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Design principles for nuclease-deficient CRISPR-based transcriptional regulators

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1. Abstract

The engineering of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated proteins (Cas) continues to expand the toolkit available for genome editing, reprogramming gene regulation, genome visualization, and epigenetic studies of living organisms. In this review the emerging design principles on the use of nuclease-deficient CRISPR-based reprogramming of gene expression will be presented. The review will focus on the designs implemented in yeast both at the level of CRISPR proteins and gRNA, but will lend due credits to the seminal studies performed in other species where relevant. In addition to design principles, this review also highlights applications benefitting from the use of CRISPR-mediated transcriptional regulation and discuss the future directions to further expand the toolkit for nuclease-deficient reprogramming of genomes. As such this review should be of general interest for experimentalists to get familiarised with the parameters underlying the power of reprogramming genomic functions by use of nuclease-deficient CRISPR technologies.

Keywords: CRISPR, dCas9, transcriptional regulation, gRNA, scRNA, dCpf1

2. Introduction

Living cells regulate gene expression through coordinated actions of DNA-binding transcriptional regulators, RNA polymerases and an arsenal of auxiliary co-activators (Hahn 2004). The complex network of the transcriptional machinery controls essential functions, such as cell differentiation, cell division, responses to environmental conditions, and metabolism. Our mechanistic understanding of the genes and pathways corroborating the timely and adequate execution of these essential functions have largely relied on functional genomics studies, often accommodated by efficient methodologies for accurate control of gene expression perturbations (Khalil *et al.* 2012; Si *et al.* 2015).

RNA interference, a post-transcriptional gene-silencing mechanism triggered by small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) formed from RNase III endonuclease-mediated degradation of double-stranded RNAs is one such methodology (Drinnenberg *et al.* 2009). By the use of iterative RNAi, knock-down of multiple genes related to chemical tolerance and production of heterologous metabolites have been optimized in microbes (Crook, Schmitz and Alper 2013; Si *et al.* 2015). Another method used to alter the expression of hundreds of genes, termed global transcription machinery engineering (gTME), relies on introducing mutant libraries of general transcription factors regulating promoter specificity and then screen for defined phenotypes followed by characterization and validation of the mutant context of the transcription factor and transcriptome analysis (Alper *et al.* 2006). Moreover, for targeted gene regulation, bottom-up engineering of synthetic transcription factors based upon hybrid zinc-finger (ZFs) proteins and promoters for orthogonal control of gene expression has elucidated the parameters of importance for coordinated, tuned, and spatial regulation of gene expression (Khalil *et al.* 2012). Taken together, the development of techniques for conditional loss-of-function studies by expression perturbations of multiple genes have proven to be important for our understanding of gene function, especially when studying the function of essential genes, and polygenic traits (eg. chemical tolerance). However, though the above-mentioned methods support the simple targeting of multiple genes for knockdown and overexpression, drawbacks are evident. This includes lack of specificity and limited regulatory potential (RNAi), the need to introduce synthetic genomic material (ZFs), or the need for a screening system to select for global transcriptional changes not imagined *a priori* (gTME).

Since 2013, the bacterial CRISPR–Cas system has inspired the rational development of orthogonal synthetic transcriptional reprogramming strategies founded upon RNA-mediated targeting of nuclease-deficient Cas proteins to predefined genomic loci (Larson *et al.* 2013; Qi *et al.* 2013). In brief, CRISPR-Cas systems are founded on an ancient bacterial adaptive immune system in which the CRISPR-associated protein (Cas) is guided to genomic loci by a guide RNA (gRNA) with 20 nt sequence-complementarity to the genomic target site (Jinek *et al.* 2012; Cong *et al.* 2013). From this platform, two basic systems have emerged for i) genome editing by the use of guide RNA (gRNA) directed endonuclease-mediated DNA double-strand breaks (DSB) to assist both gene knock-in and knock-out (Jinek *et al.* 2012; Cong *et al.* 2013), and ii) nuclease-deficient dCas-mediated transcriptional and post-transcriptional regulation, elucidation of epigenetic landscapes, and DSB-deficient base editing to name a few (Qi *et al.* 2013; Lenstra *et al.* 2015; Fu *et al.* 2016; Nishida *et al.* 2016; Cox *et al.* 2017). In terms of transcriptional regulation, the nuclease-deficient forms of the type II CRISPR-associated protein Cas9, termed dCas9, from *Streptococcus pyogenes*, has been acknowledged as a potent platform for reprogramming gene expression and genomic function. Basically, dCas9 is a Cas9 mutant which have had its nuclease activity ablated by mutations in the RuvC and HNH nuclease domains, while still maintaining DNA binding proficiency as programmed by gRNAs (Qi *et al.* 2013). Initially, it was demonstrated that dCas9 and a gRNA could mediate efficient gene repression in bacteria when dCas9 was guided to promoter proximal positions downstream the transcription start site, a mechanism coined CRISPR interference (CRISPRi)(Larson *et al.* 2013; Qi *et al.* 2013).

In more recent years it has become evident that compared with the above-mentioned conventional approaches for reprogramming genome function through non-native transcriptional regulators, nuclease-deficient variants of Cas9, and *Lachnospiraceae bacterium ND2006* Cpf1 are potent RNA-guided technologies for genome regulation in yeast. Specifically, the convenience, specificity, robustness, and scalability for endogenous gene activation and repression has been widely adopted (Gilbert et al. 2013; Farzadfard et al. 2013; Zalatan et al. 2015; Lian et al. 2017). Additionally, CRISPR-mediated transcriptional regulation is a powerful approach for targeted, combinatorial and tunable transcriptional reprogramming interface, especially considering the ease of synthesizing and expressing gRNAs without time-intensive genetic modification of host genomes of species recalcitrant to transformation and targeted genome editing.

In this review, the tremendous progress of CRISPR-mediated systems applied for reprogramming transcriptional regulation in yeast will be reviewed, including the expansive list of factors that influence gRNA efficacy, and the design principles for optimal reconfiguration of dCas9 and dCpf1. At the end of the review, future perspectives on the use of nuclease-deficient Cas proteins in combination with other complementary emerging technologies for reprogramming genome functions without the need for exogenous nuclease-activity will be highlighted. While this review will focus mostly on dCas9-mediated reprogramming of gene expression in yeast, a more host-agnostic review on nuclease-deficient CRISPR-dCas technologies has also recently been published (Mitsunobu *et al.* 2017).

3. CRISPR-based transcriptional regulation

3.1 Modulation of dCas9 activity

3.1.1. Regulation of CRISPR protein activity by protein fusions

Transcriptional regulators are by design globular. Most often regulators include two modular domains enabling i) DNA binding and ii) a regulatory domain supporting transcriptional activation or repression (Jensen *et al.* 2010; Khalil *et al.* 2012). Due to this modularity, domain-swapping experiments have proven successful for the generation of synthetic transcriptional regulators with defined DNA-binding specificities fused to various regulatory domains in order to potentiate transcriptional activation or repression of both native and synthetic promoters (Khalil *et al.* 2012; Folcher *et al.* 2013). The modularity of the regulatory domains has allowed the design of transcriptional regulators which can regulate gene expression to much higher levels compared to regulators only relying on the native design (Folcher *et al.* 2013).

