rhamnose-inducible dCas9 for gene repression. The authors showed that the tool was capable of balancing expression of key enzymes, thereby increasing the production of selected terpenoids. Moreover, Li and colleagues explored dCas9-mediated repression of gene expressions responsible for cell growth in *E. coli*, and thereby developed genetic switches to increase production [50^{*}]. Here four genes were tested to decouple production from cell growth. Repression of each gene individually decreased the cell growth significantly and increased the production of mevalonate up to 41%.

Similarly, by varying the proximity of gRNAs to the transcription start site, Deaner and Alper recently reported a method for systematic testing of enzyme perturbation sensitivities via graded dCas9 modulation in *Saccharomyces cerevisiae* (STEPS) [51^{*}]. Using this method, the authors identified rate limiting steps in glycerol biosynthesis pathway and optimized the pentose phosphate flux for xylose catabolism, ultimately resulting in a 7.8-

fold increase in 3-dehydroshikimate titer and the identification of unique targets for xylose catabolism, respectively [51*].

Zalatan *et al.* have developed an RNA scaffold and dCas9-based system for gene activation and repression and applied it to balance expression of bacterial biosynthetic violacein pathway in yeast *S. cerevisiae* [4*]. This approach allowed for simultaneous activation of *VioA* and *VioC*, and repression of *VioD* to achieve redirection of the product output [4*]. Likewise, another recent study has developed a method called SWITCH, which also allows gene transcription activation and repression in yeast *S. cerevisiae* [21*]. Here, the authors demonstrated the usability of dCas9-based systems for activation of the *ADE2* gene expressed from the *GAL2* promoter and repression of the *TSC13* gene to achieve an increased production of naringenin [21*].

Finally, Cress *et al.* have been the first to report model-guided metabolic engineering in *E. coli* to predict multiple dCas9 gene targets supporting improved chemical production. In this study, the authors combined OptForce [52] and CRISPR arrays for repressing three enzyme-encoding genes and thereby improving the production of the plant flavonoid naringenin by 2,5 fold [53**](Figure 2c).

In relation to metabolic engineering, dCas9 was recently adopted for photoautotrophic cyanobacteria – a promising organism for production of biofuels and other valuable chemicals [54]. In a first study, dCas9 was employed for repressing the formation of the carbon storage compounds polyhydroxybutyrate (PHB) and glycogen [55]. A 10-fold repression of PHB was achieved by targeting *phaE* gene, encoding polyhydroxyalkanoate synthase, which was proposed to result in higher abundance of acetyl-CoA, a key precursor for many chemicals. Similarly, by repressing *glgC* gene, encoding ADP-glucose pyrophosphorylase, the glycogen accumulation was reduced by 25%, thereby providing cells with additional carbon during starvation. In another study, the authors explored dCas9's ability to repress *glnA*, encoding glutamine synthetase I, for redirection of central carbon flux. Through this, lactate production was improved 2-fold [56*].

In addition to growth, cell morphology or shape can also have a profound effect on chemical production. Elhadi *et al.* investigated effects of dCas9 controlled gene repression affecting cell morphology for production of PHB [57]. The genes *ftsZ* and *mreB*, encoding bacterial fission ring and skeletons, were down-regulated to achieve longer and larger *E. coli* cells, with 13% to 36% increased capacity to accumulate PHB's.

In summary, dCas9 has been shown to be efficient and promising tool for metabolic engineering purposes, and it has been applied in various organisms for balancing expression of genes and both endogenous and heterologous pathways.

Perspectives and conclusion

The emerging studies on systems-level genome engineering, as evidenced by the use of Perturb-Seq, CRISP-Seq and CREATE, address the need for an improved understanding of higher-order genetic interactions in order to enable the mapping of phenotypes onto genotypes [45^{''},46^{''},48^{''}]. These technologies allow for genome-scale readouts in the form of next-generation sequencing in relation to essentially any environmental condition or selection pressure of interest. Moreover, as the methods use DNA as input, they easily scale with both the cost of DNA synthesis and sequencing. In addition to the technical advances offered by these methods, we envision that systems-level genome and metabolic engineering will be useful for validating metabolic models by experimentally mapping genome-wide genetic interactions and transcript perturbations to phenotypes of interest. Though not targeting massive genetic interactions, the experimental validation of model-guided OptForce outputs by Cress *et al.* perfectly exemplifies this emerging trend [53^{''}]. *Vice versa*, we envision that access to experimental data from systems-level engineering efforts should allow refinement of genome-scale metabolic models.

Also, we envision that transcriptional regulation driven by dCas9 will be used more and more to elucidate complicated metabolic pathways, balance the dosage of key enzymes and point out bottlenecks arising when heterologous pathways are expressed. In addition, it can be used to understand gene-to-gene relations to uncover inhibitory or activating effects of enzymes by up- or down-regulation of gene expression. Importantly, dCas9's ability to regulate multiple targets resembles native transcriptional reprogramming and thereby allows for testing and redirecting metabolic states with much higher impact compared to earlier single-gene regulatory control mechanisms.

Finally, just as for Cas9-mediated knock-out and knock-in studies, we also envision that dCas9 will become widely used tool to elucidate and improve model-guided predictions. Here special attention could be given to kinetic metabolic modeling [58], ultimately enabling predictions of overall cell factory productivity in relation to a pathway's enzyme expression levels.

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Figure Legends

Figure 1. CRISPR/Cas9 usability for metabolic engineering purposes.

Usability of CRISPR/Cas9 for metabolic engineering purposes is depicted in the figure, showing its wide application in metabolic pathway engineering and genome editing in general, genome-wide and large library screening as well as transcriptional regulation of pathway or competing pathway genes and overall combinatorial expression balancing of metabolic pathways. GOI – gene of interest; HR – homologous recombination; dsOligo – double stranded oligo.

Figure 2. CRISPR/Cas9 usability for model-guided and library screening approaches

(a) dCas9 fused with Mxi1 and inducible gRNA expression system was used for screening the gRNA library targeting 4 genes to determine rules for best gRNA design. Screen was used to test chemical-genetic interaction, which showed that silencing of 4 genes in the presence of different small molecule inhibitors resulted in decreased cellular growth [43"].

(b). CREATE cassettes, containing gRNA and homology arm used for integration by homologous recombination, are used for protein engineering, reconstruction of adaptive laboratory evolution experiments and identification of stress tolerance and antibiotic resistance genes in bacteria. Experimental setup consists of: *in silico* design and synthesis of oligos (CREATE cassettes), subsequent PCR amplification and multiplex cloning of editing vectors, vectors are transformed to the cells and after selection, frequency before and after selection is determined by sequencing [48"]

(c) Model-guided approach was used to predict targets for expressional down-regulation of genes for increased naringenin production. CRISPR array was used to achieve transcriptional silencing of 3 genes simultaneously, which resulted in 2.5-fold increase in naringenin production [53"].