FEMS Microbiology Reviews, 2024, **48**, fuae020
DOI: 10.1093/femsre/fuae020
Advance access publication date: 31 July 2024
Review Article

The rise and future of CRISPR-based approaches for high-throughput genomics

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Editor: [Bart Thomma]

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) has revolutionized the field of genome editing. To circumvent the permanent modifications made by traditional CRISPR techniques and facilitate the study of both essential and nonessential genes, CRISPR interference (CRISPRi) was developed. This gene-silencing technique employs a deactivated Cas effector protein and a guide RNA to block transcription initiation or elongation. Continuous improvements and a better understanding of the mechanism of CRISPRi have expanded its scope, facilitating genome-wide high-throughput screens to investigate the genetic basis of phenotypes. Additionally, emerging CRISPR-based alternatives have further expanded the possibilities for genetic screening. This review delves into the mechanism of CRISPRi, compares it with other high-throughput gene-perturbation techniques, and highlights its superior capacities for studying complex microbial traits. We also explore the evolution of CRISPRi, emphasizing enhancements that have increased its capabilities, including multiplexing, inducibility, titratability, predictable knockdown efficacy, and adaptability to nonmodel microorganisms. Beyond CRISPRi, we discuss CRISPR activation, RNA-targeting CRISPR systems, and single-nucleotide resolution perturbation techniques for their potential in genome-wide high-throughput screens in microorganisms. Collectively, this review gives a comprehensive overview of the general workflow of a genome-wide CRISPRi screen, with an extensive discussion of strengths and weaknesses, future directions, and potential alternatives.

Keywords: CRISPR/Cas; CRISPR interference; genome-wide screens; genotype–phenotype; complex microbial traits

Introduction

Microorganisms have developed complex traits that facilitate adaptation to different environments and interactions with various species. A microbial trait can be defined as a phenotypic characteristic, including physiology, morphology, or behaviour, and is determined by genetic loci. Often, traits are controlled by multiple genetic loci, resulting in complex genetic interaction networks. Moreover, environmental conditions and interactions with other species can modify traits, contributing to increased complexity (Martiny et al. 2015). Such trait modifications can occur when microorganisms modify their DNA or alter their expression levels through (post-)transcriptional or (post-)translational changes. These effects can radically alter phenotypes, for example by adapting the growth rate, shifting metabolic processes to match changing environmental conditions, or by providing resistance against certain stressors (Tanwar et al. 2014, Nguyen et al. 2020, Lu et al. 2023). Nevertheless, the transient nature of some phenotypes can render certain traits even more complex to study. A well-known transient phenotype is tolerance, which can emerge through phenotypic switching in the absence of genetic changes. For example, microorganisms can shield themselves against unfavourable conditions by temporarily adopting a metabolically inactive state, resulting in the formation of spores or persister cells (Wells-Bennik et al. 2016, Balaban et al. 2019, Bollen et al. 2023). To investigate the entry and exit from this metabolically inactive state, it is important to consider temporal regulation of gene expression. Furthermore, environmental factors are also important. For example, tolerance is influenced by nutrient availability, as exemplified by triggered sporulation upon entering stationary phase (Serra et al. 2014) or by the increased presence of persister cells during stationary phase compared to exponential phase (Keren et al. 2004). Additionally, species interactions influence traits. For example, tolerance of *Staphylococcus aureus* to antibiotics is increased in the presence of *Pseudomonas aeruginosa* (Orazi and O'Toole 2017). In short, microbial traits can be time-, environment-, and interaction-dependent, rendering the study of underlying genetics inherently complex.

Although advances in several high-throughput screening methods have tremendously facilitated the investigation of genotype–phenotype relationships, deciphering the genetics behind complex microbial traits remains challenging. Important impediments in many high-throughput genetic screening methods

Received 8 May 2024; **revised** 19 July 2024; **accepted** 30 July 2024

include: (i) the lack of a reversible system, (ii) the limitation to nonessential genes, (iii) the labour-intensive nature of developing genome-wide libraries, and (iv) the challenge of constructing manageable-sized libraries to study phenotypes under bottleneck conditions, for example in *in vivo* studies. This shows the need for an optimized high-throughput genome-wide screening platform coping with these limitations.

A remarkable evolution in recent years has been the development and implementation of clustered regularly interspaced short palindromic repeats (CRISPR)-based approaches, with CRISPR interference (CRISPRi) emerging as an important gene-silencing technique. This innovative technique addresses many of the previously mentioned challenges in high-throughput genetic screening approaches, thereby unlocking new opportunities for exploring complex microbial traits. In this review, we will compare CRISPRi to currently used high-throughput approaches and illustrate why it stands out as a promising tool to study complex microbial phenotypes. We will provide a comprehensive overview of its mechanism and use for genome-wide screening, discuss important limitations and how they can be overcome, and review promising improvements that have enhanced its capabilities. Likewise, we discuss other CRISPR-based approaches that are currently being developed or optimized and that may overcome persisting limitations in genome-wide high-throughput screening or offer complementary strategies for studying the genetic basis of phenotypes.

Gene-perturbation techniques that underly high-throughput genetic screens

The development of high-throughput genetic screens has revolutionized microbial research and has paved the way for unravelling microbial traits. These methods were designed to gain a better understanding of genotype–phenotype associations by performing genome-wide screens in a high-throughput manner.

General workflow of high-throughput genetic screens

For a high-throughput genetic screen, it is essential to develop a comprehensive library containing numerous perturbed genes, ideally spanning the entire genome. These perturbations are usually distributed among microbial cells with each individual cell harbouring only a single gene perturbation. Mutants can either be cultured individually or in pools. Depending on the phenotype under study, cells are subjected to a selective pressure of which the effect can be assessed through morphology-, viability-, or growth-based screens. In viability and growth-based screens, the effect of the pressure is studied through varying abundances of cells harbouring different perturbations across individual compartments for arrayed screens or throughout the population for pooled screens. By comparing the abundances of cells for each perturbed gene in this library before and after applying a selective pressure or across different conditions, a genotype–phenotype association can be established. Pooled screens are favoured for whole-genome studies as they are less labour-intensive compared to arrayed screens. Conversely, arrayed screens are more often used for morphology-based screens and for validation or follow-up experiments involving a smaller number of targets (Schuster et al. 2019, Cain et al. 2020, Bock et al. 2022).

Approaches for implementing gene perturbations

Genotypes can be linked to phenotypes by altering (the expression level of) genes and observing the resulting phenotype under specific conditions. Genes can be perturbed through various methods: (i) gene deletion, (ii) gene mutation, (iii) gene silencing, or (iv) gene overexpression, each coming with a distinct set of advantages and limitations (summarized in Table 1).

A commonly used approach to eliminate a gene function is by deleting the coding region of a gene. Gene deletion libraries are generated by replacing the gene with a marker or by creating a clean deletion, the latter allowing for read-through in operons (Winzeler et al. 1999, Baba et al. 2006). As these techniques are targeted approaches, the genome must be annotated and the construction of whole-genome libraries is a labour-intensive and cumbersome process (Winzeler et al. 1999, Baba et al. 2006, Giaever and Nislow 2014). Furthermore, gene deletion is an irreversible method that complicates the interpretation of transient phenotypes and does not allow interrogation of the function of essential genes (Baba et al. 2006).

Collectively referred to as gene mutations, point mutations and insertions or deletions (indels) in the genome can cause either loss-of-function, gain-of-function, or neutral effects (Greener et al. 1997, Trehan et al. 2016, Cain et al. 2020). Gene mutation is a permanent, irreversible technique by which the function of essential genes can be assessed, provided that the mutation does not completely inhibit the essential function of the gene product. Depending on the genome annotation, either random or targeted approaches may be employed (Trehan et al. 2016, Cain et al. 2020, van Opijnen and Levin 2020). A well-known nontargeted mutagenesis strategy makes use of transposons that are randomly integrated into the genome (Cain et al. 2020, van Opijnen and Levin 2020). As a random approach does not require *a priori* genomic information, it is less predictable compared to targeted techniques and can lead to unwanted perturbations and polar effects (Dong et al. 2018, Cain et al. 2020, van Opijnen and Levin 2020). Additionally, libraries created by a random approach are typically large to ensure coverage of the entire genome (Cain et al. 2020, van Opijnen and Levin 2020). Alternatively, genome-wide gene mutation libraries can be created using a targeted mutagenesis approach, a well-known example of which is CRISPR (Dong et al. 2018). The CRISPR technique induces DNA breaks that can undergo repair via homologous recombination in the presence of a DNA template or, in some species, nonhomologous end joining (NHEJ), a process known for its error-prone nature, often resulting in indels (Barrangou and Marraffini 2014, Arroyo-Olarte et al. 2021). As CRISPR is a targeted approach, it circumvents many limitations associated with random mutagenesis. Furthermore, compared to other targeted approaches, creating a whole-genome library using CRISPR is often more efficient, making it less time-consuming and thus less labour-intensive (Dong et al. 2018, Cain et al. 2020, Arroyo-Olarte et al. 2021).

Gene silencing is a loss-of-function approach that can be achieved via RNA interference (RNAi), a method for post-transcriptional gene expression control. Genome-wide RNAi gene-silencing libraries can be created by cloning synthetic or randomly fragmented genomic or complementary DNA under the control of a constitutive or inducible promoter (Good and Stach 2011, Ishchuk et al. 2019, Chen et al. 2020). Using an inducible promoter, RNAi is a reversible method, which is advantageous for studying phenotypes that are time- or condition-dependent. Furthermore, as it is a gene-silencing technique, it is possible to assess the function of essential genes. However, RNAi is associated with polar and extensive off-target effects, leading to false positive results (Good and Stach 2011, Ishchuk et al. 2019, Chen et al. 2020).

Gene overexpression is an approach to study gain-of-function effects. Libraries containing individual or multiple genes downstream of an inducible or constitutive promoter, or transposons

Table 1. Characteristics of gene-perturbation methods for studying the genetic basis of phenotypes. Advantages and limitations of most historically used gene-perturbation techniques and CRISPR-based approaches are included.

	Non-CRISPR-based approaches				CRISPR-based approaches		
	Gene deletion: knockout	**Gene mutation: point mutations and indels[a]**	**Gene silencing: RNAi[b]**	**Gene overexpression**	**Gene mutation: CRISPR[c]**	**Gene silencing: CRISPRi[d]**	**Gene overexpression: CRISPRa[e]**
Genomic annotation needed	Yes	Random approach: no Targeted approach: yes	Random approach: no Targeted approach: yes	Random approach: no Targeted approach: yes	Yes	Yes	Yes
Able to target essential genes	No	Yes (if mutation does not eliminate function)	Yes	Yes (unless overexpression is toxic)	Yes (if mutation does not eliminate function)	Yes	Yes (unless overexpression is toxic)
Reversibility	Irreversible	Irreversible	Reversible (with inducible promoter)	Reversible (with inducible promoter)	Irreversible	Reversible (with inducible promoter)	Reversible (with inducible promoter)
Library size to cover genome[f]	1 × #genes	1–100 × #genes	5–20 × #genes	1–20 × #genes	5 × #genes	5 × #genes	5 × #genes

[a]indels: insertions and deletions.
[b]RNAi: RNA interference.
[c]CRISPR: clustered regularly interspaced short palindromic repeats.
[d]CRISPRi: CRISPR interference.
[e]CRISPRa: CRISPR activation.
[f]N × #genes: amount multiplied by the number of genes.

with outward-facing promoters have been used for gene overexpression (Kitagawa et al. 2005, Ho et al. 2009, Prelich 2012, Dong et al. 2018, Mutalik et al. 2019, Cain et al. 2020, He et al. 2023). Depending on the overexpression technique used, such as cloning individual genes downstream of a promoter, screens using gene overexpression libraries can bypass operon effects. Additionally, overexpression libraries can be used to study the functions of essential genes (Mutalik et al. 2019). However, a major limitation of gene overexpression libraries is that overexpression may result in unnaturally high levels of the gene product, which may not reflect physiological conditions and may result in artifacts and/or toxicity (Prelich 2012, Mutalik et al. 2019, Rai et al. 2022).

To conclude, developing a whole-genome-targeting library using the discussed techniques is often labour-intensive or requires a large library size. Furthermore, many loss-of-function methods are unsuitable to study the function of essential genes and are irreversible. Finally, all techniques are limited by perturbations that are bypassed by other cellular mechanisms, such as redundant genes.

Whereas all methods discussed here have provided valuable insights into a variety of microbial phenotypes, not all are equally well-suited to study complex traits. Ideally, a high-throughput method should be a targeted approach that avoids a labour-intensive library construction process, while also being reversible and capable of investigating the functions of both essential and nonessential genes. A technique that meets these requirements is CRISPRi.

CRISPRi is revolutionizing high-throughput screening

Following the discovery of CRISPR, different types of CRISPR-based systems were identified in microorganisms, targeting both DNA and RNA (Makarova et al. 2020). These CRISPR systems proved to be versatile genome-editing tools applicable across different species. Based on CRISPR, a gene-silencing method, CRISPRi, was developed, which is currently revolutionizing the gene-perturbation era as a programmable repression mechanism (Qi et al. 2013, Rousset and Bikard 2020, Vigouroux and Bikard 2020, Todor et al. 2021, Bock et al. 2022, Call and Andrews 2022, Sun et al. 2023). Genome-wide CRISPRi screening has many advantages over other screening platforms: (i) it is a targeted approach, (ii) it can be used to study essential gene function, (iii) it is a reversible system that can be selectively activated under desired conditions by inducing catalytically dead *cas* (*dcas*) and/or guide RNA (gRNA) expression, (iv) library sizes can be relatively small, (v) libraries can be made at a low cost, rendering the system highly affordable, and (vi) they can be developed in a high-throughput manner.

The mechanism of CRISPR

CRISPR was initially discovered as a defence mechanism against foreign nucleotide sequences in bacteria and archaea and has since been repurposed as a gene-editing technique (Doudna and Charpentier 2014). In nature, CRISPR arrays comprise alternating repeat elements and variable sequences, known as CRISPR spacers, which are acquired from invading genetic material. These loci can be transcribed and processed into small CRISPR RNAs (crRNAs). Together with CRISPR-associated (Cas) proteins, crRNAs enable the targeting and cleavage of homologous sequences in invading genetic materials (Fig. 1). This property of natural CRISPR

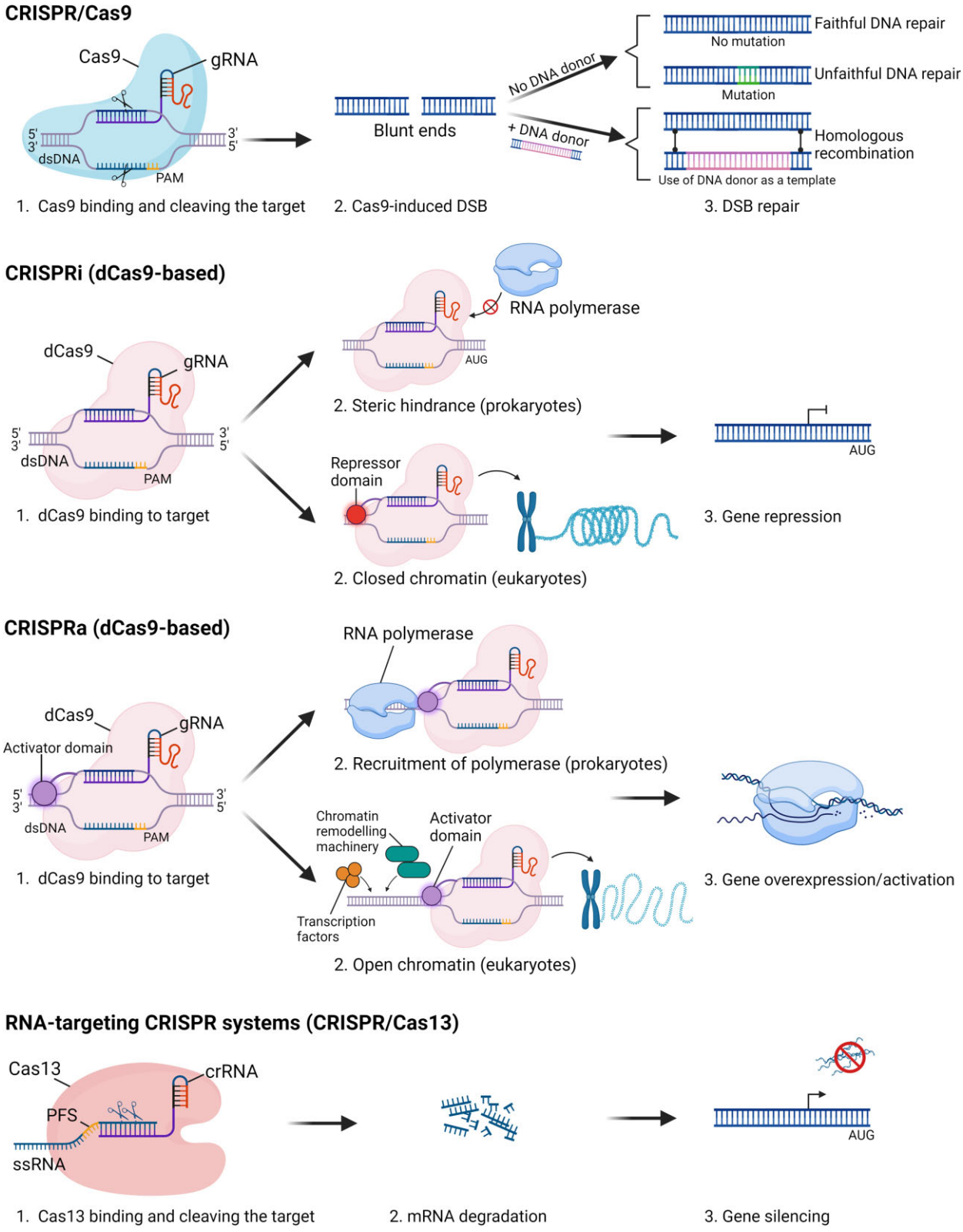

Figure 1. Mechanisms of CRISPR/Cas systems. CRISPR/Cas9 induces DNA double-strand breaks (DSBs), repairable through homologous recombination or, in some species, alternative repair pathways such as nonhomologous end-joining. CRISPRi causes steric hindrance in prokaryotes, resulting in gene repression, while repression in eukaryotes relies on dCas fusions to repressor domains that induce a closed chromatin conformation. Gene overexpression or activation via CRISPRa relies on recruitment of RNA polymerase in prokaryotes and of transcription factors and chromatin remodelling machinery in eukaryotes. CRISPR/Cas13 induces mRNA degradation, resulting in gene silencing. CRISPRi and CRISPRa systems can rely on either dCas9 or dCas12 as an effector. However, to simplify the figure, only dCas9-based CRISPRi and CRISPRa platforms are represented. gRNA: guide RNA; dsDNA: double-stranded DNA; DSB: double-strand break; PAM: protospacer-adjacent motif; INDEL: insertion/deletion; dCas9: dead Cas9; PFS: protospacer-flanking sequence; and ssRNA: single-stranded RNA.