When nuclease-deficient dCas9 was initially used in bacteria, gene repression by up to 99.9% was reported (Qi *et al.* 2013). However, when using only dCas9 and a single gRNA in yeast to target gene expression regulation, only modest repressions ranging from no effect to 2-3 fold repressions have been reported (Farzadfard, Perli and Lu 2013; Deaner, Mejia and Alper 2017; Vanegas, Lehka and Mortensen 2017), although a single study has reported up to 18-fold down-regulation of reporter gene activity (Gilbert *et al.* 2013). This level of regulation is comparable to studies in other eukaryotes, and suggest that the single gRNA complex with dCas9 is not sufficient for sterically hindering RNA progression and/or blocking of transcription initiation (Gilbert *et al.* 2013; Lawhorn, Ferreira and Wang 2014). Inspired by the modular design of other synthetic transcriptional regulators, and acknowledging that gRNA-bound CRISPR proteins are analogous to simple DNA-binding moieties, studies using dCas9- or dCpf1-mediated expression perturbations nowadays therefore include additional regulatory domains fused to dCas9 and/or dCpf1 in order to improve repression and activation potentials (Fig. 1a-b).

In their seminal study on dCas9-mediated transcriptional regulation in eukaryotes, Gilbert *et al*. compared the effect of fusing the mammalian transcriptional repressor domain, Mxi1, reported to interact with the chromatin modifying histone deacetylase Sin3 homolog in yeast, to dCas9 (Schreiber-Agus *et al.* 1995; Gilbert *et al.* 2013)(Fig. 1a). Targeting the *TEF1* promoter, dCas9-Mxi1 repressed reporter gene activity by 53-fold compared to the above-mentioned 18-fold using only dCas9. This finding is comparable to the effect recently reported in *Yarrowia lipolytica* (Schwartz *et al. 2017)*. Here, Schwartz *et al*. reported up to 10-fold repression of *MIH1* transcript levels when using dCas9, yet when directly comparing the effects of using dCas9 versus dCas9-Mxi1 on the *Ku70* and *Ku80* genes, related to non-homologous end joining, Schwartz *et al*. observed the highest level of repression (87%) for

Ku80 when the dCas9-Mxi1 fusion was compared to dCas9 (38%)(Schwartz *et al.* 2017). To further investigate dCas9 fusion designs for optimal repression, Schwartz *et al.* and Gander *et al*. (Gander *et al.* 2017) also tested fusions between dCas9 and the Krüppel-associated box (or KRAB domain) from tetrapod vertebrate genomes (Witzgall *et al.* 1994). Here Schwartz *et al.* found comparable levels of transcript abundances in the order of 2-3 fold repression for dCas9-KRAB as also observed for dCas9, while Gander *et al.* observed approx 2.5-fold repression for dCas9-KRAB compared to approx 12-fold when using dCas9-Mxi1 to control the expression of a synthetic *CYC1*-based promoter (Gander *et al.* 2017). These findings are also corroborated by mathematical models predicting that repression via dCas9 alone leaks more than repression via dCas9-Mxi1 (Gander *et al.* 2017). In addition to Mxi1 and KRAB, Gander *et al.* also tested repression domains GAL80, LUG, TPLRD1, TUP1, and XTC1 (Flick and Johnston 1990; Pierre-Jerome et al. 2014; Wu et al. 2001; Traven et al. 2002; Edmondson et al. 1996), with LUG and TPLRD1 showing similar repressing potential as KRAB, while neither GAL80, TUP1 and XTC1 fusions showed any repression (Fig. 1a). Similarly, Lian *et al.* (2017) tested variants of repressor domains TUP1, MIG1, CRT1, XTC1 and UME6 (Edmondson et al. 1996; Ostling et al. 1996; Zhang and Reese 2005; Traven et al. 2002; Kadosh and Struhl 1997), and reported a tri-partite repression domain engineered from UME6, MIG1 and TUP1 to be the most successful design for dCas9-mediated repression (up to 5-fold stronger repression compared to dCas9-Mxi1), whereas fusions to dCpf1 was not effective for CRISPRi (Lian et al. 2017).

In addition to fusion of repressor domains, several studies have worked on fusing CRISPR proteins with single and multiple transcriptional activation domains to allow for CRISPR-mediated gene expression activation, termed CRISPRa (Gilbert *et al.* 2014). In yeast, Farzadfard *et al.* were the first to show that dCas9 could be used as a transcriptional activator when fused to an activation domain (Farzadfard, Perli and Lu 2013). Here they initially tested dCas9-VP64 guided to either sense or antisense strand of the minimal *CYC1* promoter and found several positions of gRNAs enabling statistical significant upregulation of reporter fluorescence in the order of 1.5-3.0-fold (Farzadfard, Perli and Lu 2013). Similar fold-changes have been observed for dCas9-VP64 targeting the *GAL1* and *ADE2* promoters (Farzadfard, Perli and Lu 2013; Vanegas, Lehka and Mortensen 2017), while Naranjo *et al.*, reported >100- and >250-fold increases in transcript levels when using dCas9-VP64 and GAL4-dCas9-VP64, respectively, to target *FRM2* (Naranjo et al. 2015) Contrastingly, dCas9 did not increase reporter gene activity when guided by any of the tested gRNAs. In addition to testing dCas9-VP64 for CRISPRa, Farzadfard *et al*. also tested the potential of guiding multiple copies of dCas9-VP64 and thereby tune reporter promoter activity. From this, the authors observed that reporter gene activity increased by up to 70-fold when targeting dCas9-VP64 to a maximum of 12 identical operator positions using a single gRNA (Farzadfard, Perli and Lu 2013). One interesting observation, acknowledged already at this early stage of CRISPR-mediated transcriptional reprogramming, was the strong influence exerted by the position of the gRNA relative to the impact dCas9-based regulation. Specifically, Farzadfard *et al.* found that although dCas9-VP64 could serve as a transcriptional activator when gRNAs were positioned upstream of the TATA box, significant repression of reporter gene activity in the order of 2-3-fold was observed when the fusion protein was guided to positions overlapping or downstream of the TATA box (Farzadfard, Perli and Lu 2013). The gRNA positions-specific effects will be covered in more detail in section 3.2.