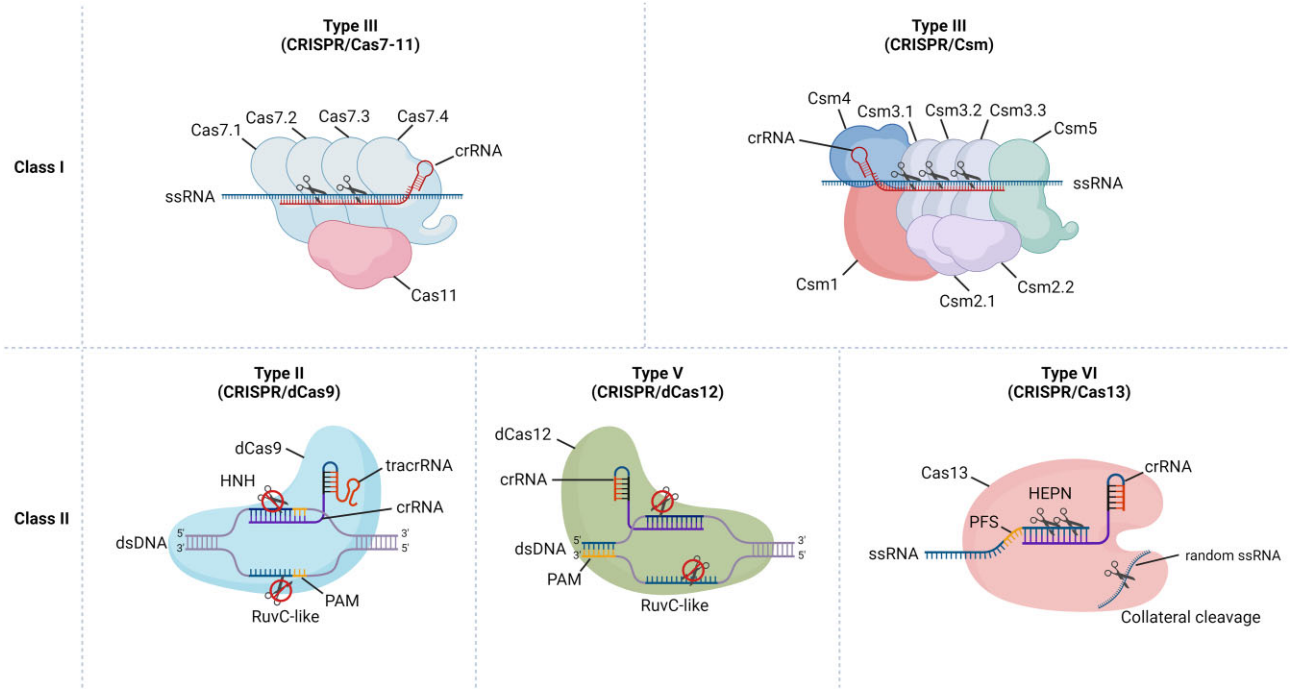

Figure 2. Comparison of CRISPR/Cas effector proteins enabling gene knockdown. Class I CRISPR systems involve multiple Cas effector proteins, while class II utilizes a single Cas effector protein for crRNA-target binding and target cleavage. Among them, type III and type VI directly target RNA while type II and V recognize DNA. In this figure, only Cas proteins mentioned in this review are shown. Furthermore, the nuclease-dead version of Cas9 and Cas12, dCas9 and dCas12, respectively, are depicted as they are the versions used in CRISPRi/a systems. PAM: protospacer-adjacent motif; ssRNA: single-stranded RNA; crRNA: CRISPR RNA; tracrRNA: trans-activating CRISPR RNA; dsDNA: double-stranded DNA; and PFS: protospacer-flanking sequence.

systems can be harnessed for programmable genome perturbation by transcribing either a single crRNA or an array of crRNAs that target specific sequences of interest (Barrangou and Marraffini 2014, Doudna and Charpentier 2014). CRISPR/Cas systems are classified into two categories: class 1, characterized by multiprotein effector complexes, and class 2, which utilizes a single Cas protein for crRNA and target binding, as well as target cleavage (Makarova et al. 2015). Within each class, there are three types of CRISPR/Cas systems, each distinguished by a signature Cas protein. Class 1 includes type I, type III, and type IV, while class 2 consists of type II, type V, and type VI systems (Makarova et al. 2015, 2020). Type II, such as CRISPR/Cas9, and type V systems are widely used due to their simple mechanism and DNA-targeting capability. Additionally, the development of novel gene-editing techniques based on type III and VI have increased, primarily for their ability to target RNA (Abudayyeh et al. 2016, Xu et al. 2021a, Colognori et al. 2023, Wei et al. 2023). Hence, our review will focus on these four types: (i) type III, consisting of multiple Cas proteins, (ii) type II, using Cas9, (iii) type V, with Cas12 as an effector protein, and (iv) type VI, which involves Cas13 (Fig. 2).

Type II (Cas9) and certain subtypes of type V (Cas12) need RNase III, a non-Cas-related protein, and an additional trans-activating crRNA (tracrRNA) molecule to process the crRNA transcript into single mature crRNAs. The crRNA and tracrRNA hybridize to form a functional gRNA before Cas9 or Cas12 can associate with this complex and be guided to target sites via the crRNA spacer sequence (Fig. 2) (Makarova et al. 2020). To simplify the crRNA–tracrRNA complex in CRISPR/Cas9 and subtypes of CRISPR/Cas12, both RNAs can be expressed as a single chimeric transcript, often referred to as the single-gRNA (Jinek et al. 2012). In type III CRISPR/Cas system, subtypes of CRISPR/Cas12, such as Cas12a, and CRISPR/Cas13 systems, only the crRNA molecule is required to guide the Cas nuclease to the target DNA (Makarova et al. 2020), and thus the crRNA itself constitutes the gRNA (Fig. 2). Since both the crRNA–tracrRNA complex and the crRNA alone function to guide Cas proteins to the correct nucleotide sequence, we will refer to them collectively as gRNAs throughout the text, without differentiation. When designing gRNAs, it is essential to consider that the Cas nuclease requires a short protospacer-adjacent motif (PAM) bordering the target sequence to bind and cleave the DNA (Bikard et al. 2013). The PAM sequence consists of a very short nucleotide motif (2–6 nt) and varies between Cas effectors (Shah et al. 2013, Miao et al. 2019). Once bound to the DNA, Cas9 induces primarily blunt-end double-stranded breaks (DSB) using its two endonuclease domains, the HNH domain and RuvC-like domain, that cleave the complementary and noncomplementary strands, respectively. In contrast, Cas12 only contains the RuvC-like domain, which induces staggered-end DBS (Jinek et al. 2012, Jeon et al. 2018, Makarova et al. 2020).

The development of CRISPRi

The nuclease activity of Cas proteins can be inactivated by introducing point mutations in the endonuclease domains, resulting in DNase-dead Cas (dCas) proteins. To deactivate Cas9, mutations are introduced in both the HNH domain and RuvC-like domain (Table 2) (Jinek et al. 2012, Zhang et al. 2017). In contrast to Cas9, Cas12a can be deactivated using either one or two mutations solely within the RuvC-like domain (Jinek et al. 2012, Leenay et al. 2016, Zhang et al. 2018a). These mutations abolish the DNA endonuclease activity of the Cas effector but do not affect its DNA-binding capacity (Fig. 2). dCas has been repurposed as a programmable RNA-guided transcription regulation

Table 2. Inactivating mutations for Cas-effector proteins.

Protein	Species	Mutation	Reference
Cas9	*Streptococcus pyogenes*	H840A, D10A	Jinek et al. (2012), Zhang et al. (2017)
Cas12a	*Francisella novicida*	D917A E1006A D917A, E1006A	Zetsche et al. (2015)
	Lachnospiraceae bacterium	D832A E925A D832A, E925A	Zhang et al. (2018b)
	Acidaminococcus sp.	D908A E993A D908A, E993A	Yamano et al. (2016)
	Moraxella bovoculi	D864A	Knott et al. (2019)

system in bacteria, called CRISPRi (Bikard et al. 2013, Qi et al. 2013, Zhang et al. 2017). Depending on the gRNA sequence, dCas causes steric hindrance by either binding to the promoter region, thereby inhibiting transcription initiation, or by binding the open reading frame, preventing transcription elongation in prokaryotes (Fig. 1) (Qi et al. 2013).

Starting from single-gene targeting, CRISPRi screens have progressed to encompass all essential genes in the genome (Peters et al. 2016) and subsequently extended to whole-genome screens (Table 3). A whole-genome CRISPRi screen can be performed with a gRNA library comprising multiple gRNAs per gene for each gene in the genome, as demonstrated in prokaryotes (Wang et al. 2018a, Dhamad and Lessner 2020). In contrast to prokaryotes, eukaryotes require the fusion of dCas with a nuclear localization signal to facilitate its localization to the nucleus (McInally et al. 2019, Ciurkot et al. 2021). Additionally, eukaryotes have a more complex transcriptional regulation system, hampering gene silencing via simple steric hindrance. Increased silencing levels were achieved for the model yeast *S. cerevisiae* by fusing a transcriptional repressor domain to dCas (Gilbert et al. 2013), which was then used to perform whole-genome screens in *S. cerevisiae* (Momen-Roknabadi et al. 2020).

In addition to the classical mechanism, alternative CRISPRi approaches are emerging. Instead of dCas, a catalytically active Cas effector is able to repress gene expression using gRNAs shortened below a certain threshold (Ramesh et al. 2020, Auradkar et al. 2023). For Cas9, shortening of gRNAs from the full 20 nt to 14 nt mediates efficient repression without any detectable target site mutations (Auradkar et al. 2023). Similarly, active Cas12a fused to a transcriptional repressor domain and combined with gRNAs of 16 nt or less (compared to the usual 20–23 nt) efficiently represses transcription (Ramesh et al. 2020). This approach enables the integration of both CRISPR and CRISPRi in a single experiment, using the same Cas effector.

General workflow of a CRISPRi screen

High-throughput screens to correlate genotypes with phenotypes can be conducted using CRISPRi. CRISPRi screens consist of four steps: (i) introduce *dcas* and the gRNA library into the species of interest, (ii) perform a phenotypic assay, (iii) record the read-out, and (iv) perform data analysis (Fig. 3).

To perform a CRISPRi screen, a dCas enzyme and a library of gRNAs are required. Determining the appropriate dCas effector, the number of gRNAs per gene and their sequence depends on the species and experimental setup (Information Box and Section *Genome-scale efficacy prediction for improved gRNA library design*). The dCas enzyme and gRNA library must first be transferred to the microbial cell. There are three options to do so: integrating both *dcas* and gRNA into the genome (Peters et al. 2016, Dhamad and Lessner 2020, Silvis et al. 2021, de Bakker et al. 2022), cloning both components on replicative plasmids (Wang et al. 2018a, Lee et al. 2019, Momen-Roknabadi et al. 2020, Rousset et al. 2021, Spoto et al. 2022), or integrating one into the genome while cloning the other one into a replicative plasmid (Liu et al. 2017c, 2021, Yao et al. 2020, Miao et al. 2023). Genomic integration provides a more stable system compared to a plasmid-based approach and avoids the need for additional selection markers to maintain the plasmids. Furthermore, the transfer of the CRISPRi system into the microbial cell can occur either in a pooled or arrayed manner, utilizing bulk processing or individual cell manipulation, respectively (Bock et al. 2022, de Bakker et al. 2022).

Following insertion of the CRISPRi system in the microbial cell, a phenotypic assay is performed. Most often, this is done on pooled CRISPRi libraries by applying a selection pressure, such as using selective media to assess survival in specific conditions or drug treatment to identify resistance-related genes (Bock et al. 2022, de Bakker et al. 2022). In this case, differences in fitness are recorded across the CRISPRi mutants, ultimately linking genes to increased or decreased growth in specific conditions. Control conditions, such as omitting the applied pressure, should be included for comparison. However, some assays, like studying morphological phenotypes, do not depend on fitness effects (Otten and Sun 2020, Bock et al. 2022). In this case, arrayed screens can be considered (Shields et al. 2020, Silvis et al. 2021) or pooled CRISPRi libraries can be subjected to fluorescence-activated cell sorting to sort out phenotypes of interest (Dewachter et al. 2022). Additionally, depending on the phenotype under study, timing of the induction of the CRISPRi system might be important, particularly for studying transient phenotypes (Bock et al. 2022).

To evaluate the effect of gene silencing on the phenotype, it is necessary to determine either the abundances of gRNAs for pooled screens, or the specific gRNA target associated with a compartment for arrayed screens. Both the target of the gRNA and its abundance are determined by amplification of gRNA sequences via PCR followed by short-read sequencing (Bock et al. 2022, de Bakker et al. 2022).

Bioinformatic tools are used for analyzing the sequencing data and comparing the selective conditions with control conditions to identify hit genes (de Bakker et al. 2022). A detailed overview

Table 3. Overview of microbial traits studied with high-throughput CRISPRi screening.

Gene types	Species	Traits studied	Reference
Core genome	*Escherichia coli*	Gene essentiality in various strains and conditions	Rousset et al. (2021)
Essential genes	*Acinetobacter baumannii*	Drug targets and responses to antibiotic treatments	Ward et al. (2024)
	Bacillus subtilis	Growth and terminal death phenotypes, cell morphology, and drug targets	Peters et al. (2016)
		Transfer of conjugative elements	Harden et al. (2022)
	Escherichia coli	Morphological and growth phenotypes	Silvis et al. (2021)
	Klebsiella pneumoniae	Drug targets and virulence	Zhu et al. (2023)
	Mycobacterium smegmatis	Mode of action of a drug	de Wet et al. (2020)
	Streptococcus pneumoniae	Growth phenotypes, competence regulation, and morphological phenotypes	Liu et al. (2017c)
Essential and growth-supporting genes	*Streptococcus mutans*	Growth and morphological phenotypes	Shields et al. (2020)
		Biofilm formation and the mode of action of an antimicrobial drug	St. Pierre et al. (2023)
Essential and respiratory growth-essential genes	*Saccharomyces cerevisiae*	Acetic acid tolerance	Mukherjee et al. (2021)
		Growth in fermentative and respiratory growth conditions	Smith et al. (2017)
Genes of a diverse range of biological processes	*Escherichia coli*	Expression-growth landscapes	Otto et al. (2024)
	Mycobacterium tuberculosis	Drug targets vulnerability and lethality	McNeil et al. (2021)
Genome-wide	*Corynebacterium glutamicum*	R-protein production and secretion	Yu et al. (2023)
	Escherichia coli	Gene essentiality in rich medium and infection by phage lambda, T4, and 186	Rousset et al. (2018)
		Genes essentiality in rich medium, auxotrophy in MOPS medium, and tolerance to furfural and isobutanol	Wang et al. (2018a)
		The decoupling of growth from protein production	Li et al. (2020)
		Infection and resistance of 14 double-stranded DNA phages	Mutalik et al. (2020)
	Eubacterium limosum	Heterotrophic and auxotrophic growth	Shin et al. (2023)
	Mycobacterium tuberculosis	Bedaquiline susceptibility and resistance	Yan et al. (2022)
		Vulnerability differences between antibiotic-resistance and -sensitive strains	Eckartt et al. (2024)
	Mycobacterium tuberculosis and *smegmatis*	Drug targets	Bosch et al. (2021)
		Drug resistance mechanisms of nine different drugs	Li et al. (2022)
	Saccharomyces cerevisiae	Furfural tolerance and protein surface display	Lian et al. (2019)
		Growth in bioreactor cultures	McGlincy et al. (2021)
		Haploinsufficiency and adenine and arginine biosynthesis	Momen-Roknabadi et al. (2020)
		α-amylase secretion	Johansson et al. (2023)
	Staphylococcus aureus	Aminoglycoside sensitivity	Jiang et al. (2020)
		Antibiotic susceptibility and tolerance	Liu et al. (2024a)
		Mode of action of an antimicrobial drug	Zhang et al. (2023)
	Staphylococcus epidermis	Survival different stress and nutrient-limiting conditions	Spoto et al. (2022)

Table 3. Continued

Gene types	Species	Traits studied	Reference
	Streptococcus pneumoniae	Bacterial cell cycle control, both in the presence and absence of the cell cycle regulator protein CcrZ	Gallay et al. (2021)
		Bottleneck sizes and virulence	Liu et al. (2021)
		Resensitization of amoxicillin-resistant cells	Dewachter et al. (2022)
		Competence	Minhas et al. (2023)
		Virulence	Liu et al. (2024b)
	Streptococcus salivarius	Competence	Knoops et al. (2022)
	Synechocystis sp.	Photoauxotrophic growth, growth in the presence of L-lactate and increased L-lactate production	Yao et al. (2020)
		Fitness effects in different conditions, including varying light regimes and carbon sources	Miao et al. (2023)
	Vibrio natriegens	Genes essentiality for rapid growth in rich medium	Lee et al. (2019)
	Zymononas mobilis	Aerobic and anaerobic growth	Enright et al. (2023)
		Increased D-lactate production	Peng et al. (2024)
L-proline transporters	*Corynebacterium glutamicum*	L-proline exporters	Liu et al. (2022b)
Metabolism-related genes	*Escherichia coli*	Increased free fatty acid production	Fang et al. (2021)
Nonlethal cell cycle-related and unannotated genes	*Escherichia coli*	Cell cycle replication initiation and cell division sizes	Camsund et al. (2020)
Transcription factors and protein kinases	*Saccharomyces cerevisiae*	Enhanced tolerance to Spruce hydrolysate and lignocellulosic toxins	Gutmann et al. (2021)

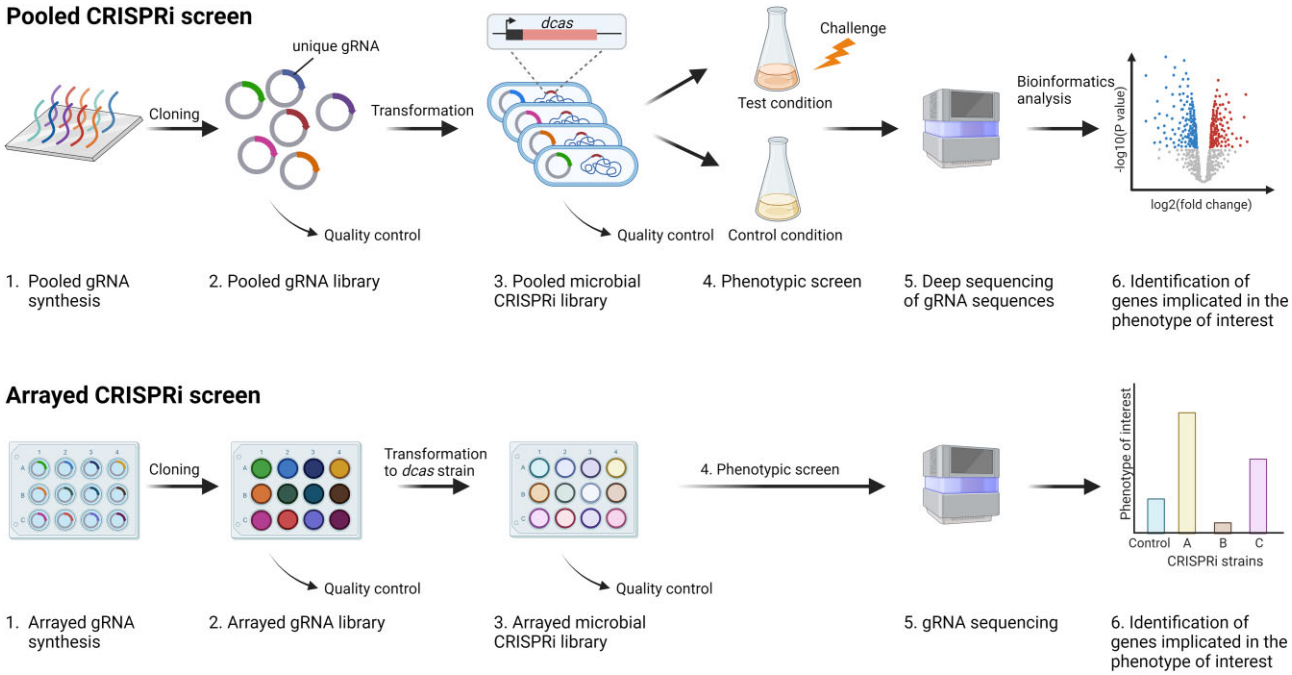

Figure 3. General workflow of a CRISPRi screen. A CRISPRi screen can be performed either in a pooled, where the gRNAs are pooled together, or arrayed format, where the gRNAs are maintained separately, and involves the following steps: (1) Designing and creating gRNAs, (2) constructing a gRNA library, (3) introducing *dcas* and gRNAs into the cell, (4) performing a phenotypic screen, (5) identifying gRNA abundances and their target, and (6) analysing the results. Quality control of the gRNA and CRISPRi libraries (2 and 3) is performed to ensure sufficient library representation and minimal skew in abundance of gRNA sequences before the screen.

Information box: requirements for a high-throughput CRISPRi screen

This information box serves as a guideline for implementing CRISPRi in a new species, detailing factors to take into account.

1. Genome annotation. Is the genome of the species well-annotated? Genomic knowledge is essential for designing gRNAs. If genomic information is lacking, we recommend an untargeted approach, such as random insertional mutagenesis, to create gene-perturbation libraries.
2. Microorganism. What is the domain of the species: bacteria, archaea, yeast, or protozoa? Since yeasts and protozoa have a nucleus, fusion of a nuclear localization signal to dCas is often necessary. Simple dCas roadblock systems are not very efficient in yeasts, but fusion of a transcriptional repressor domain to dCas enhances efficacy.
3. Endogenous CRISPR system. CRISPR is a defence mechanism against foreign DNA, leading to the presence of endogenous CRISPR systems in some prokaryotes. If this is the case, endogenous systems might interfere with CRISPRi, necessitating their deactivation.
4. dCas. Which dCas protein should be used? For DNA targeting, dCas9 or dCas12 are typically used. While dCas9 is better characterized, dCas12a can be less toxic, is more specific and easier to multiplex, and facilitates targeting of T-rich regions and genomes.
5. gRNA design rules. What factors contribute to designing an ideal gRNA? Key considerations include the positioning on the target, the target strand, the PAM sequence, and the GC content. In prokaryotes, the gRNA should target the gene body or maximum −400 bp upstream of the start codon, with substantially stronger repression closer to the start codon. In yeasts, dCas–repressor fusions mediate efficient knockdown in a window between −200 and +50 bp relative to the TSS. In coding regions, the choice between targeting the template or nontemplate strand depends on the specific dCas protein being used. Additionally, each dCas protein prefers particular PAM sequences. Finally, the GC-content of gRNAs should not be extreme.
6. gRNA library. How many gRNAs per gene are needed in a whole-genome screen? Generally, multiple gRNAs per gene (3–10, depending on availability of prediction models) are recommended to enhance reliability of hit-calling. However, when bottleneck effects are anticipated, such as in infection studies, smaller gRNA libraries of one to three gRNAs per gene may be necessary. Alternatively, the screen can be conducted in multiple subpools, each targeting only a fraction of all genes but with >3 gRNAs for greater reproducibility.
7. Delivery of CRISPRi system. Are CRISPRi components encoded genomically, on a plasmid, or a mixture of both? Chromosomal integrations are advantageous for reducing leakiness and enhancing stability as only one copy of the system is present per cell. Conversely, plasmid-based systems allow greater flexibility, ease of use, and higher abundance, but may increase cell-to-cell variability due to differences in plasmid copy number. Additionally, too high expression of CRISPRi components can be toxic.
8. Screen. What is the phenotype of interest and is an inducible system required? For studying phenotypes emerging under selective conditions, an inducible CRISPRi system is necessary, achievable by regulating either dCas, gRNA or both through an inducible promoter.

of bioinformatic tools, e.g. MAGeCK and others, can be found in reviews of Colic and Hart (2021) and Zhao et al. (2022).

Applications of CRISPRi in microorganisms to study genotype–phenotype relationships

Following the introduction of *dcas* and CRISPRi in *Escherichia coli* (Jinek et al. 2012, Bikard et al. 2013, Qi et al. 2013), this technique has been implemented and optimized in various species, including bacteria, yeast, archaea, and protozoa. CRISPRi has already been utilized to target small specific subsets of genes, either for mapping gene functions or for engineering metabolic pathways in industrially relevant microorganisms [reviewed by Call and Andrews (2022) and Sun et al. (2023)]. In contrast to targeting small subsets of genes, CRISPRi can also be used for larger subsets and genome-wide screens by increasing the number of genes targeted. These high-throughput screens have been conducted for bacteria and yeast, but not yet for archaea and protozoa. We, here, focus on examples of high-throughput CRISPRi screens (Table 3), while references to optimization and small-scale screens can be found in the reviews by Todor et al. (2021), Call and Andrews (2022), and Sun et al. (2023).

Initially, CRISPRi was used to screen for genes influencing growth or viability, primarily under standard laboratory conditions, but its applications have since broadened to encompass various other growth conditions (Peters et al. 2016, Smith et al. 2017, Rousset et al. 2018, Wang et al. 2018a, Lee et al. 2019, Momen-Roknabadi et al. 2020, Shields et al. 2020, Yao et al. 2020, Liu et al. 2021, McGlincy et al. 2021, Silvis et al. 2021, Enright et al. 2023, Miao et al. 2023, Shin et al. 2023, Otto et al. 2024). Additionally, genes underlying the tightly regulated replication system could be elucidated using CRISPRi (Camsund et al. 2020, Gallay et al. 2021). When opting for an arrayed approach over a pooled approach, variation in morphological phenotypes can be studied (Liu et al. 2017c, Shields et al. 2020, Silvis et al. 2021).

Furthermore, CRISPRi has been used to unravel resistance mechanisms against phage infection and antibiotics (Peters et al. 2016, Rousset et al. 2018, Jiang et al. 2020, Mutalik et al. 2020, Dewachter et al. 2022, Yan et al. 2022, Eckartt et al. 2024). Additionally, CRISPRi serves as a tool to prioritize drug targets, specifically by identifying genes essential for survival or reproduction (Bosch et al. 2021, McNeil et al. 2021, Zhu et al. 2023, Ward et al. 2024). Moreover, the mode of action of a drug can be elucidated by correlating different morphotypes resulting from gene silencing with those induced by drug administration (de Wet et al. 2020).

In addition to examining growth in stressful conditions, exploring other survival mechanisms is of significant interest. Microorganisms can withstand a variety of harsh environments through tolerance mechanisms, which were uncovered by CRISPRi (Wang et al. 2018a, Gutmann et al. 2021, Mukherjee et al. 2021, Spoto et al. 2022, Liu et al. 2024a).

DNA transfer and uptake are prevalent among microorganisms, serving as a strategy for rapid adaptation to new environments. Microorganisms transfer integrative and conjugative elements from donor to acceptor cell via conjugation. CRISPRi screening has revealed host genes that are important for DNA transfer (Harden et al. 2022). Furthermore, microorganisms can activate and regulate competence, which is a synchronized process whereby multiple cells take up DNA. Competence activation and regulation have been studied using CRISPRi (Liu et al. 2017c, Knoops et al. 2022, Minhas et al. 2023).

In biotechnology, microorganisms are used as cell factories for the industrial production of chemicals. Enhancing the yield of these chemicals necessitates metabolic engineering of the microorganisms, directing the metabolic flux towards specific pathways while minimizing the production of undesired byproducts. Hence, CRISPRi screens have been used to identify genes that enhance the production of compounds in industrially relevant strains (Li et al. 2020, Yao et al. 2020, Fang et al. 2021, Liu et al. 2022a, Johansson et al. 2023, Yu et al. 2023, Peng et al. 2024).

The examples mentioned illustrate that CRISPRi represents a novel approach with unique advantages for conducting high-throughput screens across diverse microorganisms. CRISPRi enables the study of various phenotypes beyond fitness, including complex and context-dependent phenotypes. With the CRISPRi system being implemented in an increasing number of species, including archaea and protozoa, it is expected that more high-throughput screens will be conducted in the future.

The strengths and weaknesses of CRISPRi

CRISPRi has recently been introduced in the field of genome-wide high-throughput screening due to its ability to overcome limitations of the previously discussed gene-perturbation approaches. First, as is the case for PCR-based methods, CRISPRi is a targeted approach and consequently relies on genomic information (Baba et al. 2006, Zaslaver et al. 2006, Qi et al. 2013, Trehan et al. 2016). CRISPRi screens are possible with relatively small libraries—ranging from 3–10 gRNAs per gene, each with a length of 20–23 nt depending on the dCas protein used (Wang et al. 2018a, Calvo-Villamañán et al. 2020, Rousset et al. 2021). In contrast, high-quality transposon insertion libraries are considerably larger to ensure complete coverage of coding regions. With the use of compact CRISPRi libraries, multiple conditions can be simultaneously examined while also reducing population bottlenecks by increasing the number of microbial cells per gRNA in experiments, resulting in a higher coverage (Wang et al. 2018a, Rousset and Bikard 2020). Increased coverage further facilitates the application of CRISPRi for screening subpopulations, such as persister cells, or for *in vivo* experiments. CRISPRi applications *in vivo* encompass screens for studying virulence at the genome-scale in *Streptococcus pneumoniae* (Liu et al. 2021, 2024b) and for a small subset of virulence genes in *Streptococcus mutans* during waxworm *Galleria mellonella* infection (Shields et al. 2020) and in *Legionella pneumophila* during macrophage infection (Ellis et al. 2021, 2023). Second, constructing whole-genome CRISPRi libraries is less labour-intensive in comparison to other targeted approaches, as gRNA plasmids can be generated in parallel through pooled cloning strategies. This characteristic renders CRISPRi a high-throughput gene-perturbation technique (Peters et al. 2015). Third, CRISPRi silences genes without altering the genome, distinguishing it from irreversible methods such as gene deletion or mutation (Good and Stach 2011, Bikard et al. 2013, Qi et al. 2013). To establish a reversible system, CRISPRi should be used as an inducible system by either cloning *dcas*, gRNA, or both, downstream of an inducible promoter, thereby allowing for control over the timing of CRISPRi activation and thus the regulation of gene silencing. By exerting control over timing, CRISPRi facilitates the screening for genetic mechanisms underlying transient phenotypes, which was demonstrated for virulence (Liu et al. 2021). In addition to high-throughput screening for virulence, CRISPRi has been utilized to investigate other transient phenotypes, albeit on a smaller scale. For example, microorganisms can switch from the planktonic to a sessile state, thereby forming a biofilm, which is a complex microbial community embedded in an extracellular matrix. CRISPRi has been employed to silence genes associated with

biofilm formation, thereby elucidating their role in biofilm production (Noirot-Gros et al. 2019, Afonina et al. 2020, Ghosh et al. 2022, St. Pierre et al. 2023). Finally, the inducible system enables the interrogation of the role of both essential and nonessential genes (Bikard et al. 2013, Qi et al. 2013).