In addition to single-domain VP64, CRISPR proteinshave also more recently been successfully fused to combinations of transcriptional activators, including the VPR, which is constructed from quadruple copies of the Herpes simplex viral protein (VP16), the transactivation domain of NF-kB p65 subunit (p65AD), and the Epstein-Barr virus R transactivator (Rta)(Chavez *et al.* 2016; Deaner and Alper 2017; Jensen *et al.* 2017). As evidenced by Chavez *et al*., comparing reporter gene expression levels using gRNAs targeting the yeast *GAL7* and *HED1* promoters, dCas9-VPR mediated approx 100- and 40-fold upregulation, respectively, compared to the modest 14- and 8-fold increases observed when guiding dCas9-VP64 (Fig. 1b)(Chavez *et al.* 2015). Beyond the use of nuclease-deficient Cas9 from *S. pyogenes,* Lian *et al.* systematically tested novel CRISPR-mediated transcriptional activators by fusing several nuclease-deficient CRISPR proteins to activation domains (Lian et al. 2017). Here the authors found that the optimal activation domain was dependent on the Cas protein tested with the best-performing *S. pyogenes* dCas9 variant showing up to 12-fold activation of reporter gene activity when fused to VPR, while the best-performing dCpf1 variant induced up to 8-fold activation of gene activity when fused to VP64-p65AD (Lian et al. 2017).

Acknowledging the findings from dCas9 fusion studies, several groups have since then successfully applied dCas9-VPR for CRISPRa in yeast (Deaner and Alper 2017; Deaner, Mejia and Alper 2017; Jensen *et al.* 2017). Even though most upregulations observed are in the 2-10-fold range, Deaner *et al*. observed more than 160-fold changes in *NDE2* gene expression when comparing the best gRNA position for mediating CRISPRi using dCas9-Mxi1 versus the most potent gRNA position for mediating CRISPRa using dCas9-VPR (Deaner and Alper 2017). In alignment with the findings from Farzadfard *et al*., Deaner and Alper also reported the position-specific potential of dCas9-VPR to modestly repress gene expression (Farzadfard, Perli and Lu 2013; Deaner and Alper 2017).

Taken together, several studies have reported CRISPRi/a in yeast yielding changes in gene expression and activity in the order of >50-fold down-regulation and >100-fold upregulation, with dCas9-Mxi1 and dCas9-VPR currently being the most often adopted regulators. In general, dCas9 is a versatile fusion partner for both activation and repression domains, yet the optimal choice of regulatory domain(s) to be used for transcriptional reprogramming can depend on the CRISPR protein. This opens up opportunities for multi-functional CRISPR-mediated reprogramming using orthogonal PAM sequences of different CRISPR proteins as analogues for upstream activating or repressing sequences (UAS and URS, respectively)(Lian et al. 2017).

3.1.2. Regulating expression of genes encoding CRISPR proteins

When using nuclease-deficient CRISPR-based synthetic regulators, it is important to acknowledge that the regulatory potential of transcriptional regulators inherently depend on the expression level of the transcriptional regulator itself, with higher expression most often providing the highest repression and/or activation level of the target gene(s) in question (Skjoedt *et al.* 2016). In line with this, most studies in yeast make use of strong constitutive or glycolytic promoters to drive the expression of the gene encoding dCas9 and variants thereof (Gilbert et al. 2013; Lian et al. 2017). From *S. cerevisiae* this includes the *TDH3* (or GDP1), *TEF1* and *PDC1* promoters for use in *S. cerevisiae* and *Kluyveromyces marxianus* (Gilbert *et al.* 2013; Chavez *et al.* 2015; Smith *et al.* 2016; Nambu-Nishida *et al.* 2017), whereas in *Yarrowia lipolytica* Schwartz *et al.* used a previously engineered strong constitutive promoter based on a truncated core *TEF1* promoter fused to 8 copies of a 105-bp UAS element from the *TRX2* promoter, named pUAS1B8-TEF(136), to drive the expression of dCas9 (Blazeck et al. 2011; Blanchin-Roland et al. 1994; Schwartz et al. 2016; Schwartz et al. 2017).

In addition to constitutive expression of dCas9, the ability to program the onset of target gene regulation has prompted the use of inducible expression of dCas9 for conditional transcriptional reprogramming in yeast. By the use of a synthetic promoter originally developed by Ellis *et al*., galactose- and anhydrotetracycline-inducible (aTc) expression of dCas9-VP64 was achieved (Ellis, Wang and Collins 2009; Farzadfard, Perli and Lu 2013) allowing for 70-fold inducible expression of a minimal *CYC1* promoter with outputs comparable to the activation potential of other commonly used endogenous *GAL1* and *CUP1* promoters (Farzadfard, Perli and Lu 2013). Together with the complementary set of light- and allosterically regulated CRISPR/Cas9 systems reported in mammalian cells (Gao *et al.* 2016; Oakes *et al.* 2016), such galactose- and aTc-inducible expression of dCas9 and its variants allows control over the onset of target gene expression.

3.2. Modulation of gRNA activity

3.2.1. Recruitment of effectors by aptamer-fused gRNAs

The inherent one-to-one relationship between dCas9 and the gRNA constrains dCas9-mediated programming of multi-gene transcription-based gene circuits to only one direction of regulation (ie. repression or activation) at the single-cell level. This is not levelling the complexity and sophistication underpinning native transcriptional networks. However, in analogy to the fusion of regulatory domains to dCas9, the engineering of the gRNA itself has proven a modular and tunable platform for diversifying not only the genomic target sites (seed sequence), but also the function of CRISPR-mediated transcriptional regulation.

Taking advantage of the 3´-end of gRNAs, Zalatan *et al*. and Kiani *et al.* were the first to engineer gRNAs with protein-interacting RNA aptamers (Kiani *et al.* 2015; Zalatan *et al.*

2015). In yeast, this included gRNAs which indeed could control not only localization of dCas9 (and Cas9), but also function. In their seminal studies they showed that fusing RNA aptamers to the tracr-part of gRNAs enabled binding of RNA-binding proteins and thereby control of regulatory potential depending on the protein-interaction partner anchored to the RNA-binding protein (Fig. 2a)(Kiani *et al.* 2015; Zalatan *et al.* 2015). More specifically, in order to refactor both target sequence specificity and function into these scaffolding RNAs (scRNAs), Zalatan *et al*. tested i) different aptamers, ii) 5'- vs 3'-end fusions, iii) different numbers of aptamers, iv) linker length between gRNA 5'-end and aptamer, and v) orthogonality between aptamer and their cognate RNA binding interaction partners. The systematic characterization uncovered three potent RNA binding modules each consisting of the aptamer and its RNA-binding protein partner fused to either a VP64 activation domain or an Mxi1 repression domain (Fig. 2a). Moreover, the authors showed that several aptamers could be introduced into single scRNAs and no crosstalk was observed between the components of the RNA binding modules, ultimately enabling both dCas9-mediated activation and regulation in single cells only depending on the seed sequence and aptamer encoded in the scRNA(s) (Zalatan *et al.* 2015). Most importantly, when using the scRNA strategy together with VP64-based RNA-binding modules in yeast more than 50-fold activation of a synthetic reporter promoter was observed, compared to modest 2-3-fold activation observed for dCas9-VP64. Using two different scRNAs for targeted gene activation together with dCas9-mediated repression, Zalatan *et al.* enabled synthetic control over branchpoint fluxes in the violacein biosynthetic pathway (Fig. 2b), while Jensen *et al.*, demonstrated combinatorial reprogramming of mevalonate and carotenoid pathway genes using the MCP:VPR activation and PCP:Mxi1 repression modules, ultimately enabling significant changes in carotenoid levels (Fig. 2c)(Zalatan *et al.* 2015; Jensen *et al.* 2017).