Despite these many advantages, CRISPRi has some unique limitations. When performing CRISPRi screens, expression of the dCas effector and gRNA design need to be optimized to minimize undesired effects. High levels of dCas protein are associated with toxicity in some organisms, resulting in severely reduced growth rates (Cho et al. 2018). The expression of *dcas* can be adjusted to an optimal level, which maintains dCas binding while alleviating toxicity (Nielsen and Voigt 2014, Cho et al. 2018). Furthermore, toxicity is dependent on the compatibility between the dCas effector and species under study, as shown for dCas12a, which is less toxic in some bacteria compared to dCas9 (Knoot et al. 2020, Kuo et al. 2020). In addition, it is essential to carefully design gRNAs for CRISPR/dCas9, as sequence-specific toxicity, known as the bad-seed effect, can occur due to off-target binding in the promoter of essential genes by minimal 4 nt adjacent to a PAM sequence (Cui et al. 2018). Mitigating sequence-specific toxicity can be achieved by avoiding the strongest bad-seed sequences and reducing the dCas9 concentration (Cui et al. 2018, Rostain et al. 2023). However, as gene essentiality differs among conditions, strains and species, and promoter sequences are typically not well conserved, bad-seed sequences are not fixed (Rostain et al. 2023). Their prediction based on genomic data or gRNA-target binding strengths alone is not yet possible as 4–5 nt of complementarity is not always sufficient to cause a bad-seed effect and dCas9 binding via a bad-seed sequence is generally weak (Rostain et al. 2023). Instead of dCas9, dCas12a can be used, as no bad-seed toxicity has been reported yet. This might be due to the longer seed sequence required for stable dCas12a target binding, reducing the target space available for off-target binding, though more investigation is necessary (Jiang et al. 2015, Singh et al. 2016, Swarts et al. 2017). In addition to essential genes, gRNAs may also induce off-target effects in nonessential genes. This becomes more prevalent in larger genomes, such as those in eukaryotes. It is therefore crucial to use multiple gRNAs per gene to ensure reliable hit-calling (Rostain et al. 2023). Although CRISPRi suffers from off-target effects, these effects are lower compared to RNAi (Schuster et al. 2019).

While bad-seed and off-target effects should be avoided, PAM sequences must be considered while designing gRNAs. Specifically, the dCas–gRNA complex requires a PAM recognition site to initiate DNA unwinding starting from the PAM, facilitating subsequent hybridization of the gRNA with the DNA. Each type of dCas possesses its own PAM preference, with the commonly used dCas9 from *S. pyogenes* favouring NGG. Hence, genome characteristics, like GC-content, determine the frequency and distribution of potential targets for a given dCas type. By varying the dCas effector according to genome traits, a broader range of target positions can be accessed (Leenay et al. 2016, Vigouroux and Bikard 2020). For instance, dCas12a expands the targeting space of CRISPRi with its T-rich PAM (compared to the G-rich PAM of Cas9) (Jinek et al. 2012, Zetsche et al. 2015). This is of particular importance for organisms with a T-rich genome, like certain *Clostridium* species and fission yeasts (Zhao and Boeke 2020, Joseph and Sandoval 2023), and in AT-rich genome regions, such as promoters. While CRISPRi necessitates a PAM sequence, potentially resulting in fewer available gRNAs for small genes, it can handle small genes more effectively compared to random gene disruption methods, which have a bias towards longer genes (Wang et al. 2018a). Furthermore, similar to other gene-perturbation techniques, perturbations can be compensated by other cellular mechanisms, such as redundant genes and buffering of transcriptional perturbations at the protein level, as cells attempt to maintain homeostasis. Due to this compensation, the perturbed cells might exhibit a reduced or no phenotypic effect, indicating that the observed outcome is a combination of gene perturbation and cellular buffering (Flatt 2005, Blevins et al. 2019).

Finally, DNA-targeting systems, such as CRISPR/dCas9 and CRISPR/dCas12, have their own DNA-related limitations, including chromatin accessibility and polar effects. In eukaryotes, CRISPRi efficacy is reduced in regions with lower chromatin accessibility (Horlbeck et al. 2016a). The polar effect observed in screens using RNAi or random insertional mutagenesis libraries similarly limits CRISPRi. As CRISPRi inhibits polymerase progression, targeting of upstream operon genes leads to the transcriptional blockade of any downstream-encoded genes (Nakashima et al. 2006, Peters et al. 2015, Hutchison et al. 2019, Vigouroux and Bikard 2020). Additionally, a reverse polar effect may occur, leading to the repression of upstream genes. The effect potentially occurs through destabilization of the transcript, although its extent varies depending on the species (Peters et al. 2016, Zhao et al. 2017, Cui et al. 2018).

CRISPRi evolved over time to extend its capabilities

Over time, improvements and extensions of the original CRISPRi system increased its capabilities for a variety of applications, including genome-wide high-throughput screens. These advancements include the possibility to perturb multiple genes simultaneously, the development of inducible and tunable systems for more precise control over the timing and extent of knockdown, the establishment of design rules for efficient knockdown across the entire genome, and the extension of these techniques beyond the classical model species. These improvements and extensions are discussed in more detail in the sections below.

From single- to multigene perturbations

To determine the genetic basis of a phenotype it is important to consider genes in the broader cellular context, as the phenotype of some mutants can be modified by the presence or absence of other genes. Such genetic interactions are common and can reveal how (functionally redundant) genes interact to perform key biological functions (Babu et al. 2014, Costanzo et al. 2016). As biosynthetic pathways rely on the coordinated action of many different genes, gene-perturbation techniques that target multiple genes simultaneously are invaluable in metabolic engineering. Besides studying interactions, the ability to target multiple sites in a gene gives access to a greater dynamic range of repression.

The first strategies for multiplex targeting relied on separate expression of gRNAs from individual promoters, usually a copy of the same promoter (Qi et al. 2013, Peters et al. 2016, Jensen et al. 2017, Kim et al. 2017, Schwartz et al. 2018, Batianis et al. 2020). However, this can result in interference between the different copies of the promoter and comes at the cost of a large DNA footprint and higher instability due to the presence of repetitive sequences. The latter are especially problematic in high-throughput screens, where efficient transformation and stability of gRNA plasmids is essential. To mitigate these issues a single gRNA array encoding multiple gRNAs separated by repeats, similar to CRISPR arrays found in nature, can be expressed from a single promoter. By separating the spacers with a direct repeat and expressing a tracrRNA separately, the array can be processed

into individual mature gRNAs by a host enzyme with RNase activity, such as RNase III. This method has been used in several microorganisms, including *S. cerevisiae*, *E. coli*, *L. pneumophila*, and *Pseudomonas putida* (Liao et al. 2019, Batianis et al. 2020, Ellis et al. 2021). Other possibilities to process a CRISPR array are adding self-cleaving ribozymes between gRNAs (He et al. 2017), adding Csy4 recognition sites and expressing Csy4, which can cleave RNA 20 nt from its recognition site (Ferreira et al. 2018), or adding tRNAs, which can be transcribed from Pol II and Pol III promoters and cleaved by RNase P or Z to form mature gRNAs (Zhang et al. 2019). We refer to McCarty et al. (2020) for an in-depth discussion of these methods.

While alleviating the need for cumbersome constructs with multiple promoters and terminators, CRISPR arrays can still be large and are repetitive in nature. The DNA footprint can be reduced by shortening the direct repeats at the 3′ end (as the 5′ end is important for processing) without impairing dCas9-mediated repression efficiency (Gawlitt et al. 2023). In addition to reducing genetic instability associated with repeat-rich regions, shorter repeats can be of particular interest if the array is used as a barcode in pooled screens. A shorter barcode will reduce sequencing cost and shorter repeats reduce the likelihood of PCR chimeras, i.e. spurious combinations of gRNA barcodes arising from repeat-driven mispriming during PCR. Such chimeric barcodes lead to wrong assignment between phenotype and applied perturbation and generate noise in resulting data. Alternative to shortening repeats, shortening spacers from the native 30 nt to as little as 24 nt maintained repression efficiency for most tested spacers (Gawlitt et al. 2023). As individually expressed gRNAs maintain repression even if further shortened, factors relating to array processing, gRNA stability, or interaction with dCas9 may explain why further shortening reduced knockdown (Gawlitt et al. 2023). Earlier studies indicated at least 12 nt of complementarity are required for efficient gene knockdown (Qi et al. 2013), while more recent reports show repression even with very short gRNAs of 9 nt in *E. coli* (Cui et al. 2018, Jeong et al. 2023). This suggests that further spacer shortening may be possible for array-encoded gRNAs. Apart from reducing array length if translatable to a multiplex setting, shortened gRNAs could enable substantial cost savings in oligo library synthesis by also encoding several gRNAs on the same oligo. Repetitiveness can further be reduced by using different variants of the direct repeat, which enabled simultaneous expression of 20 gRNAs with a maximum shared repeat of only 16 nt in *E. coli* (Reis et al. 2019).

More recently, the intrinsic RNase activity of some Cas effectors to process the primary transcript of a CRISPR array into individual mature gRNAs, without relying on a tracrRNA or host factors, has been leveraged for multiplex targeting. To this end, dCas12a has been used extensively (Zhang et al. 2017, Li et al. 2018, Miao et al. 2019, Choi and Woo 2020, Knoot et al. 2020, Schilling et al. 2020, Wu et al. 2020, Zhao and Boeke 2020, Fleck and Grundner 2021, Magnusson et al. 2021, Joseph and Sandoval 2023). Its intrinsic RNase activity facilitates multigene targeting with a reduced DNA footprint and higher efficacy, which has been demonstrated in a wide range of microorganisms ranging from the model organism *E. coli* to mycobacteria, cyanobacteria, and fission yeast (Zhang et al. 2017, Choi and Woo 2020, Zhao and Boeke 2020, Fleck and Grundner 2021). Similar to dCas9, the dCas12a array can be shortened to further decrease array length. Knockdown remains strong with direct repeats of 19 nt, matching its length in the mature gRNA (Zetsche et al. 2015), and spacers of 16–18 nt. In contrast to dCas9 no further spacer shortening is possible as knockdown is completely abolished for spacers shorter than 16 nt (Miao et al. 2019). This is in line with observations that dCas12a requires perfect complementarity of at least 16 nt to form a stably bound state (Jeon et al. 2018, Specht et al. 2020).

To maximize multigene targeting efficacy, overall array design needs to be considered carefully. The relative abundance of gRNAs coexpressed from an array, and hence the number of dCas–gRNA complexes for each target in the cell, can vary up to 100-fold (Campa et al. 2019, Liao et al. 2019, Creutzburg et al. 2020, Magnusson et al. 2021). While the position of a gRNA within an array as well as array secondary structure have been linked to abundance differences, secondary structure elements interfering with array processing likely explains this effect. Proper formation of a hairpin from the direct repeat is necessary for proper array processing by dCas12a, and processing is impaired by base-pairing of the direct repeat with other parts of the array (Liao et al. 2019, Creutzburg et al. 2020). Spacers downstream of spacers with high GC content at their 3′ end, which are prone to RNA secondary structure formation, are particularly inefficient in mediating knockdown (Magnusson et al. 2021). Encouragingly, insertion of short AT-rich separator sequences between individual gRNAs, mimicking the architecture of natural CRISPR arrays, is sufficient to disrupt secondary structures and improve performance of gRNA arrays in mammalian cells (Magnusson et al. 2021). Separators should similarly improve dCas12a array processing in microbial organisms and reduce differences in repression efficacy resulting from varying gRNA abundance. The potential for RNA secondary structure formation should also be taken into account for dCas9 arrays, as repression efficacy can vary substantially depending on gRNA and flanking sequences and their propensity for base-pairing interactions with upstream repeats (Gawlitt et al. 2023).

A general challenge in multiplexing is a dilution of the dCas effector molecule between multiple intracellular targets, as the different gRNAs directly compete for dCas effector binding. This in turn impacts the efficiency of knockdown with each gRNA: in *E. coli*, repression was reduced from 60x with a single gRNA to less than 10x once >6 gRNAs were coexpressed (Zhang and Voigt 2018). Reliable prediction of gRNA efficacy based on sequence is consequently more difficult, and extrapolation from efficacy measurements of single gRNAs to a multiplex setting may not be possible. In reality, this is less of a limitation for genome-scale screening applications, as the majority of screens will likely focus on pairwise or triple interactions, where these effects are less pronounced. Testing higher order interactions exhaustively is not readily feasible due to the large combinatorial space and consequently large numbers of cells that need to be assessed.

Another potentially serious challenge is that as the number of gRNAs in a cell increases so does the number of off-targets, which may enhance the negative impact of bad-seed sequences. Lowering dCas9 expression level to mitigate bad-seed effects could hamper repression efficacy in a multiplex setting due to the competition between gRNAs. While a lot of efforts have focused on dCas variants with lower off-target cleavage activity (Anzalone et al. 2020), it is unclear whether any of these modifications would reduce off-target binding. For some dCas9 variants, the improvement is at least partially through lowered intrinsic cleavage activity (Singh et al. 2018), which would not reduce promiscuous binding. Given cleavage requires more extensive pairing with target DNA (Wu et al. 2014), while as little as 4 nt are sufficient for binding, it may be substantially more difficult to develop variants with higher binding specificity. As dCas12a binding is more specific than dCas9 due to the more stringent targeting requirements and bad-seed effects have so far not been described, dCas12a may be a safer option for multiplex applications.

Enhanced control over CRISPRi components with inducible and tunable systems

Inducible and titratable CRISPRi systems offer the possibility to study the effect of temporal changes and quantitative differences in gene expression level. In library-scale applications, inducible knockdown prevents dropout of essential genes during cloning or preculturing steps. This reduces library skew and allows more precise measurements of the impact of losing essential gene functions in various growth conditions and on phenotypes other than growth. Tunable CRISPRi systems further enable studying the effect of partial repression of a gene, which is not only interesting for essential genes given that many mutations do not lead to complete loss of gene function (Vande Zande et al. 2022).

A simple strategy is to control expression of the dCas effector protein or gRNA via inducible promoters. Inducible promoters not only allow flexible temporal control over gene expression but can achieve a broad and continuous range of expression simply by varying the amount of inducer (Gordon et al. 2016, Li et al. 2016, Fontana et al. 2018, Müh et al. 2019, García-Huerta et al. 2022). As the amount of dCas effector protein and gRNA correlate with the knockdown efficiency, varying their expression levels results in a readily tunable system. Several inducible promoters are available for microorganisms, including an anhydrotetracycline (aTc)-responsive $P_{tetR/tetA}$ promoter (Gordon et al. 2016, Ellis et al. 2021, Gauttam et al. 2021, Shaw et al. 2022), an arabinose-inducible P_{BAD} promoter (Li et al. 2016, DeLorenzo et al. 2018, Gauttam et al. 2021), a rhamnose-inducible P_{rha} promoter (Kim et al. 2017, Hogan et al. 2019), a xylose-inducible P_{xyl} promoter (Peters et al. 2016, Müh et al. 2019, Batianis et al. 2020), a doxycycline inducible P_{ran} promoter (García-Huerta et al. 2022), a cumate-inducible $P_{CymRP21}$ promoter (Jiang et al. 2024), and an IPTG-inducible P_{lac} promoter (Miao et al. 2019, Knoot et al. 2020, Gauttam et al. 2021). While all these promoters are inducible, they differ markedly in not only their titratability but also their leakiness—that is, the level of basal transcription in absence of inducer. The latter can be problematic as the dCas/gRNA complex remains bound to its target for an extended time (Sternberg et al. 2014, Ma et al. 2016, Jeon et al. 2018) such that even a small amount of repressor complex could mediate substantial repression. All reported inducible promoters have a certain degree of leakiness, ranging from 10% repression (P_{lac}, P_{BAD}, and P_{rha}) to up to 30% repression ($P_{tetR/tetA}$ and P_{xyl}) in absence of inducer (Li et al. 2016, Peters et al. 2016, Batianis et al. 2020, Knoot et al. 2020, Gauttam et al. 2021). A wide range of quantitative repression up to 90% relative reduction can be achieved with these promoters. The relationship between inducer and repression level is generally linear, with the strongest correlation for the $P_{tetR/tetA}$ and P_{BAD} promoters (Gordon et al. 2016, Li et al. 2016, Miao et al. 2019, Batianis et al. 2020, Knoot et al. 2020, Gauttam et al. 2021). While the P_{BAD} promoter is normally not titratable, engineering the arabinose transporter systems in *E. coli* enables tunable and strong induction (Li et al. 2016). Leakiness can be reduced by controlling expression of the dCas effector and the gRNA with two different inducible promoters (Dhamad and Lessner 2020), by expressing the inducible dCas effector from the genome instead of a plasmid (Peters et al. 2016, Yao et al. 2020) or by combining positive and negative regulation for control of expression. An example of the latter is a combined tet-ON and tet-OFF system developed for *S. cerevisiae*, which reduces leakiness from more than 10% to less than 4% (Shaw et al. 2022). While leakiness can be managed, inducible promoters have other limitations. Some inducers, like xylose and arabinose, are metabolized by microorganisms, resulting in changes in inducer concentration and variability in repression strength over time. ATc is light sensitive, limiting its use in long-term experiments, while other inducers limit the conditions in which a library can be screened. Synthetic inducible promoters with broad applicability (Chen et al. 2018b, Liu et al. 2019a) could overcome these limitations and be adapted for high-throughput CRISPRi screens in the future. Other strategies to control CRISPRi activity include small-molecules and engineered conditional gRNAs using RNA nanotechnology (Hanewich-Hollatz et al. 2019, Maji et al. 2019). While gRNAs relying on RNA triggers are already tested in *E. coli*, small-molecule inducible systems are not, and both show relatively high leakiness and offer less precise control compared to the previously discussed methods (Hanewich-Hollatz et al. 2019, Maji et al. 2019).

Controlling repression via altering repressor complex abundance has other drawbacks, due to the substantial cell-to-cell variability in gene expression capacity between isogenic cells. To address this limitation Vigouroux et al. (2018) proposed a strategy relying on design of the gRNA to tune repression. As RNA polymerase dissociates from the target DNA at a rate controlled by the level of gRNA:target DNA complementarity, knockdown can be tuned via gRNAs with varying levels of target complementarity (Vigouroux et al. 2018). This strategy not only reduces cell-to-cell variability but also allows programming of different levels of repression for individual genes in multiplexing applications, increasing the range of interactions that can be studied in high-throughput screens (Vigouroux et al. 2018). Titration of CRISPRi efficacy via mismatches between the gRNA and target sequence ranges from no reduction to almost full knockdown (Hawkins et al. 2020, Wang et al. 2023, Otto et al. 2024). While mismatches throughout the gRNA base-pairing region impact knockdown strength (Wang et al. 2023), their effect on effector complex–target interaction becomes negligible beyond position 17 relative to the PAM for dCas12a, and the absolute number of mismatches in the seed region is more important than their position (Specht et al. 2020). Not only the position but also the type of mismatch plays a role (Jost et al. 2020, Specht et al. 2020), with possible differences between nucleases (Specht et al. 2020). While mismatches in the seed region generally abolish repression with a dCas–repressor fusion in mammalian cells (Jost et al. 2020), these gRNAs maintain measurable activity in bacteria, likely due to different mismatch tolerance of dCas9 and dCas9–repressor fusions (Gilbert et al. 2014, Hawkins et al. 2020). By using mismatches to titrate knockdown, it is also easy to screen the impact of different levels of reduction in a single pool. As gRNAs are used as barcodes, sequencing will identify both the gene target and level of reduction. Finally, the advantages of inducible promoters and gRNA design can be combined for optimal temporal control of knockdown at well-defined, titrated levels.

Sources of variable CRISPRi efficacy across the genome

With the availability of more CRISPRi datasets it has become possible to shed light on variables that impact CRISPRi repression strength across different contexts (Fig. 4). A better understanding of these dependencies is key for optimizing gRNA design and experimental parameters to maximize efficacy of a genome-scale CRISPRi screen. A first important consideration is the effective targeting window. Gene repression via a simple dCas roadblock is possible by directing the repressor complex to gene promoters, interfering with transcription initiation, or by targeting the coding region, blocking elongation (Bikard et al. 2013, Qi et al. 2013, Zhang et al. 2017). For promoter regions, targeting either DNA strand supports good repression with both dCas9 and dCas12a (Bikard et al. 2013, Qi et al. 2013, Miao et al. 2019), while knockdown in coding

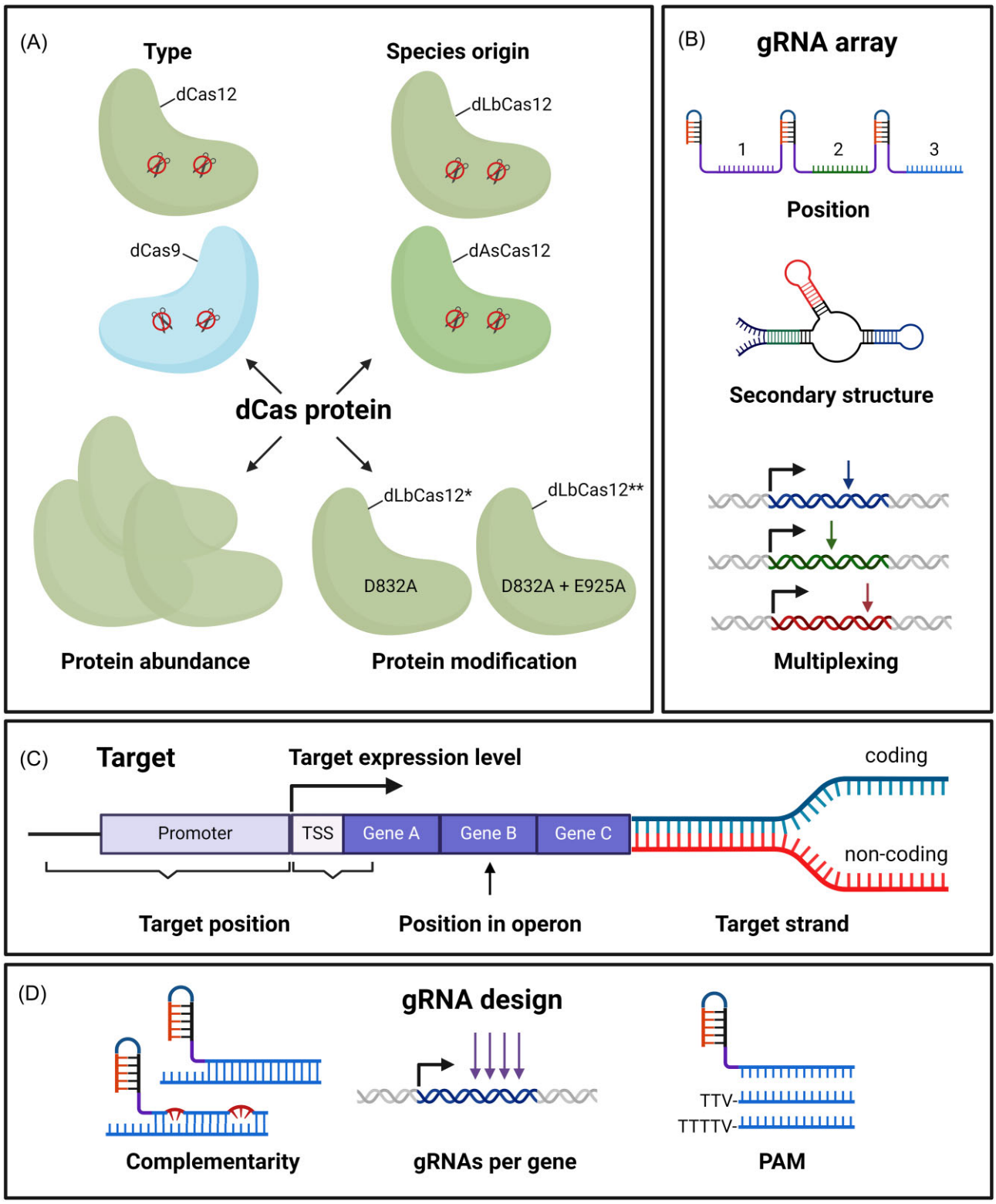

Figure 4. Factors impacting CRISPRi repression efficacy. The efficacy of CRISPRi knockdown is impacted by various factors. (a) dCas protein. The efficiency and toxicity of different dCas effectors varies among microbial species. Higher dCas protein abundance can increase repression but may simultaneously enhance toxicity. For dCas12a, single inactivating mutations support stronger repression than two inactivating mutations. (b) gRNA array. In multiplex applications, the number of simultaneously encoded gRNAs, their position within a gRNA array, and array secondary structure can influence relative gRNA abundance and knockdown efficacy. (c) Gene target. Knockdown is generally stronger when targeting close to the TSS and with higher expression of the target. Moreover, in bacteria, repression efficacy varies depending on the position of a target within an operon and the targeted strand (for coding regions). (d) gRNA design. Lower complementarity between gRNA and target reduces knockdown strength, while optimization of the PAM sequence can increase knockdown efficiency. Knockdown efficacy can alternatively be titrated by altering either dCas protein or gRNA abundance via inducible and titratable promoters. Finally, using multiple gRNAs per gene can enhance repression. dCas = deactivated Cas; TSS = transcription start site; PAM = protospacer-adjacent motif; and gRNA = guide RNA.

regions depends on the orientation of the gene and the targeted strand. With dCas9, repression is substantially stronger when targeting the nontemplate strand (i.e. the gRNA is complementary to the coding sequence) (Cui et al. 2018, Calvo-Villamañán et al. 2020, Hawkins et al. 2020) while the opposite applies to dCas12a (Miao et al. 2019). Repression is generally efficient when targeting throughout the reading frame (Cui et al. 2018), although some studies suggest slightly higher repression when targeting the first 5% or 60 bp of the open reading frame (Wang et al. 2018a, Yu et al. 2024). In promoter regions good efficacy is observed as far as −400 bp upstream of the start codon in *E. coli*, but repression is substantially stronger closer to the start codon (Cui et al. 2018). For dCas–repressor fusions, repression is most efficient when targeting upstream or a very short region downstream of the transcription start site (TSS), with an optimal targeting window of −200 bp to TSS for a dCas9-Mxi repressor in *S. cerevisiae* (Smith et al. 2016, Momen-Roknabadi et al. 2020, McGlincy et al. 2021, Mukherjee et al. 2021).

Not only position and target strand, but also the PAM and gRNA sequence contribute to variation in gRNA efficacy (Fig. 4). The optimal PAM sequence for dCas9 and dCas12a, respectively, is CGGH (Calvo-Villamañán et al. 2020) and TTTV (Miao et al. 2019, Specht et al. 2020). Compared to dCas9, target binding by dCas12a is less dictated by the specific gRNA sequence but rather by the PAM and possible mismatches between the gRNA and target sequence (Specht et al. 2020). Consequently, dCas12a will bind matching gRNA sequences that only differ in their PAM with different efficacies, which could translate to variable repression (Miao et al. 2019). As the impact of PAM was only examined among gRNAs with a fixed GC content of 50%, gRNA sequence may show a stronger contribution when GC content is varied (Specht et al. 2020). The GC content of the gRNA is an important feature due to increased thermodynamic stability of G–C compared to A–T base-pairing, but typically only explains a small portion of variation in gRNA efficiency (Calvo-Villamañán et al. 2020, Yu et al. 2024). Extreme GC content should be avoided, however, and most screens use gRNAs with a GC content of 30%–85% (Calvo-Villamañán et al. 2020, Spoto et al. 2020, Yu et al. 2024). Low GC content leads to weak gRNA–DNA base pairing, while high GC content increases the chances of gRNA secondary structure formation. Besides GC content, also nucleotide homopolymers negatively impact gRNA efficacy (Gilbert et al. 2014). Long stretches of T are particularly unfavorable when expressing gRNAs from Pol III promoters, leading to lower gRNA abundance, likely because Pol III aborts transcription at T-rich sequences (Roy et al. 2018).

A potential caveat for CRISPRi screening applications is that target expression level correlates with repression efficacy, with increased repression observed for—counterintuitively—more abundant targets (Yu et al. 2024). To level out potential differences in efficacy or further increase knockdown strength, the same gene can be targeted by multiple gRNAs simultaneously expressed from an array. Effects can range from additive to multiplicative compared to single targeting and up to complete silencing (>300x reduction) with three gRNAs, highlighting the importance of careful design (Qi et al. 2013, Miao et al. 2019, Spoto et al. 2020, Ciurkot et al. 2021). Another important factor to consider is the dCas effector protein (Fig. 4). As described previously, some dCas effectors can be highly toxic in certain species but repression efficacy also varies for different dCas orthologs, ranging from almost no to more than 150-fold repression (Rock et al. 2017). Even different versions of the same effector can yield varying results; for instance, dCas12a effectors with single inactivating mutations facilitate more effective repression compared to variants with two inactivating mutations (Miao et al. 2019). Many of the discussed factors have only been tested in isolation and it is not trivial to predict how their effects might combine to impact repression strength. In addition, host-dependent variables can also impact CRISPRi efficacy. In single target or small-scale applications this uncertainty can be overcome by screening multiple systems and gRNAs and choosing the best performing one. However, due to the large numbers of gRNAs used in a genome-scale screen, it is not practical to test each gRNA's impact on target expression individually. Reliable *in silico* prediction of repression efficacy is therefore needed to ensure efficient knockdown across the entire genome.

Genome-scale efficacy prediction for improved gRNA library design

While CRISPRi gRNA design tools exist for eukaryotes (Smith et al. 2016, Horlbeck et al. 2016a, Hoberecht et al. 2022, Yao et al. 2024), they are not readily transferrable to prokaryotes due to some important differences. For example, nucleosome positioning impacts CRISPRi efficacy in mammalian cells and *S. cerevisiae* (Horlbeck et al. 2016a,b, Smith et al. 2016), but bacteria lack histones. Bad-seed toxicity needs to be taken into account in at least some bacteria (Cui et al. 2018), while this phenomenon has not been observed in eukaryotes. Polar effects can similarly bias results, particularly as their range and extent varies among species (Peters et al. 2016, Cui et al. 2018). It is also not possible to infer repression efficacy from models predicting cleavage efficacy, as the latter have only poor predictive accuracy for CRISPRi datasets, even for the same species (Calvo-Villamañán et al. 2020).

While CRISPRi efficacy prediction tools have so far only been developed for *E. coli*, they have revealed a number of important factors, besides target strand and optimal targeting window, correlating with strong repression. Important predictive features include the gRNA seed sequence (particularly the most PAM proximal base, where T and C are favoured and A should be avoided), the N in the NGG PAM (where C is favoured), and the base immediately after the PAM (where C is favoured, and G should be avoided) (Calvo-Villamañán et al. 2020, Yu et al. 2024). Interestingly, bases downstream of the PAM—corresponding to the sequence read by RNA polymerase when encountering dCas9–seem to also play a role (Calvo-Villamañán et al. 2020). One difficulty is that typically only indirect measurements of guide efficacy, such as the depletion of gRNAs targeting essential genes, can be obtained in sufficiently high numbers to inform prediction. Efficacy measures are thus confounded by other variables, including the fitness impact of depleting the target (Calvo-Villamañán et al. 2020) and gene-specific features like expression level, position in an operon and number of downstream essential genes (Yu et al. 2024). To separate these effects two approaches can be applied: using a mixed-effect random forest regression model to remove the gene contribution and extract gRNA design rules based on gRNA sequence alone (Yu et al. 2024), or normalizing gRNA effects by the average effect of gRNAs targeting the same gene (Calvo-Villamañán et al. 2020). Encouragingly, at least some features that predict gRNA activity seem to be shared among species, as a model for gRNA efficacy prediction in *E. coli* performed similarly well on a small set of gRNAs in *B. subtilis* (Calvo-Villamañán et al. 2020). Apart from reducing uncertainty in screens, good prediction models help to save costs and increase accuracy and reproducibility. For example, as few as three well-designed gRNAs per gene yield more accurate information on gene effects than a larger library with randomly designed gRNAs (Calvo-Villamañán et al. 2020). Prediction algorithms are not only useful for design of maximal repression libraries but can also inform gRNA design for titration. One

prediction model uses different 6 nt PAM sequences for dCas12a to predictably titrate repression in *E. coli*. By changing the PAM sequence from the least to the most optimal PAM (TTTV) for dCas12a, a CRISPRi screen can range from medium to maximal repression (Miao et al. 2019).

A caveat of many predictors is that they perform well on the dataset used for training but worse on other datasets, due to differences in the exact CRISPRi system used and other experimental parameters. Inclusion of more and diverse datasets in training improves generalizability (Yu et al. 2024), and further improvements should be possible with the availability of more data in the future. Currently, most bacterial CRISPRi prediction models are developed using data from *E. coli* (Cui et al. 2018, Wang et al. 2018a, Miao et al. 2019, Rousset et al. 2021, Yu et al. 2024). While some features may translate to other organisms, as shown by similar efficacy and titration of CRISPRi efficacies with the same gRNAs in *E. coli* and *B. subtilis* (Calvo-Villamañán et al. 2020, Hawkins et al. 2020), other features like bad-seed and polar effects are less universal (Peters et al. 2016, Cui et al. 2018, Rostain et al. 2023). Organism-specific data will greatly increase the accuracy of prediction models in each species and will facilitate implementation of efficient high-throughput CRISPRi approaches with minimal and well-designed libraries across organisms.