In summary, the engineering of gRNAs into scRNAs offer CRISPR-based multi-directional reprogramming of gene expressions, and is of particular relevance for studying, and improving our understanding of, polygenic traits and combined effects of key metabolic pathway branch points.

3.2.2. Regulating gRNA expression

The expression levels of gRNAs have been shown to correlate with CRISPR/Cas9-mediated genome engineering efficiency in mammalian cells (Hsu *et al.* 2013). To match the stoichiometries of dCas9 or dCpf1 expressed from strong constitutive polymerase II promoters (see section 3.1.2.), optimizing the expression of gRNA and scRNAs have been investigated vigorously. In general, polymerase III promoters are used to drive expression of gRNAs because RNA polymerase II promoters add extra nucleotides to the 5′- and 3′-ends of gRNAs, and thereby are believed to interrupt gRNA function (Yoshioka et al. 2015). Originally, the polymerase III promoters *SNR52* and *RPR1* were adopted for constitutive delivery of gRNAs in yeast (Fig. 3)(DiCarlo *et al.* 2013; Farzadfard, Perli and Lu 2013; Gilbert *et al.* 2013). Especially, the use of *SNR52* promoter has been used extensively because of its native transcript cleavage sites that result in the excision of gRNAs from the primary transcripts (DiCarlo et al. 2013). Next, to enable larger flexibility to the design and expression strength of gRNAs, two studies immediately following the aforementioned studies on constitutive delivery of gRNAs, tested the fusion of self-cleaving hepatitis delta virus (HDV) and Hammerhead (HH) type ribozymes to the gRNA thereby enabling genome editing derived from polymerase II promoters (Gao and Zhao 2014; Ryan *et al.* 2014). Gao & Zhao were the first to highlight the use of ribozyme-flanked gRNAs to enable use of pol II promoters to drive expression of pre-gRNAs targeted for self-catalysed processing (Fig. 3)(Gao and Zhao 2014; Zhang *et al.* 2017). In addition to that study using the *ADH1* pol II promoter to drive the expression of gRNAs flanked by a 5' minimal hammerhead (mHH) and a 3' hepatitis delta virus (HDV) ribozymes at the 5' and 3' ends, respectively, Ryan *et al*., tested a total of eleven pol III promoters for delivery of functional gRNAs (Ryan *et al.* 2014). The study concluded that while tRNA promoters were compatible with the HDV ribozyme fusion yielding nearly 100% engineering efficiency, the snoRNA promoter *SNR52* was the only non-tRNA promoter levelling such efficiencies when fused to the HDV ribozyme (Ryan *et al.* 2014). These findings add to the more recent benchmark of synthetic pol III fusion promoters, pol II promoters (driving expression of ribozyme-flanked gRNAs (RGRs), and non-tRNA pol III promoters driving expression of gRNAs in *S. cerevisiae* and *Y. lipolytica* (Fig. 3)(Schwartz *et al.* 2016; Deaner, Mejia and Alper 2017). Here, expression levels were found to largely correlate with the engineering efficiency of the various designs, with the synthetic fusion promoters between truncated pol III promoters and tRNA promoters yielding the highest scores (>90%) in *Y. lipolytica*, while the strong pol II TEF1-RGR approach produced almost 4-fold more gRNA compared to SNR52 correlating with a stronger regulatory potential as well (Schwartz *et al.* 2016; Deaner, Mejia and Alper 2017). Also, Gander *et al.*, used the minimal *CYC1* promoter to build a set of gRNA-responsive Pol II promoters (pGRR) driving the expression of RGRs (Gander *et al.* 2017). In their study a library of 400 dual-target site gRNA-responsive polymerase II promoter (pGRRs) were constructed together with 20 RGRs totalling 8000 NOR (either one or both) logic gates, including both constitutive and estradiol inducible pol II promoters to drive the expression of RGRs, ultimately yielding up to 12-fold regulation from single gRNA controlled reporter promoters (McIsaac *et al.* 2013; Gander *et al.* 2017).

Apart from native pol III and inducible pol II promoters controlling the expression of gRNAs and RGRs, other groups have made use of an engineered native *RPR1* pol III promoter to include a TetO binding site for aTc inducible depression of gRNA expression when co-expressing the constitutively expressed TetR repressor thereby enabling expression perturbations in the order of 2-20-fold (Fig. 3)(Farzadfard et al. 2013; Smith et al. 2016; Jensen et al. 2017; Ferreira et al. 2018). Interestingly, in the study by Ferreira *et al.*, 3 gRNA cassettes were expressed from a single engineered *RPR1* pol III promoter, and subsequently the Csy4 endoribonuclease was used to digest the transcript into subelements and boost dCas9-VPR-mediated expression of *HMG1*, *OLE1* and *ACS1* promoters approx 2-fold (Ferreira et al. 2018). This elegant approach easily circumvents the need for re-use of the same promoter, or the need for multiple different promoters, when aiming to reprogram transcription of multiple genes (Fig. 3).

In summary, though native pol III promoters were originally the design of choice, the simple engineering of pol II promoters driving the expression of self-cleaving RGRs allows for control of genome reprogramming founded on basically any pol II promoter has gained attention (Zhang *et al.* 2017). Also, the sterical hindrance offered by inducible repressors can be used to engineer pol III promoters for functional, timely and potent gRNA delivery.

3.2.3. Multiple gRNAs for reprogramming of genomic functions

Regulating native and synthetic promoters by the use of endogenous or engineered transcription factors is dependent on their ability to bind cognate TF binding sites in such promoters (Khalil *et al.* 2012). In analogy to this, and as mentioned earlier (section 3.1.2.), Farzadfard et al. showed that synergistic effects on transcriptional regulation can be observed when using multiple gRNAs directing dCas9-mediated control of target promoters. For instance two separate gRNAs conferred each 2-fold repression, whereas a combination of the two showed 7-fold repression. Moreover, Farzadfard *et al*. also tested the guiding of multiple copies of dCas9-VP64 and thereby tune reporter promoter activity, and hereby observed that reporter gene activity increased by up to 70-fold when targeting dCas9-VP64 to a maximum of 12 positions (Farzadfard, Perli and Lu 2013). Likewise, Gilbert *et al*. tested 7x gRNAs on *TetO* promoter showing the highest ever reported repression in reporter gene activity by the use of dCas9-Mxi1 (153x, Fig. 1), while Deaner *et al.* used dual-gRNAs expressed from both *SNR52* and *TEF1* derived promoters to boost the regulatory potential of dCas9-VPR (Gilbert *et al.* 2013; Deaner, Mejia and Alper 2017). Contrastingly, Schwartz *et al.*, also used two gRNAs in the -120 bp transcription start site (TSS) region to test if this enhanced repression of *Ku70* and *Ku80*, yet they found only marginal effects from using two gRNAs compared to the perturbations observed when only using one gRNA (Schwartz *et al.* 2017).

Taken together, as in native and other synthetic transcription regulatory networks, the number of regulators tethered to the target regulon offer a modular valve to tune the impact of CRISPR-dCas9-mediated reprogramming. However, the use of multiple gRNAs should be carefully designed with particular focus on the position of existing regulatory elements and nucleosomes in order to tune regulatory potential by simple increases in gRNA numbers targeting such regions (see sections 3.2.5 and 3.2.6.).

3.2.4. Strand-bias vs regulatory potential

The mechanistic understanding of CRISPRi in relation to gRNA positioning has attracted a lot of attention. Initially, the underlying mechanism of dCas9-mediated transcriptional repression was elucidated using NET-seq in *E. coli* (Churchman and Weissman 2011; Qi *et al.* 2013). In *E. coli*, Qi *et al.* identified that gRNAs induced strong transcriptional pausing upstream of the gRNA target locus on the non-template strand, leading to the hypothesis that physical collision between the elongating RNA polymerase and the dCas9:gRNA complex conferred a transcriptional block (Qi *et al.* 2013). In yeast, however, Farzadfard *et al*. where the first to show that placing gRNAs at similar positions downstream TSS, but on different strands of a promoter, had similar negative effects on gene expression. Moreover, placing the gRNAs on either strand upstream the TATA box and the TSS lead to similar dCas9-VP64 mediated gene activation (Farzadfard, Perli and Lu 2013). Likewise, Gilbert *et al*. later reported that the targeted DNA strand and guanine-cytosine content of gRNA were not determining factors for successful CRISPRi in their study (Gilbert *et al.* 2014). Finally, in a more recent study, adopting a much larger gRNA library approach to deduce chemical-genetic interaction, Smith *et al.*, designed 383 gRNAs to the +500 bp to -500 bp of the TSS region window of 5 genes (Smith et al. 2016). Here the authors found no strand-bias in relation to gRNA efficacy along the 1 kb window tested.

In line with these findings, it has recently been further elucidated that, in contrast to the findings from CRISPRi in *E. coli* (Qi et al. 2013), dCas9 in yeast may not act as a simple transcriptional road-block mechanism for the RNA polymerase in a strand-specific manner, but rather that the gRNA:dCas9 complex supports the formation of a permissive transcript formations, including premature termination and formation of novel transcript, in both sense and antisense orientation (Howe *et al.* 2017). Taken together, this highlights that not only is yeast recalcitrant to potential CRISPRi strand-bias, but also that conclusions drawn from CRISPRi studies should consider the integrity of the transcripts targeted.

3.2.5. Position effects of gRNAs

In contrast to studies on potential strand-specific effects, there is much stronger evidence from bigger data sets on the position-specific effects of gRNAs in promoters.

In general, gRNAs targeting the region upstream of the TATA box and TSS have largely correlated with both dCas9-VP64- and dCas9-VPR-mediated gene activation, while positioning dCas9 variants downstream of, or in close proximity to, TATA boxes negatively impacts gene expression (Farzadfard, Perli and Lu 2013; Deaner and Alper 2017). For instance, targeting of dCas9-VP64 to a position upstream the TATA box provided almost 5-fold upregulation of a minimal *GAL1* promoter, while targeting gRNAs to the TATA box or the kozak element downstream thereof led to CRISPRi, likely due to interference with the transcriptional initiation complex, as also observed by Deaner *et al*. when using dCas9-VPR for CRISPRi (Figure 1)(Farzadfard, Perli and Lu 2013; Deaner, Mejia and Alper 2017). Moreover, Deaner and Alper provided a detailed study on the systematic testing of enzyme perturbation sensitivities (STEPS) by positioning gRNAs in an approx 0-750 bp window upstream the TATA box of various native yeast promoters. By observing changes in gene expression as dCas9-Mxi1 is positioned further away fromthe TATA box and dCas9-VPR is positioned closer towards the TATA box, the authors were able to infer flux sensitivity maps by plotting changes in glycerol formation as a function of the 5 genes' graded expression (Deaner and Alper 2017). Application-wise, the authors used STEPS to show that *GPD1* and *TPI1* gene expression levels positively and negatively correlate with glycerol titers, respectively. Ultimately, these interrogations lead to a simple over-expression strategy for

GPD1/*GPP1* yielding more than 5-fold increase in glycerol titers (4.89-28.0 g/L). Likewise, using STEPS on 5 key pentose phosphate pathway genes to increase flux through the aromatic amino acid pathway yielded approx 8-fold increase in 3-DHS titers (to 126.4 g/L) in a *zwf1* deletion background (Deaner and Alper 2017).

The abovementioned studies on gRNA position effects are largely corroborated by another recent study. Here, Smith *et al*. used CRISPRi based on dCas9-Mxi1 to test approx. 1,000 gRNAs directed against 20 genes whose expression levels are predicted to influence sensitivity to specific growth inhibitors (Smith *et al.* 2016). Here, the authors found that the median guide effect for dCas9-Mxi1 was maximal in the window of -200 bp to TSS, while gRNAs positioned outside the -200 bp to TSS window only in some cases could effectively repress transcription, but less effectively (Smith *et al.* 2016). These findings differ from the studies performed in mammalian cells in which the -50 to +300 region relative to TSS was found to be the most impactful for CRISPRi (Gilbert *et al.* 2014). Still, for yeast, Smith *et al.* developed a tool for gRNA design (http://lp2.github.io/yeast-crispri/) taking into considerations both genome position, chromatin accessibility (section 3.2.6.), nucleosome (section 3.2.6), gRNA length and sequence (3.2.7.), as well as transcription factor occupancy of the target site (3.2.8.)(Smith *et al.* 2016). Based on these findings and others, Schwartz *et al.* identified gRNAs for efficient repression of gene expression in *Y. lipolytica* (Schwartz *et al.* 2017). In the largest-to-date study, Smith *et al.* targeted dCas9-Mxi1-mediated repression of >1,500 genes essential for growth (Smith et al. 2017). By analysing >9,000 strains containing a unique sequence-verified gRNA, the authors refined their earlier findings (Smith et al. 2016), now highlighting gRNA positions in the region between TSS and approx. 125 bp upstream TSS to be particularly effective for CRISPR-mediated repression (Smith et al. 2017).

Having this said, , even though Jensen *et al.* targeted 88 gRNAs to the -200 bp to TSS window of 12 native yeast promoters, the authors found several gRNAs to be non-functional when using dCas9-VPR and dCas9-Mxi1 for transcriptional reprogramming (Jensen *et al.* 2017).

Summarizing, the positioning of gRNAs relative to TATA and TSS offers a easy tunable and portable strategy to perturb gene expression activity for both CRISPRi and CRISPRa, thoughspecific positioning should also take into consideration other local sterical and regulatory features of eukaryotic promoters (see sections 3.2.6. and 3.2.7).