High-throughput CRISPRi approaches beyond model species

Genome-scale CRISPRi screens remain largely limited to well-studied organism like *E. coli* and *S. cerevisiae* (Wang et al. 2018a, Lian et al. 2019, Momen-Roknabadi et al. 2020, McGlincy et al. 2021, Mukherjee et al. 2021, Johansson et al. 2023). CRISPRi approaches in other microorganisms have typically focused on a smaller subset of genes, where knockdown efficiency can easily be validated individually. As mentioned, host-specific factors may impact CRISPRi efficacy and hence optimal gRNA design, complicating the use of prediction models developed for other species. Many challenges beyond optimal gRNA design impede translating CRISPRi-screening approaches to other organisms. CRISPRi components first need to be constructed and optimized for function and minimal toxicity in each new organism, which can be time-consuming. Transferrable CRISPRi tools can speed up this process (Peters et al. 2019, Wang et al. 2019, Ke et al. 2022).

One approach, Mobile-CRISPRi, uses modular CRISPRi components that can be transferred between and integrated into the genomes of diverse bacterial species, alleviating the need for stable replicative plasmids. The modularity of the system enables easy swapping of organism-specific promoters and gRNA libraries. Transfer efficiencies are sufficiently high for genome-wide screening in many organisms, and transfer and integration introduce only minimal skew into the library. Importantly, integrations are stable and functional for >50 generations without selection, paving the way for studying microbial and host–microbe interactions in pathogenic or microbiome contexts (Peters et al. 2019). Knockdown efficiencies ranged from 8x in *P. aeruginosa* to 150x in *S. aureus* when targeting a red fluorescent protein.

Another high-efficacy transfer method is Chassis-independent Recombinase-Assisted Genome Engineering (CRAGE) (Wang et al. 2019, Ke et al. 2022). CRAGE utilizes a two-step integration system, first integrating a Cre-LoxP cassette in a random position in the genome via transposons, followed by Cre-LoxP-mediated integration of both the dCas effector protein and gRNA (Ke et al. 2022). CRAGE offers many of the same advantages as Mobile-CRISPRi. However, as random genomic insertions can affect the phenotype and cassette expression may differ across genomic contexts, Mobile-CRISPRi will likely enable better reproducibility in high-throughput screens.

Apart from the need for optimization of different CRISPRi components, PAM restrictions limit genome-scale efforts in some microorganisms. The different PAM preferences of natural dCas orthologs like dCas9 (G-rich PAM) and dCas12a (T-rich PAM) already increase flexibility and can be harnessed to target a greater fraction of genome sequences. Moreover, recent efforts are focused on relaxing PAM requirements to the extent of entirely PAM-less dCas variants (Gao et al. 2017, Chatterjee et al. 2020, Legut et al. 2020, Walton et al. 2020). While increasing the available target space, the simultaneous increase in the number of putative off-target binding sites could worsen the impact of toxic seed sequences (Rostain et al. 2023). This risk will likely vary depending on the mechanisms underlying the relaxed PAM requirement and the nuclease, as some PAM-relaxed Cas9 variants have increased specificity despite a larger number of potential off-target sites (Hu et al. 2018). Further information on the development of dCas variants with less restrictive PAM requirements is detailed in the review by Collias and Beisel (2021).

Factors like culturing conditions, a lack of reliable genetic parts and potential triggering of the native immune response further complicate implementation of CRISPRi in many organisms. Moreover, interference with putative endogenous CRISPR or anti-CRISPR systems should be avoided. Online tools that predict the presence of such systems can be helpful in this regard (Couvin et al. 2018, Chai et al. 2019, Wang et al. 2020, 2021a). In organisms where CRISPRi with a non-native CRISPR system is unsuccessful, endogenous systems offer an opportunity as they can be repurposed for gene repression (Luo et al. 2015, Zheng et al. 2019, Shields et al. 2020, Qin et al. 2021, Xu et al. 2021b). We refer to Call and Andrews (2022) for a more in-depth review of challenges surrounding CRISPRi implementation in nonmodel bacteria, which covers many of these aspects. CRISPRi systems developed in conventional bacteria can be transferred to archaea with high efficiencies (Nayak and Metcalf 2017, Dhamad and Lessner 2020), while translation to many eukaryotic microorganisms, like protozoa and nonmodel yeasts, faces similar challenges as for nonmodel bacteria (Smith et al. 2016, McInally et al. 2019, Barcons-Simon et al. 2020, Dhamad and Lessner 2020, Momen-Roknabadi et al. 2020, Ciurkot et al. 2021, Mukherjee et al. 2021, Saini et al. 2023).

Remaining challenges

CRISPRi is a powerful tool to study complex phenotypes in high-throughput screens across different microorganisms, and numerous improvements to the original system have increased its efficacy, specificity, and flexibility. However, some challenges remain. Off-target binding is an inherent property of CRISPR systems and a source of confounding in screening applications, as even short stretches of complementarity between the guide and target can mediate some degree of repression (Rostain et al. 2023). Besides this bad-seed effect, other yet unknown mechanisms may contribute to dCas9 toxicity (Rock et al. 2017, Cho et al. 2018) and may bias screening results. Furthermore, due to prolonged targeting of essential genes and/or the high metabolic burden associated with expression of CRISPRi components, suppressor mutations in the promoter or coding region of the dCas effector protein can occur (Zhao et al. 2016, Liu et al. 2017c, Cui et al. 2018, Batianis et al. 2020). To reduce false positive results from this and other sources, gene effects should always be inferred from multiple gRNAs per genes, with 3–10 gRNAs per gene as a rule of thumb, depending on available prediction models.

To study multiple gene perturbations simultaneously, individual gRNAs can be expressed from different promoters or as a gRNA array. dCas12a is especially interesting for multiplex applications as it can process arrays without relying on host factors and reduces the DNA footprint. However, proper design is important due to the negative impact of array secondary structure on processing and the possible enhancement of off-target and bad-seed effects when multiple gRNAs are used. Tight control over expression of CRISPRi components is crucial for screening and can be achieved via inducible and titratable promoters. The main limitation of this approach is the leakiness of most inducible promoters, although recent developments of new tightly regulated systems could alleviate this problem. Other features related to the dCas effector, PAM, gRNA and target impact CRISPRi repression efficiency, highlighting the need for reliable gRNA design tools to enable robust and efficient knockdown across the entire genome. Some recent attempts to develop these tools show promising results, although incorporation of more data will improve their predictive potential in different contexts and species. Finally, to study genotype–phenotype relationships in nonmodel microorganisms and microbial communities, easy transfer of CRISPRi modalities across different organisms is needed. Besides issues surrounding cultivability and availability of well-characterized genetic parts, a major challenge lies in the development of reliable prediction tools in these species, especially without a better understanding of the impact of host-specific factors on CRISPRi efficacy.

Beyond CRISPRi—alternative CRISPR-based approaches for high-throughput investigation of phenotypes

To further increase the capabilities for studying the genetic basis of phenotypes at scale, other methods exploiting the programmable nature of CRISPR/Cas have been developed. A first extension is the use of CRISPR activation (CRISPRa), to increase gene expression rather than reducing it. Overexpression of genes or expression of genes that are normally silenced can improve our knowledge of gene function in different conditions. Tools building on the more recently discovered RNA-targeting CRISPR systems can overcome some limitations of CRISPRi, including less precise targeting in dense genomes and potential off-target effects, such as the polar-effect discussed previously. Finally, not only the expression level of a gene is important, but more subtle changes to the DNA and RNA can affect a phenotype. To study their impact, tools that enable precise genome manipulation with single nucleotide resolution at scale are instrumental.

CRISPRa for programmable activation of genes

A complementary approach to CRISPRi is the use of CRISPRa systems for programmable activation of genes (Fig. 1). Some genes only show a phenotype when overexpressed, and their identification can lead to more complete genome function annotations and a better understanding of the genetic basis of phenotypes. CRISPRa holds a lot of promise for microorganisms in particular, as genes involved in the metabolism of complex substrates or synthesis of valuable compounds in industrially relevant microorganisms are often silent or poorly expressed. Similarly, medically important phenotypes like antifungal resistance are often achieved though chromosome or gene duplications and gene overexpression (vanden Bossche et al. 1992, Marichal et al. 1997, Li et al. 2004, Luesch et al. 2005, Shin et al. 2007, Abbes et al. 2013, Begolo et al. 2014, Vale-Silva et al. 2016, Forche et al. 2018, Todd et al. 2019). These examples highlight the need for genetic tools to not only study consequences of gene loss but also of gene overexpression.

Gene activation is achieved via targeting a transcriptional activator for recruitment of RNA polymerase and a dCas protein to promoter regions via a gRNA (Fig. 1) (Casas-Mollano et al. 2020, Fontana et al. 2020b). Existing systems encode a transcriptional activator domain either fused to the dCas effector or recruited via a modified gRNA. While most CRISPRa systems rely on the dCas9 protein, more recent dCas12-based systems have increased the targeting space (Schilling et al. 2020).

Many of the advantages that make CRISPRi superior to other repression tools also apply to CRISPRa. Additionally, it is more scalable and portable than cumbersome promoter engineering and provides greater flexibility compared to plasmid-based (c)DNA overexpression libraries. Similar to CRISPRi, CRISPRa enables reversible regulation of (multiple) genes in a time- and cost-efficient manner. However, in contrast to CRISPRi, CRISPRa requires more complex design rules and depends on host-specificity of the activator.

Compared to mammalian systems (Casas-Mollano et al. 2020), CRISPRa has seen less use in microorganisms and consequently, activator domains are less optimized and factors that impact its efficacy are less well-understood. In yeast, the CRISPRa system was first implemented in the model species *S. cerevisiae* to target a small number of genes (Farzadfard et al. 2013). Platforms rely on either constitutive or inducible systems, dCas9 or dCas12 effectors, and the activator domains VP64 (four tandem copies of Herpes Simplex Viral Protein 16 (VP16), a commonly used eukaryotic transcription activator domain) or Viral Protein R (VPR) (composed of three parts: VP64 and activator domains from the p65 subunit of NFκB and Epstein–Barr virus R transactivator Rta). VP64 fusions achieve generally modest (~2.5-fold) upregulation of target expression in *S. cerevisiae*, which can be boosted up to 70-fold for synthetic constructs with multiple targeting sites upstream of the promoter (Farzadfard et al. 2013). VPR leverages the synergistic effects observed from recruitment of multiple activators in eukaryotic transcriptional control and generally results in stronger activation in *S. cerevisiae* and the industrially relevant yeast *Yarrowia lipolytica* (Farzadfard et al. 2013, Schwartz et al. 2018), analogous to higher eukaryotes (Casas-Mollano et al. 2020). The activity of both VP64- and VPR-based systems strongly depends on the targeting window, with repressive effects observed for some gRNAs (Cámara et al. 2020), highlighting the need for careful gRNA design. Efficiency generally increases with distance from the TSS, with an optimum around −300 bp upstream in *S. cerevisiae* (Farzadfard et al. 2013, Cámara et al. 2020), whereas more modest activation is occasionally seen closer to the TSS. VP16 domains can recruit the mediator complex if positioned appropriately upstream of a TSS and studies in mammalian cells see a peak of active gRNAs −400 to −50 bp upstream from the TSS (Gilbert et al. 2014). A further improvement in activity is possible with scaffold RNAs (scRNAs), modified gRNAs that serve as a scaffold to recruit activation domains fused to diverse RNA binding proteins (Zalatan et al. 2015, Yu and Marchisio 2024). The scRNA system showed up to 4.7x (Yu and Marchisio 2024) or 10–30x (Zalatan et al. 2015) higher activation compared to direct VP64–dCas fusions. The difference in activation between studies could be due to differences in scRNA architecture, gene targets and target windows, dCas fusions (dCas9 and dCas12e), yeast strains, or experimental parameters, highlighting the difficulty in achieving consistent results even with the same CRISPRa system. Interestingly, optimal activity also depends on the activator–dCas combination, with dCas9 from

S. pyogenes performing best with the strongest activator (VPR), while dLbCas12a gives better activation with a medium-strength activator (VP) (Lian et al. 2017).

Despite often only modest activation and a lack of reliable rules for prediction of target sites supporting maximal activation, CRISPRa has been used successfully in a variety of applications in yeasts. In the methylotrophic yeast *Pichia pastoris*, a scRNA CRISPRa system was used to increase riboflavin (vitamin B2) production through overexpression of the *RIB1* gene (Baumschabl et al. 2020). This CRISPRa-programmed riboflavin production exceeded the titers of a conventional *RIB1* overexpression with a P_{GAP} promoter, highlighting the powerful on-demand control of specific biological processes enabled by CRISPRa. In *Y. lipolytica*, VPR-based CRISPRa overexpression of cellobiose degrading genes enabled growth on this sugar as the sole carbon source (Schwartz et al. 2018). Furthermore, CRISPRa systems have been a powerful tool to discover new genes whose overexpression is linked to stress or antifungal drug tolerance in the human pathogens *Candida albicans* and *Nakaseomyces* (*Candida*) *glabrata* (Gervais et al. 2022, Maroc et al. 2024). Larger-scale screens are rare and remain limited to *S. cerevisiae*. A CRISPRa screen using dCas9–VP64 and a gRNA library targeting 52 genes was used to screen for improved thermotolerance (Li et al. 2019). qPCR of five targets showed modest upregulation of four (1.62–2.56 x) and strong upregulation of one (21.37x) target, despite the same window being targeted. A genome-scale screen using the more efficient dCas9–VPR system identified both known and new factors for increased protein secretion in industrial *S. cerevisiae* strains (Johansson et al. 2023). The >300 identified hits were associated with components of the secretory pathway, and 9 out of 10 hits also increased secretion in independent validation experiments. Another genome-scale screen using dCas12a–VP (a fusion of only VP64 and p65 activation domains) identified new factors for furfural tolerance (Lian et al. 2019). Both genome-scale screens employed a combinatorial approach using CRISPRa and CRISPRi (Johansson et al. 2023) or CRISPRa, CRISPRi, and CRISPR-KO libraries (Lian et al. 2019). The complementarity of identified hits across the different libraries highlights the power of combining gene perturbation approaches.

The implementation of CRISPRa platforms in bacteria has been more challenging due to a lack of activation domains that remain effective when coupled synthetically with DNA binding domains and an even stronger sensitivity to the targeting position (Fontana et al. 2020b). CRISPRa was first implemented in *E. coli* using fusions of the RNA polymerase subunit ω (encoded by *rpoZ*) to dCas9 (Bikard et al. 2013). Upregulation relies on interaction between the complex and a σ70 promoter, resulting in a narrow targeting window, and while upregulation is strong for weaker promoters (23x), it is almost negligible for stronger promoters (1.4x). Similar to eukaryotes, repression is observed for some target sites—most notably when targeting immediately upstream of the −35 region (Bikard et al. 2013). Despite these limitations, the RpoZ system was successfully applied to study effects of gene perturbation on bacterial adaptation in *E. coli* (Otoupal et al. 2017) and to enhance production of anti-MRSA antibiotics in *Lysobacter enzymogenes* (Yu et al. 2018) and epothilone A in *Myxococcus xanthus* (Peng et al. 2018). In *B. subtilis*, it enabled a significant increase in amylase BLA production compared to the traditional promoter replacement strategy (Lu et al. 2019). Interestingly, the active window in *B. subtilis* was broader and further upstream relative to the TSS compared to *E. coli* (Bikard et al. 2013), suggesting optimal design rules may be host-specific even if the same effector is used.

To address the problem of a lack of effective activation domains, Dong et al. (2018) screened a broad set of candidate proteins for transcriptional activity in *E. coli* and identified several more potent activators—most notably the transcriptional regulator SoxS and the transposon effector TetD. SoxS is also a potent activator when fused directly to dCas12a with stronger target activation compared to RpoZ fusions across various TSS distances in *Paenibacillus polymyxa* (Schilling et al. 2020). Further optimizations, including an optimized scRNA for recruitment and a mutant SoxS with reduced binding to endogenous SoxS targets further improved efficacy while reducing off-target potential (Dong et al. 2018, Fontana et al. 2020a). Targeting requirements for the scRNA based SoxS system are the best studied to date (Fontana et al. 2020a). Activation is strongest for moderately weak promoters and gradually decreases with promoter strength, with no activation seen for very weak promoters. In addition, the sequence between the gRNA target site and the −35 region influences potency, possibly due to competition for binding with other factors (Fontana et al. 2020a). This suggests a strong sequence dependence even outside the gRNA base-pairing region. Importantly, the system is broadly active on promoters dependent on various σ factors, which is advantageous for genome-scale screening. Similar to RpoZ, optimal targeting is limited to a relatively narrow window, corresponding to −60 to −100 bp upstream of the TSS for SoxS (Dong et al. 2018, Fontana et al. 2020a). However, not the entire region allows efficient targeting due to strong periodic position-dependence with peak activities every 10–11 bp, corresponding to full DNA helix turns (Fontana et al. 2020a). Unfortunately, despite a better understanding of the factors that drive efficient upregulation, only three out of the seven endogenous genes targeted using these principles exhibited activation levels greater than 2-fold (Fontana et al. 2020a), indicating other factors remain to be elucidated.

A more recent and very promising system relies on the phage protein AsiA fused to dCas9 and was already introduced in *E. coli*, *Salmonella enterica*, and *Klebsiella oxytoca* (Ho et al. 2020). Direct fusion of wild-type AsiA to dCas9 has a broader targeting range than previous systems, with activation ranging from 2.5 to 12x upregulation. An evolved and more potent variant of AsiA can enhance upregulation of even strong promoters, achieving a 5-fold increase compared to 1.4-fold increase observed with RpoZ (Ho et al. 2020). The broader activity window and higher potency across target expression levels make this an interesting tool for genome-scale screening applications.

All systems described above are effective on σ^{70}-dependent promoters, which control most but not all *E. coli* genes. To also enable targeting of σ^{54}-dependent promoters, which include many stress-responsive genes, a CRISPRa platform relying on bacterial enhancer binding proteins (bEBPs) was developed. bEBPs interact with more distant upstream activating sequences (UAS) to activate expression of σ^{54}-dependent promoters (Liu et al. 2019b). The activator consists of a truncated version of the bEBP PspF from *E. coli*, which is tethered to the gRNA via an RNA binding peptide. While highly active on a synthetic construct (78x upregulation with a fully complementary compared to a mismatched gRNA), upregulation of endogenous *E. coli* and *K. oxytoca* promoters was more modest. As targeting is also restricted by the availability of a PAM near the UAS this system is potentially more limited in its application for control of endogenous genes. Genome-scale CRISPRa screens have yet to be performed in prokaryotes.

In summary, despite encouraging progress several limitations still complicate implementation of CRISPRa in all

microorganisms, particularly for genome-scale screening. Compared to CRISPRi, design of CRISPRa systems is more complex and difficult to generalize, as different activators are optimal for different dCas molecules, activation domains are organism- and even promoter-specific, and targeting windows are much narrower, particularly in prokaryotes. In most CRISPRi systems, repression is efficient in a relatively large window upstream of the TSS as well as in the beginning of (eukaryotes) or throughout the entire gene body (prokaryotes). However, with CRISPRa, an effective activator domain needs to be brought to a narrow targeting window upstream of the TSS or next to UAS for upregulation to occur. The stronger positional requirements with respect to distance from TSS also means that TSSs must be carefully identified for efficient gRNA design, which is information that is still lacking in many organisms and can differ between conditions. Consequently, PAM limitations are a major problem for CRISPRa but orthogonal dCas effectors and PAM flexible systems (Fontana et al. 2020a, Kiattisewee et al. 2022) can expand the targeting space. In addition, newer generation systems based on, e.g. AsiA are promising due to their less restrictive activation windows.

First-generation CRISPRa systems showed only very modest activity, which could be boosted by targeting multiple effectors to a single target. However, genome-scale screens require efficient perturbation of a target gene with a single gRNA. Fortunately, newer activators are substantially more potent, and additional improvements may be possible by emulating strategies used for optimization of CRISPRa in higher eukaryotes and with a better understanding of cellular factors that impact CRISPRa efficacy. A major limitation is a lack of reliable design rules that allow predicting the effect of even single gRNAs. Moreover, optimal design rules may vary for different nucleases, activators, and organisms and the dependence on promoter activity will complicate genome-scale screening. Nevertheless, as screens in *S. cerevisiae* show, CRISPRa tools can already provide substantially new insights into the genotype–phenotype space, and availability of more data will aid in the development of better prediction tools. It is of course feasible that predicting CRISPRa activity will always remain difficult due to the more complicated mechanism and effects, ranging from repressive to activating depending on position. However, while this can lead to false-positive hits, it also offers an opportunity to screen inhibition and activation with a single effector via varying gRNA design (Lu et al. 2019, Schilling et al. 2020).

The strong host dependence and variation in optimal windows between organisms also suggest that CRISPRa will be more difficult to translate to other microorganisms. In addition, while some systems do not require strain modification to achieve their highest levels of activation (e.g. AsiA or SoxS), others require host engineering to be fully functional, limiting their portability. For example, the native copies of *rpoZ* (Bikard et al. 2013) and *pspF* (Liu et al. 2019b) genes have to be deleted to avoid interference from competing endogenous functions.

These constraints come with additional ones, shared with CRISPRi, such as compensation of transcriptional perturbation by other cellular mechanisms, masking phenotypes, or the fact that not all genes respond to perturbation. For some genes, a strong activation is needed to observe a phenotype, and thus an optimized and powerful system must be developed. Off-targets are less studied for CRISPRa but given that targeting relies on the same mechanisms as for CRISPRi, similar issues regarding off-target gene repression and bad-seed toxicity will likely apply. In addition, off-target effects attributed to the activation domains need to be considered, although protein evolution can help to reduce these effects (Dong et al. 2018, Fontana et al. 2020a).

For all these reasons, CRISPRa platforms have been more difficult to implement than CRISPRi in microorganisms. However, some encouraging progress has already been made and more research will pave the way for using these systems more broadly for studying the genetic basis of various microbial phenotypes.

Direct transcript targeting via RNA-targeting CRISPR systems

Most CRISPR-based tools are built on Cas9 or Cas12 whose natural target is DNA. The discovery of novel types of Cas effectors that recognize and cleave RNA (types III and VI from classes 1 and 2, respectively) (Fig. 2) has spurred the development of a new generation of technologies that directly act on transcripts (Abudayyeh et al. 2016, Özcan et al. 2021, Xu et al. 2021a, Colognori et al. 2023, Wei et al. 2023). Since RNA and not DNA is targeted, type III and VI systems could overcome limitations of DNA-targeting CRISPR systems such as chromatin accessibility, a limited targeting window, polar effects, and the repression of all isoforms of a transcript. When RNA is targeted, the expression of other nearby gene products arising from bidirectional promoters, antisense transcripts or partially overlapping genes will also not be perturbed. This is important given the dense genomes of many microorganisms and recent findings showing that altering transcription at one locus can influence isoform length and expression levels of nearby transcripts (Brooks et al. 2022). Beyond these advantages RNA-targeting CRISPR systems open up new and unique capabilities for deciphering the role of noncoding transcripts and alternative RNA isoforms. We refer to Van Beljouw et al. (2023) for an in-depth review of the biological function and specific properties of different RNA-targeting CRISPR/Cas systems and will limit our discussion to a brief introduction of types that have been leveraged for programmable RNA degradation and their potential as genome-scale screening tools.

CRISPR/Cas13 systems

The type VI CRISPR/Cas systems are characterized by single protein Cas13 effectors that target RNA exclusively (Figs 1 and 2) (Abudayyeh et al. 2016, 2017, Cox et al. 2017, Konermann et al. 2018). For RNA cleavage activity, most Cas13 proteins require a protospacer flanking sequences (PFS), analogous to the PAM of DNA targeting systems (Abudayyeh et al. 2016, 2017). However, knockdown is efficient when the complex is directed to sites throughout the transcript (Wessels et al. 2020, 2023, Wei et al. 2023), increasing the chances that a suitable gRNA can be found. Targeting is mostly limited by target and gRNA structure and interaction, along with positional sequence preferences in the seed region (Wessels et al. 2020, 2023, Wei et al. 2023). The newly discovered effectors LwaCas13a (Cas13a from *Leptotrichia wadei*) or RfxCas13d (Cas13d from *Ruminococcus flavefaciens*) have no PFS-requirement, further increasing the targeting space (Abudayyeh et al. 2017, Konermann et al. 2018, Yan et al. 2018). Cas13 effector proteins, similar to Cas12a, can process their own gRNA arrays (Zhang et al. 2018c), making them suitable for multiplex applications. RfxCas13d is currently the most widely used Cas13 effector due to its robust and high knockdown efficiencies (Konermann et al. 2018, Mahas et al. 2019, Wessels et al. 2020). However, it is mostly used in organisms other than microorganisms, like mammals (Konermann et al. 2018, Kushawah et al. 2020, Ai et al. 2022, Li et al. 2023, Shi et al. 2023), fish (Kushawah et al. 2020), fruit flies (*Drosophila melanogaster*) (Buchman et al. 2020, Huynh et al. 2020, Ai et al. 2022) and plants (Mahas et al. 2019). This is largely due to the high cytotoxicity of RfxCas13d in

microorganisms (Yan et al. 2018, Meeske et al. 2019). Upon binding to its cognate target RNA and activation of target-specific RNA cleavage, Cas13 proceeds to indiscriminately cleave nearby RNA, termed *trans*-cleavage or collateral cleavage (Fig. 2) (Liu et al. 2017a,b, Zhang et al. 2018c, Slaymaker et al. 2019). This function is key for its role in bacterial immunity, where host transcript depletion eventually triggers cells to enter dormancy (Van Beljouw et al. 2023), and likely underlies its high toxicity in microorganisms. Collateral cleavage is also observed in higher eukaryotes but to a lesser extent (Ai et al. 2022, Li et al. 2023, Shi et al. 2023). Despite the downsides associated with collateral cleavage, Cas13 has been implemented for targeted RNA knockdown in *E. coli* (Abudayyeh et al. 2016, Yan et al. 2018, Zhang et al. 2020), *Corynebacterium glutamicum* (Zhang et al. 2020), and *Schizosaccharomyces pombe* (Jing et al. 2018), while both LwaCas13a and RfxCas13d appear nonfunctional in *S. cerevisiae* (Zhang et al. 2022). More recently, other highly efficient naturally occurring (Wei et al. 2023) and engineered (Charles et al. 2021, Kelley et al. 2022, Tong et al. 2023) Cas13 variants with reduced collateral cleavage have been described, but whether these variants perform better when they are tested more broadly and in other organisms is not yet clear. In summary, while promising due to their high efficacy, collateral cleavage currently limits the use of Cas13 for precise genome-scale RNA knockdown, particularly in microorganisms.

To avoid toxicity, a nuclease deactivated Cas13 effector (dCas13) can be used for translational regulation (Otoupal et al. 2017, Charles et al. 2021, Montagud-Martínez et al. 2023, Cardiff et al. 2024). Binding of dCas13 to the ribosomal binding site blocks translation by interfering with ribosomal binding (Charles et al. 2021, Montagud-Martínez et al. 2023) and achieves up to 95% reduction in protein levels (Charles et al. 2021). Like for CRISPRi/a, additional translational regulation domains can be fused to dCas13 for enhanced repression or activation. Interestingly, targeting the 5′ UTR of a gene with or without an activation domain can result in up to 16-fold increased expression (Otoupal et al. 2022), outperforming many CRISPRa systems. dCas13 enables single gene repression even for operon encoded genes (Cardiff et al. 2024), and activation of single genes is possible by combining CRISPRa to increase the expression of the whole operon with dCas13-mediated repression of the other operon-encoded genes (Cardiff et al. 2024).

Although dCas13 is a promising tool with no apparent toxicity, its high mismatch tolerance could result in off-target effects, similar to the bad-seed effect (Montagud-Martínez et al. 2023). Moreover, unlike CRISPRi/a, high levels of dCas13 are necessary for efficient knockdown, as mRNA is continuously transcribed. Consequently, dCas13-mediated knockdown is likely most efficient for transcripts with low transcription but high translation rates (Montagud-Martínez et al. 2023), which could skew results in a high-throughput screen. Finally, dCas13-mediated knockdown has so far only been demonstrated in *E. coli* (Charles et al. 2021, Otoupal et al. 2022, Montagud-Martínez et al. 2023, Cardiff et al. 2024), and further investigation is necessary to assess its broader applicability. In the meantime, other programmable RNA-targeting systems that do not suffer from collateral cleavage are potentially more interesting for genome-scale screening in microorganisms.

Type III RNA-targeting CRISPR/Cas systems

While class 1 CRISPR/Cas systems are typically characterized by multisubunit effector complexes (Makarova et al. 2015), the subtype III-E effector Cas7-11 is a single-protein effector capable of targeted RNA knockdown in *E. coli* and mammalian cells (Özcan et al. 2021). Cas7-11 consists of a Cas11 domain fused to four Cas7-like domains, originating from subtype III-D (Özcan et al. 2021). DiCas7-11 (Cas7-11 derived from *Desulfonema ischimotonii*) can both process its own gRNA array via the Cas7.1 domain and specifically target and cleave ssRNA via the Cas7.2 and Cas7.3 domains, making it an ideal tool for multiplexed RNA knockdown (Fig. 2) (Özcan et al. 2021, Kato et al. 2022). Efficiencies in *E. coli* vary between 15% and 60% when targeting a reporter, and no cytotoxicity was observed, but targeting of endogenous transcripts was not yet tested. Like RfxCas13d, DiCas7-11 has no apparent sequence preference for sites flanking the target site (Özcan et al. 2021). Importantly, Cas7-11 is less toxic than RxfCas13d in mammalian cells and shows both fewer off-targets and fewer differentially expressed transcripts (ca. 2.5x less), indicating reduced or no collateral cleavage (Özcan et al. 2021).

Although multisubunit effectors are rarely harnessed as tools due to their larger size and more complex composition, the well-studied biochemical and structural properties of type III CRISPR–Csm complexes (Tamulaitis et al. 2014, Guo et al. 2019, Jia et al. 2019, You et al. 2019) and the absence of collateral cleavage activity (Staals et al. 2014) make them highly promising as programmable knockdown tools. The type III-A CRISPR–Csm complex from *Streptococcus thermophilus* has already been successfully used in zebrafish embryos and human cells with knockdown efficiencies of up to 99% (Fricke et al. 2020, Lin et al. 2022, Colognori et al. 2023). Knockdown is variable for gRNAs targeting both the same and different transcripts, indicating that sequence- and/or structural, and possibly target-specific factors impact efficacy. More data is needed to elucidate these rules and leverage them for maximizing knockdown efficacy and, possibly, tunable knockdown. While CRISPR–Csm complexes naturally possess not only ribonuclease activity but also single-stranded DNase (Samai et al. 2015, Kazlauskiene et al. 2016) and cyclic oligoadenylate synthase activity (Kazlauskiene et al. 2017, Niewoehner et al. 2017), which are both key for their role in immunity, all activities are performed by independent components of the complex. This enables easy ablation of undesired activities that may globally interfere with cellular function without impacting degradation of the target RNA (Fig. 2) (Colognori et al. 2023). CRISPR–Csm has similar efficiency as RfxCas13d in human cells while affecting expression of significantly fewer nontarget transcripts (around 10-fold less), with no apparent cytotoxicity (Colognori et al. 2023). CRISPR–Csm has not yet been tested in microorganisms. However, the endogenous type III-B system of the archaeon *Sulfolobus solfataricus* was harnessed for targeted knockdown of a reporter transcript, achieving 2-fold reduction (Zebec et al. 2014). The study also highlights an alternative way of blocking type III DNA cleavage via gRNA design, without altering complex components. DNase activity is not activated when there is a perfect match between the 5′ end of the gRNA and the target, which is part of how type III systems discriminate between self and nonself (Manica et al. 2013).

Both Cas7-11 and CRISPR–Csm complexes are promising RNA-targeting CRISPR tools but require broader characterization of their on- and off-target characteristics, particularly in microbial cells. Although no cytotoxicity was apparent in mammalian cells, toxicity may be more apparent in microorganisms. As seen with Cas13, the effects of collateral cleavage may be more pronounced in microbial cells due to their smaller size (Bot et al. 2022, Vialetto et al. 2022). Currently, Cas7-11 is less efficient than RfxCas13d in mammalian cells (Wei et al. 2023) and *E. coli* (Özcan et al. 2021), but future optimization may address this issue. A perhaps more

important limitation is the large size of both Cas7-11 and CRISPR–Csm complexes, which may complicate their use in some organisms. However, recent studies show the possibility to reduce the size of Cas7-11 while maintaining on-target efficiency (Kato et al. 2022, Brogan et al. 2024), and, similar to CRISPRi, endogenous systems may offer an alternative if an orthogonal system is unsuccessful.

From gene to single nucleotide resolution

A key limitation of CRISPRi and its alternatives is the inability to assess more precise genetic perturbations, such as the effect of specific amino acid variants in proteins of interest or mutations in noncoding regions. This limitation can be overcome by leveraging the native DNA endonuclease function for gene editing or by employing engineered CRISPR-based editing agents that use effector fusions to deactivated Cas enzymes for making precise sequence changes. We will briefly introduce different CRISPR-based technologies for targeted genome modification and unbiased screening of DNA sequence variants in microorganisms, along with their limitations. We will limit our discussion to methods with strain-tracking capability via a barcode for measuring the functional impact of engineered mutations at scale and refer to other reviews for a discussion of genome-scale or single locus mutagenesis tools without strain tracking capability (Li et al. 2022, Lu et al. 2022, Zimmermann et al. 2024).