3.2.6. Nucleosome positioning and chromatin accessibility

Nucleosomes have been shown to effectively interfere with the action of DNA binding transcriptional regulators (Griesenbeck *et al.* 2003; Mao *et al.* 2011). CRISPRsystems, inherently relying on DNA binding, have been used widely in the eukaryotic kingdom, but unlike bacteria, DNA in eukaryotes is largely coiled around histones to form nucleosomes, making eukaryotic DNA more tightly packaged and less accessible to other DNA-binding proteins. As reviewed above, gRNAs targeting the same promoter can have differences in their transcriptional impact (Smith *et al.* 2016), even gRNAs positioned closely can have different efficiencies not strictly correlating with their distance from TSS (Farzadfard, Perli and Lu 2013; Jensen *et al.* 2017; Vanegas, Lehka and Mortensen 2017). This led Smith *et al.* to investigate whether chromatin accessibility and nucleosome positioning could also impact a guide's efficiency for dCas9-mediated transcriptional regulation. In analogy with transcription factors canonically binding nucleosome-free DNA within promoters crucial to the regulation of gene expression, Smith *et al.* took advantage of the study Schep *et al*. recently performed in which they identified a highly structured pattern of DNA fragment lengths and positions around nucleosomes in yeast using an assay of transposase accessible chromatin (ATAC-seq). Using the ATAC-seq data together with other genome-wide nucleosome position datasets (Lee *et al.* 2007), Smith *et al.* found a positive correlation between guide efficiency and chromatin accessibility scores in the TSS -400 bp to TSS +400 bp window. Even though studies have shown that gRNA positioning downstream TSS can be effective for transcriptional reprogramming (Farzadfard, Perli and Lu 2013; Deaner and Alper 2017), Smith *et al.* observed from testing hundreds of gRNAs that the relationship between guide efficiency and ATAC-seq read density extended into the typically nucleosome-occupied region downstream TSS (Yuan *et al.* 2005; Lee *et al.* 2007; Zaugg and Luscombe 2012), underpinning the notion that gRNA efficacy is not *sensu stricto* determined by it's TSS proximity. These observations are in line with biochemical studies showing that Cas9 and dCas9 cannot stably interact with a PAM when located in the nucleosome core, indicating PAM accessibility to be the critical determining factor for nuclease-deficient CRISPR protein activity (Hinz, Laughery and Wyrick 2015; Isaac *et al.* 2016), which again underpins the observation that guides which target regions of low nucleosome occupancy and high chromatin accessibility are likely to be more effective (Smith et al. 2016; Smith et al. 2017). Moreover, in human cells, several reports have highlighted that locations for efficacious gRNAs for dCas9-mediated transcriptional repression correlate with chromatin marks associated with active transcription and open chromatin (H3K27ac, H3K9ac, H3K4me3, H3K4me2 and H3K79me2)(Horlbeck *et al.* 2016; Radzisheuskaya *et al.* 2016).

Taken together, biochemical and *in vivo* evidence suggest that gRNA design strategies should avoid targeting gRNAs near the nucleosome core. Moreover, since several data sets exist on large scale nucleosome positioning and DNA accessibility maps (Jiang and Pugh 2009; Schep *et al.* 2015), development of future computer-aided design tools for design of specific and highly efficient gRNAs should evaluate the inclusion of such data sets when inferring gRNA selections.

3.2.7. gRNA specificity and length

The length of the gRNA is a crucial factor for target-specificity of nuclease-proficient Cas9, with 17 nt gRNAs observed to be the minimum length for targeted nuclease activity (Fu *et al.* 2014b). For CRISPRi and CRISPRa, several studies have assessed the impact of truncated gRNAs compared to full-length 20 nt spacer regions of gRNAs. Initially, Qi et al. found that for CRISPRi the strongest repression was observed when using full-length gRNAs, which is corroborated by Kiani et al. who found that dCas-VPR-mediate activation increase from 2- to 100-fold activation when seed length is shifted from 8 nt to 20 nt (Kiani *et al.* 2015). Likewise, in yeast Smith *et al*., have found that mismatches located in the seed region positioned 1-10 relative to the PAM were poorly tolerated by both full-length and truncated gRNAs (Smith *et al.* 2016), which is also in agreement with findings from Cas9-targeting *in vitro* and in mammalian cells (Hsu *et al.* 2013; Fu *et al.* 2014a; Wu *et al.* 2014), and the observation that as little as a single base-pair mismatch is sufficient to redirect dCas9 targeting in yeast (Farzadfard, Perli and Lu 2013).

In general, the conclusions drawn from these studies suggests that truncating gRNAs reduce the efficacy of CRISPR-dCas9-mediated transcriptional regulation towards both perfectly matched and imperfectly matched target sequences compared to 20 nt full-length gRNAs (Kiani *et al.* 2015; Smith *et al.* 2016), though there is some degree of flexibility in the design of the seed-distal positions of gRNAs which may be considered when designing gRNAs targeting promoter regions dense in nucleosomes and upstream-activating sequences.

3.2.8. Other features of relevance - basal promoter activity, TF binding interference, and RNA secondary structure

In the previous sections, some design principles stand out as being of particular importance for efficient CRISPR-mediated transcriptional reprogramming. For gRNAs, this includes i) the positive correlation between gRNA expression level and engineering efficiency (Deaner et al. 2017; Schwartz et al. 2016), ii) targeting of gRNAs to the window between -125 bp upstream TSS and TSS for CRISPRi, and iii) positioning of gRNAs in nucleosome-depleted regions of target promoters (Smith et al. 2016; Smith et al. 2017). In addition to these design criteria, a few additional studies deserves to be mentioned for designing optimal CRISPR-mediated probing of genome function.

First, when selecting genes of interest it is worth considering the observed inverse relationship between basal expression levels of the genes of interest and the relative expression perturbations which can be gained by dCas9-mediated reprogramming (ie. high basal expression can often only be marginally activated and *vice versa*)(Chavez *et al.* 2015; Jensen *et al.* 2017). In line with this, another factor of interest is related to the regulatory organization of the targeted promoter(s). On the use of dCas9 to block the DNA-binding of the synthetic transcriptional regulator rTA on the synthetic TetON-Venus reporter promoter, Gilbert *et al*. found that a 115-fold repression of rtTA-induced activation can be obtained when co-expressing dCas9 and gRNA, suggesting that dCas9 can sterically compete with transcription factors otherwise controlling the regulation of the target promoter, indicating that CRISPRi and CRISPRa can be used to identify regulatory functions of upstream-activating and upstream-repressive sequences (Gilbert *et al.* 2013). However, from their large-scale library approach, Smith *et al*. (2016) only found a small number of cases where overlap with a transcriptional activator binding site correlated with increased CRISPRi efficacy, indicating that this design parameter may be subject to the native regulatory context of the targeted promoters. In relation to this, Jensen *et al.* showed CRISPR-mediated up- and down-regulation of gene activity of *OLE1* over the course of 48 hrs, correlating with time-resolved quantitative analysis demonstrating that *OLE1* is highly expressed during early-phase to mid-exponential phase and downregulated from late exponential phase (Jensen et al. 2017). Finally, another important gRNA design principle to mention comes from the before-mentioned large-scale CRISPRi study performed by Smith *et al.* (2017). Here the authors identified a significant correlation between the folding energy in kCal/mol for the predicted RNA structure (leader, 20nt gRNA targeting sequence, and structural part) of the gRNA and the gRNA efficacy (ie. more folding, less efficacy)(Smith et al. 2017).