The first generation of CRISPR-based genome-editing tools leveraged the natural programmable nuclease activity of Cas effectors. DNA-targeting Cas nucleases like Cas9 and Cas12 induce a DNA DSB at sites specified by the gRNA (Jinek et al. 2012, Zetsche et al. 2015), which triggers recruitment of endogenous DNA repair factors to the break to repair the damage. This process can be exploited to install designed mutations via highly precise template-dependent repair processes (homology-directed repair, HDR) by providing cells with a DNA repair template (donor DNA) encoding a desired mutation flanked by homologies to the target site (Fig. 1) (Sander and Joung 2014). However, in eukaryotes repair preferentially occurs via religation of the break, most commonly via NHEJ, and often results in acquisition of short indels (Fig. 1) (Yeh et al. 2019). HDR is generally only active in dividing cells as it relies on proteins expressed in the S and G2 cell cycle phases, when homologous templates are available, and it is actively suppressed by NHEJ. In addition, NHEJ occurs much faster than HDR (Mao et al. 2008). Thus, while HDR-mediated editing of single loci is possible in many organisms, the generally low efficacy of this process has limited the implementation of genome-scale screening platforms in most species.

A notable exception is the yeast *S. cerevisiae*, which has a strong preference for DSB repair via HDR. Several high-throughput genome-editing methods have enabled measuring fitness effects of thousands of mutations in parallel across the yeast genome (Bao et al. 2018, Guo et al. 2018, Roy et al. 2018, Sadhu et al. 2018, Sharon et al. 2018). Analogous to a CRISPRi screen, cells are provided with a library of plasmids encoding a gRNA that targets Cas9 to a specific location, but each plasmid additionally contains a short (80–130 nt) donor DNA encoding the desired edit. Due to the mismatch tolerance of Cas effectors, the edited locus and donor are at risk of cleavage, as they differ from the wild-type sequence by only one nucleotide. To mitigate this risk, additional synonymous mutations can be encoded on the donor, and donors containing only the synonymous mutations can be included to confirm that the observed phenotype is due to the designed mutation. However, successful editing of single nucleotide variants is possible (Roy et al. 2018), demonstrating that additional mutations are not always necessary to prevent (re-)cleavage. To map mutations to cells, most methods use the plasmid-encoded gRNA-donor cassette as a barcode. While easy, this has several disadvantages. It requires keeping cells under plasmid selection, thereby limiting the conditions available for screening while also complicating the assessment of species that do not propagate plasmids stably. Due to plasmid copy number variation between cells, the resulting data is also noisier. To circumvent this problem, the barcode can be integrated into a dedicated genomic site during editing (Roy et al. 2018). As the barcode is only present in one copy per cell, this allows a one-to-one edit to barcode correspondence.

A difficulty in pooled editing is the growth-inhibitory effect of DSBs. Cells stop proliferating until a DSB is repaired, leading to a growth advantage of cells with weak or nonfunctional gRNAs over cells with potent gRNAs. As editing typically proceeds over many generations, this fitness difference results in a strong enrichment of wild-type cells in the pool after editing (Roy et al. 2018). It is difficult to address this challenge via design as we are not yet capable of efficiently telling good from bad gRNAs *a priori*. Moreover, nonfunctional gRNAs are inherently present in every library due to errors during oligo synthesis. To mitigate this problem, Roy et al. (2018) employ a strategy to directly recruit the donor repair template to DSBs, which improves HDR efficacy for weak gRNAs and reduces enrichment of nonfunctional gRNAs during editing. This strategy relies on specific protein–protein interactions at DSBs and may be difficult to translate to species where this interaction is not conserved. However, if similar specific interactions can be found, the approach could improve HDR editing efficacy and survival for other species. Editing efficiency and survival can also be improved by transforming linearized and partially overlapping plasmid parts that need to be assembled *in vivo* (Guo et al. 2018). The benefit may be similar as when synchronizing cells in late G2/S-phase prior to editing (Lin et al. 2014), selecting for cells in cell cycle stages where HDR components are expressed (Horwitz et al. 2015). CRISPR-independent HDR was increased in many fungi with a similar strategy (Tsakraklides et al. 2015). While useful for editing of single loci, this strategy may introduce skew and bottlenecks in genome-scale libraries for less HDR competent organisms as selection of transformants depends on HDR. Another possibility is to not only rely on the double-stranded DNA donors encoded on high-copy plasmids for editing but using a bacterial retron to generate many single-stranded DNA copies of the donor via reverse transcription (Sharon et al. 2018). This strategy increases the number of templates available for repair but as no direct comparison is made against plasmid-encoded donors, the benefit for gRNAs with varying efficacy and for survival is unclear. However, as retrons improve precision mutagenesis efficacy in *E. coli* and mammalian cells (Schubert et al. 2021, Lopez et al. 2022) they could prove broadly beneficial, although optimization of the retron architecture may be needed (Lopez et al. 2022). Finally, the fraction of precisely edited cells can be increased via genetic inactivation of NHEJ (Sadhu et al. 2018). While this strategy has been used in many organisms, it cannot prevent the enrichment of wild-type cells during editing and additionally comes with the risks of permanently inactivating an important cellular repair pathway.

Implementation of HDR-mediated precision editing tools is more challenging in organisms with lower HDR efficiencies. Low activity of HDR results in only a small fraction of the library being edited, while repair via alternative pathways, like NHEJ, would lead to most cells not carrying the intended mutation. These concerns have limited implementation in eukaryotic

microorganisms that preferentially use NHEJ, like *Y. lipolytica* and many other unconventional yeasts (Wagner and Alper 2016). Bacteria possess a more rudimentary NHEJ machinery compared to eukaryotes and NHEJ pathways are far less understood (Bertrand et al. 2019), and some bacteria, including *E. coli*, seem to lack NHEJ components entirely (Wilson et al. 2003). Despite generally less interference from NHEJ, achieving HDR efficacies compatible with genome-scale screening remains challenging in many bacteria due to the low activity of endogenous repair systems. Contrary to eukaryotes, Cas effectors are seen as a means to select correctly edited cells by inducing lethal DSBs in any unedited cells in bacteria (Jiang et al. 2013). Interestingly, one strategy to boost both survival and the fraction of cells with the desired edit is to adjust gRNA cleavage to the capacity for repair by native HDR systems (Collias et al. 2023, Liu et al. 2023). The underlying hypothesis is that weaker targeting leaves more time for repairing the DNA with the repair template or other copies of the genome. Less efficient cleavage prevents cutting of all genome copies, leaving some genomic DNA available for recombination and enabling bacterial survival, while repair via the donor abolishes retargeting and eventually drives accumulation of correctly edited cells in the population (Cui and Bikard 2016, Collias et al. 2023). These insights have been leveraged to design purposely less efficient (attenuated) gRNAs, for example via hairpins or mismatches. Attenuated gRNAs also improve editing and survival in other bacterial species (Collias et al. 2023), making them an interesting strategy for genome-scale precision editing screens in organism with inefficient endogenous HDR systems.

An alternative approach is the use of heterologous recombinases to assist repair. For example, combining the phage λ-Red recombinase system with a Cas-induced DSB significantly enhances the fraction of edited cells in *E. coli* (Jiang et al. 2013). Recombination is efficient with short 85–100 bp long donors, making the strategy compatible with oligo array synthesis. This was leveraged in the first microbial high-throughput editing platform, CRISPR-enabled trackable genome engineering (CREATE). CREATE edits single loci with efficiencies of 30%–100% in *E. coli* and is compatible with saturation and genome-wide editing (Garst et al. 2017). Enrichment of nonfunctional gRNAs is likely less problematic as wild-type cells are expected to be eliminated via Cas9 cleavage. However, the increased survival of cells with 'weaker' gRNAs described above could enhance library skew. Modified versions of CREATE were used to systematically identify temperature-sensitive alleles for essential *E. coli* genes (Schramm et al. 2023) and for saturation editing of essential bacterial proteins (Dewachter et al. 2023). As λ-Red improves editing in other bacteria, like *P. aeruginosa* (Chen et al. 2018a) or *Klebsiella pneumoniae* (Wang et al. 2018b), it could pave the way for large-scale efforts in these and other microorganisms. Other recombinases have also been combined with CRISPR/Cas and can improve HDR in species where λ-Red is not functional (Arroyo-Olarte et al. 2021).

In addition to low HDR activity, high activity of other cellular repair pathways can interfere with editing. In *E. coli*, methyl-directed mismatch repair (MMR) systems recognize certain DNA mismatches resulting from editing of the wild-type allele with high affinity and revert them back to the wild-type sequence. CREATE coinstalls synonymous mutations to prevent recognition by the MMR machinery but sacrifices single nucleotide resolution. Inactivation of MMR via knockout or temperature-sensitive mutants of MMR components *mutS* or *mutL* improves editing of single nucleotide variants but can increase the background mutation rate significantly (Costantino and Court 2003, Nyerges et al. 2014). Expression of a dominant-negative form of MutL (MutL-E32K) not only results in higher editing efficiencies in *E. coli* and other species, but also keeps background mutations low (Nyerges et al. 2016). Combining MutL-E32K with CREATE enables single nucleotide resolution screens in *E. coli* and could provide similar benefits in other microorganisms.

HDR-mediated repair of Cas-induced DSBs is only one strategy to engineer precise mutations. Newer approaches use the gRNA–Cas complex for targeting while the mutation is introduced by effectors fused to Cas. A major advantage of these approaches is that they do not introduce a DSB into the DNA and are hence independent of the HDR machinery. Base editors (BEs) are fusions of a catalytically impaired Cas effector to deaminases that catalyze the conversion of C•G to T•A base pairs (cytosine base editors, CBEs) or A•T to G•C base pairs (adenine base editors, ABEs) within a relatively small window in the target-gRNA binding region (Rees and Liu 2018). CBEs typically include a fusion to uracil glycosylase inhibitors (UGI) to prevent excision of uracil (resulting from T deamination), which increases editing efficiency and product purity. Some limitations of BEs, including the very narrow targeting window and low initial efficacy, have been partially addressed (Anzalone et al. 2020, Wang et al. 2021b), while others are more difficult. BEs rely on deamination reactions, confining the mutational spectrum to predominantly transition mutations. CBEs without UGI can catalyze conversion of T to other bases at low levels, somewhat increasing the mutational spectrum but simultaneously lowering product purity, and BEs containing both a cytidine and adenosine deaminase fused to a single Cas effector unite C–T and A–G editing capacities (Anzalone et al. 2020). BEs can further lead to a variety of undesired outcomes, which are particularly problematic for large-scale applications where edited cells cannot be screened carefully for presence of only the intended mutation. Unintended outcomes include bystander edits at target sites (when multiple C or A nucleotides are present), indels resulting from base excision repair and Cas-dependent or independent deamination at off-target DNA or RNA sites (Rees and Liu 2018). Bystander edits are less problematic if all As or Cs in target windows are converted all the time, but in reality a mixture of products is often generated, making it difficult to unequivocally assign a genotype to a gRNA barcode (Després et al. 2020, Schubert et al. 2022, Liu et al. 2022b).

Base-editing tools are established for many model and non-model microorganisms (Wang et al. 2021b, Li et al. 2022) but large-scale screens so far remain limited to CBEs and the microorganisms *S. cerevisiae* (Després et al. 2020, Schubert et al. 2022), *E. coli* (Gawlitt et al. 2024), and *C. glutamicum* (Liu et al. 2022b). Screens in *S. cerevisiae* with the CBEs Target-AID (Després et al. 2020) and BE3 (Schubert et al. 2022) show variable efficacies ranging from 0% to 90% editing, with many gRNAs producing a mixture of genotypes (single or multiple edits in the target window). Nevertheless, both screens enriched for mutations with stronger predicted deleterious effects, indicating BE screens can pinpoint relevant genes or positions in a protein, if not always the exact genotype (Després et al. 2020, Schubert et al. 2022). Similar conclusions were drawn for *E. coli*, but while the screen enriched for mutations in essential genes, an only modest depletion of the respective gRNAs indicates low efficacy (Gawlitt et al. 2024). Similar to HDR editing, Cas9-induced killing of nonedited cells after base editing can enrich for edited cells (Gawlitt et al. 2024). While gRNA design rules for base editing remain somewhat enigmatic, parameters like gRNA sequence and binding energy, positional and PAM sequence preferences, and target strand play a role (Després et al. 2020, Schubert et al. 2022, Gawlitt et al. 2024). More recent deep-learning based

models improve predictions for efficient base editing and mutational outcomes for different BEs in mammalian cells (Kim et al. 2024). Some of these parameters are likely conserved across organisms but, as for previously discussed tools, host-specific factors may need to be considered. Other factors, like a high spontaneous mutation rate, may complicate screening in some microorganisms (Liu et al. 2022b), particularly when designed mutations are installed with low efficiencies and strong selection is applied.

The more recently developed prime editors (PE) install edits via a reverse transcriptase using a specialized gRNA (pegRNA) that specifies the target site and serves as template for the desired mutation (Anzalone et al. 2019). PEs combine the advantages of HDR editing and BEs, as they can make all possible mutations but do not insert DSBs. While already widely used in higher eukaryotes, prime editing applications in microorganisms remain limited, and first efforts in *E. coli* showed very low efficacy (Tong et al. 2021, Zhang et al. 2024). In mammalian cells, continuous improvements to PE components (Chen and Liu 2023), including pegRNA design (Kim et al. 2021) and stability (Nelson et al. 2022), efforts to lower indel formation at target sites, and identification of host-factors impeding prime editing (Chen et al. 2021) have significantly improved efficiency and product purity and paved the way for genome-scale screens (Erwood et al. 2022). Similar efforts are on the way for microorganisms and will enable the more widespread use of PEs in these organisms. For example, while degradation of PE intermediates via exonucleases seems to be a major limiting factor for prime editing in *E. coli*, depletion of several exonuclease via dCas12a-mediated CRISPRi improves editing not only in *E. coli* but also in *K. pneumoniae* and *Acinetobacter baumannii* (Zhang et al. 2024). Prime editing, thus offers a highly versatile approach for precise manipulation of genomes with great promise for small- and large-scale investigation of specific mutations, yet it requires further characterization and optimization in microorganisms.

In summary, while genome-scale precision editing tools are currently only established in a few model microorganisms, the resulting technological advances and improved understanding of associated challenges will facilitate their implementation in other species. Different methods are available for programmable editing, each with their advantages and disadvantages, and which method is most appropriate depends on the organism and exact goal. HDR-based strategies and prime editing are less restricted by PAM availability and the types of variants that can be engineered with prime editing, offering the greatest flexibility. HDR-based editing tools currently still outperform prime and base editing in *S. cerevisiae* and *E. coli*, and some of the identified strategies for improving HDR and survival during editing in these microorganisms may enhance the efficacy of HDR editing in other species. BEs offer a powerful alternative for microorganisms with HDR systems that are inefficient or require long homology arms incompatible with library-scale synthesis. Base editing is still significantly more efficient than HDR in many organisms, and outperformed early prime editing systems, but recent improvements indicate prime editing may eventually be the method of choice for many applications and species.

Conclusion

CRISPR-based perturbation methods have revolutionized the field of genome-wide high-throughput screens to study genetic traits in microorganisms. Compared to other high-throughput gene-perturbation methods, CRISPRi is a simple, reversible technique that is cost- and time-efficient, and can be used to study both nonessential and essential genes. As CRISPRi technology has advanced, its full potential has increasingly been realized. We have explored various methods to simultaneously investigate multiple genes and to employ CRISPRi in both an inducible and titratable fashion. Additionally, the ongoing development of predictive models promises more robust knockdowns across the genome. While CRISPRi's applicability is not constrained by species, adapting CRISPRi screens for use in other nonmodel microorganisms presents ongoing challenges. Despite these hurdles, CRISPRi has significantly enhanced our ability to study microbial traits in a high-throughput setting. Recently, the advent of CRISPRa has introduced new capabilities for studying the genetic basis of various phenotypes in mammalian cells and *S. cerevisiae*, and recent advances should pave the way for its wider use in prokaryotes. Moreover, emerging techniques such as RNA-targeting CRISPR systems, and single-nucleotide resolution perturbation methods are proving to be either promising alternatives or provide complementary capabilities in genome-wide high-throughput screens. In summary, CRISPRi is readily adaptable for genome-wide high-throughput screens to dissect complex microbial traits, and its continued refinement along with the emergence of new methods will broaden the potential for biotechnological and industrial applications.

Acknowledgments

Figures were created with BioRender.com.

Conflict of interest: None declared.

Funding

The work was supported by the Research Foundation Flanders (FWO) (grant numbers G0B0420N and 11PT624N to S.F.); EOS—Excellence of Science (grant number 40007496); KU Leuven (grant numbers C16/17/006 and C16/23/007); and VIB.

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Received 8 May 2024; **revised** 19 July 2024; **accepted** 30 July 2024