Taken together, numerous design parameters have been elucidated for optimal CRISPR-mediated transcriptional regulation. Several of the parameters are defined from large-scale studies and considered to be gene-inspecific. Likewise, as evident from several studies, CRISPR-mediated regulatory potential of target promoters can be sustained over long time-spans (Jensen et al. 2017; Deaner and Alper 2017), highlighting the robustness and orthogonality of the technology.

4. Outlook

As is evident from the previous sections, there are many design considerations to be taken into account when using CRISPR to probe genome functions through CRISPRi and CRISPRa (Textbox 1). Still, for transcription perturbations, compared to other methods such as RNAi, gTEM and targeted overexpression, CRISPRi/a offer easy design, programmable RNA-mediated targeting, and regulatory direction of both individual and multiple genes at the single cell level. This is powerful and leverages the nature of multi-factored native transcriptional regulation for transcription perturbations. Indeed, for transcriptional reprogramming, dCas9-based approaches have been used to quickly assay metabolic pathway dynamics and elucidate rate-limiting enzymatic steps without the need for genome editing (Zalatan *et al.* 2015; Deaner and Alper 2017; Jensen *et al.* 2017). Also, single sets of transformation experiments (multiplex) can be easily implemented, and the one-time synthesis of gRNA sets allows rapid progression through iterative engineering cycles, namely by quickly assessing the combinatorial effects of expression perturbations in order to identify primary and secondary targets which could not be known *a priori* from single gene expression perturbations.

However, though several CRISPR proteins and gRNA versions have been tested in large-scale studies in yeast, the relative expression changes observed when using dCas9-mediated transcriptional regulation are still often observed to be at least an order of magnitude less than those observed for bacterial and mammalian re-programming efforts, often in the 100-20000-fold (Qi *et al.* 2013; Chavez *et al.* 2015), whereas highest transcript changes reported in yeast are approx. 100-250-fold .(Chavez et al. 2015; Gilbert et al. 2013; Naranjo et al. 2015) In order to improve the regulatory potential of CRISPR-dCas9 in yeast and to further potentiate the toolkit available for probing genome functions, there is still a need for further development of reprogramming technologies.

One new-in-class CRISPR technology of relevance for functional genomic studies, was recently reported using orthologs of nuclease-proficient and -deficient RNase Cas13 from Type VI CRISPR-Cas systems, which can be guided by single-effector gRNAs to target more than 70% post-transcriptional knock-down of gene expression in mammalian and plant cells with high target specificity (Abudayyeh *et al.* 2017; Cox *et al.* 2017). Also, the Zhang laboratory showed that dCas13 could be fused to enzymes of the adenosine deaminase acting on RNA (ADAR) family and thereby enable RNA editing (Cox *et al.* 2017). As such, RNA-targeted dCas13 is believed to advance functional genomics at the post-transcriptional level supporting functional studies, e.g. mRNA splice variants, base editing at the RNA level, and elucidating mRNA processing by way of dCas13 variants fused to regulatory domains, akin the design principles of dCas9 variants.

Also, though distinct from CRISPR, it should be mentioned that Barbieri *et al.* recently reported that silencing of yeast DNA repair machinery and slowing of replication enhances multiplex genome editing by 90-nt single-stranded oligodeoxynucleotides (ssODNs) in yeast, thereby enabling simultaneous integration of more than 10 ssODNs with up to 60 mutations pr transformation (Barbieri *et al.* 2017). Most importantly, this strategy is both independent of DNA double strand breaks and homologous recombination, and it should be possible in the near future to combine the multi-loci and single-base pair resolution of this approach with CRISPR-dCas9-mediated transcriptional reprogramming for fast-track identification of genome and expression imprints related to desired traits.

Finally, native transcriptional regulation rely on integrate multi-gene spatio-temporal expression perturbations. To further enable synthetic and on-demand transcriptional control of polygenic traits, especially those dependent on essential genes, research within controllable CRISPR systems should take advantage of, and further develop, reprogramming strategies compatible with optogenetics, thereby circumventing the limited reversibility of the chemical-induced (eg. aTc) systems (Xiaofeng *et al.* 2017). Likewise, allosteric regulation of CRISPR protein activity should be considered for conditional switching of cellular decision-making, e.g. growth and metabolic states (Oakes *et al.* 2016). Ultimately, such techniques are envisioned to dramatically support our understanding and orthogonal control of transcriptional and post-transcriptional regulations for desired cellular and metabolic outputs.

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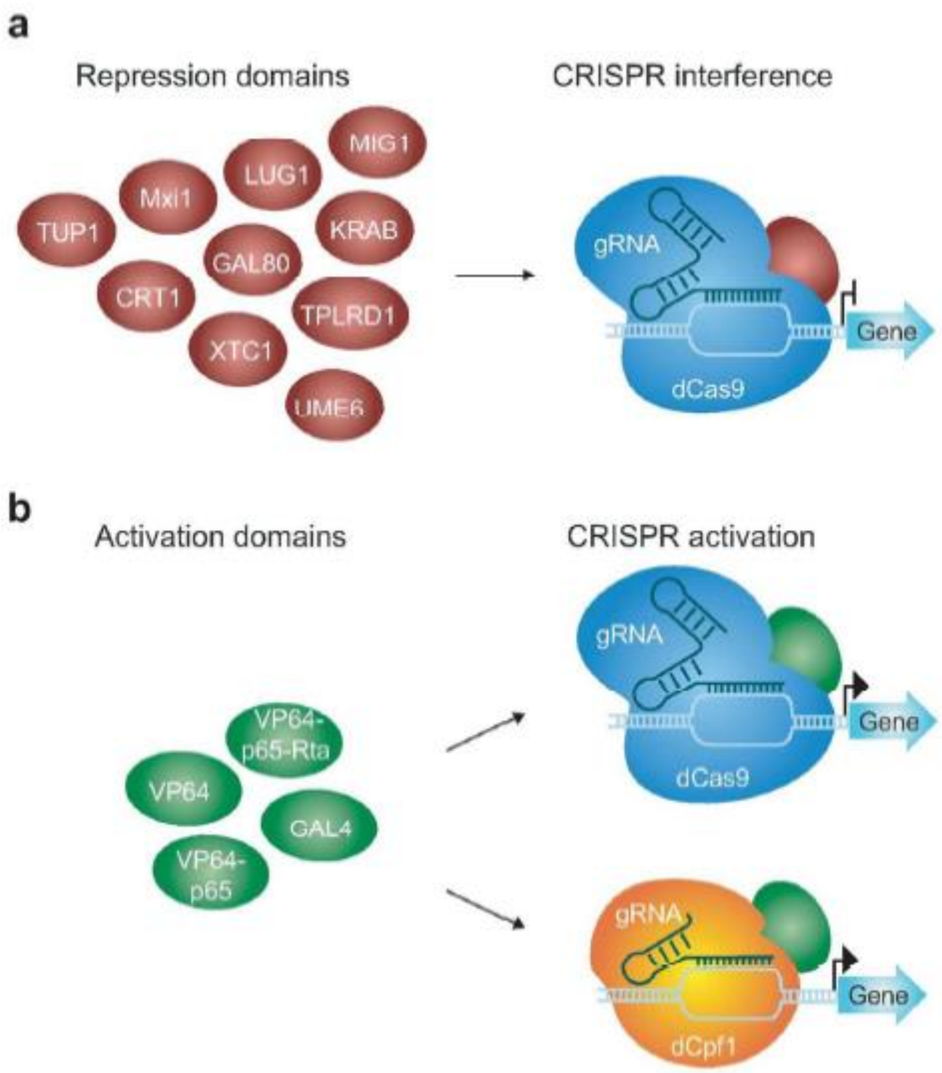

Figure 1. Modulation of nuclease-deficient Cas9 and Cpf1 activities in yeast by fusion of transcription regulatory domains. a) Schematic illustration of the transcriptional repression domains which have been successfully fused to nuclease-deficient dCas9 CRISPR activation in yeast. **b**) Schematic illustration of the transcriptional activation domains which have been successfully fused to nuclease-deficient dCas9 and dCpf1 for CRISPR activation in yeast.

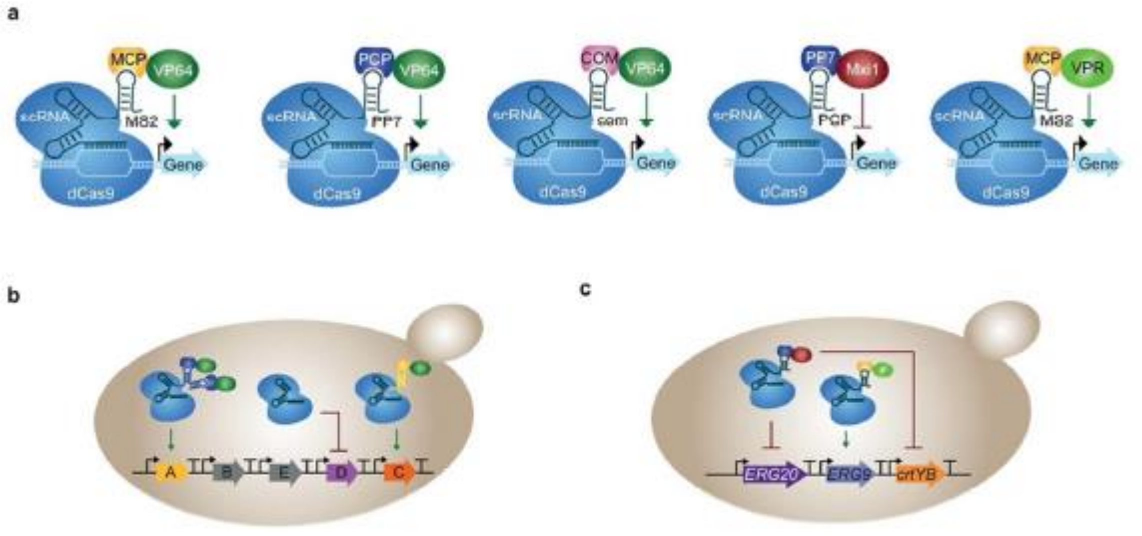

Figure 2. Design and application of scaffold RNAs controlling both genomic target sequence and regulatory function. a) Five examples of scaffold RNAs (scRNAs) used in yeast. ScRNAs are engineered gRNAs with protein-interacting RNA aptamers. The protein and aptamer is collectively referred to RNA binding modules. The aptamer-binding protein MCP, PCP, and COM interact in an orthogonal manner with the aptamers MS2, PP7 and com, respectively. MCP, PCP, and COM can be fused to activation or repression domains, thereby enabling scRNAs to specify genome target locus and regulatory function. **b**) An example illustrating single-cell reprogramming of the expression of three genes encoding part of the violacein biosynthetic pathway. **c**) An example illustrating single-cell reprogramming of the expression of three genes encoding proteins regulating metabolic flux through the mevalonate and carotenoid pathways.

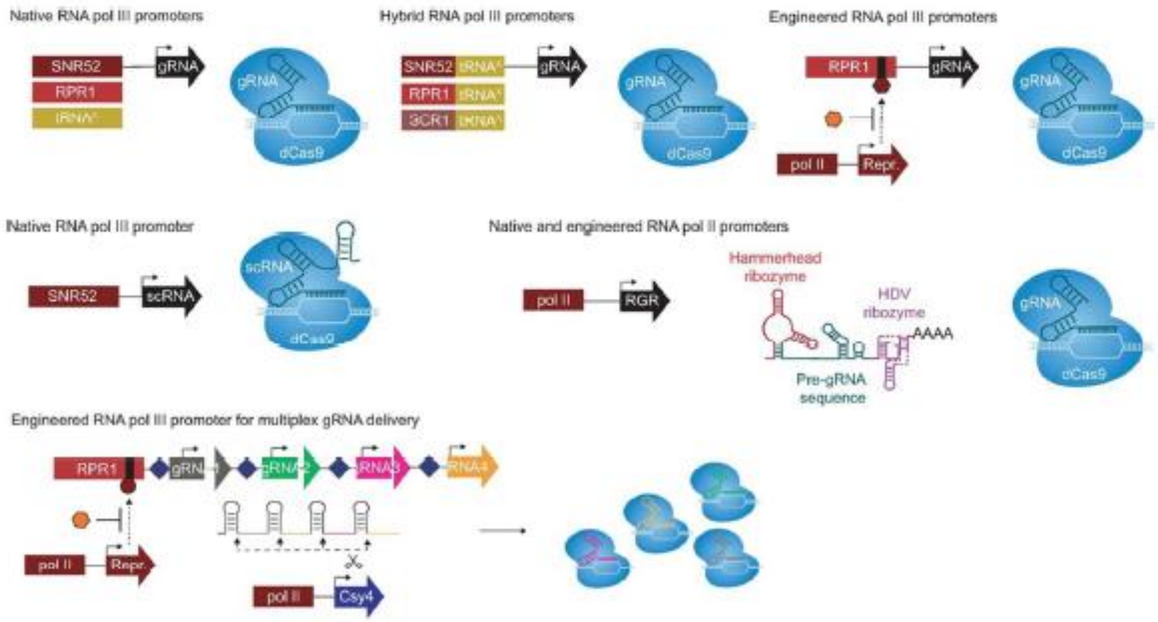

Figure 3. Expression of guide RNAs, scaffold RNAs, and ribozyme-flanked guide RNAs. Examples of native, hybrid and engineered promoters reported to drive the expression of guide RNAs, scaffold RNAs, and ribozyme-flanked guide RNAs in yeasts.