Next-Generation CRISPRi and CRISPRa Libraries

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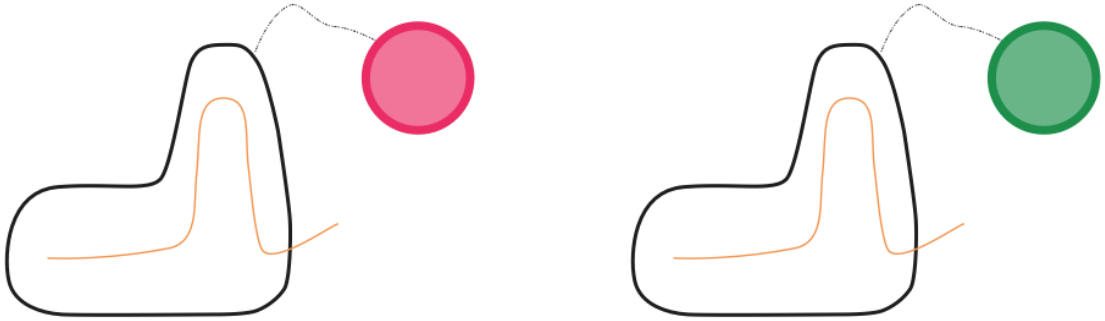

A longstanding goal in biological research is to modify gene expression without permanently disrupting the DNA sequence. This type of genetic manipulation avoids the central conceit of programmable nucleases like TALENs, ZFNs and Cas9: cytotoxic indel mutations associated with NHEJ-mediated repair of double-stranded breaks. Researchers have fused catalytically “dead” nucleases like dCas9 to effector proteins, offering a new perturbation capability beyond DNA cutting and repair. The sequence-specific nature of proteins like dCas9 means they target and bind to specific genomic loci even if their catalytic activity is deactivated.

Non-permanent editing relies on functional regulatory proteins called effector domains. These are fused to deactivated programmable nucleases and either block transcriptional machinery from accessing the target gene or alter chromatin 3D conformation by modifying histones — all without cutting the DNA strand (Thakore et al. 2016). The first effector system was first tested in TALENs by Maeder et al. in 2013 and has since been adapted to CRISPR in what is known as CRISPR interference (CRISPRi) (Larson et al. 2013). Furthermore, dCas9 can be fused to transcriptional activators for gene upregulation in a system called CRISPR activation (CRISPRa). The technology has since been expanded into effector constructs in a wide range of applications (**Table 1**).

APPLICATION	EFFECTOR	REFERENCE
GENE REPRESSION	dCas9, KRAB	Gilbert et al. 2013, Mandegar et al. 2016
GENE ACTIVATION	VPR	Guo et al. 2017
DNA METHYLATION	DNMT3A	McDonald et al. 2016
GENOME-WIDE ACTIVATION SCREEN	SunTag	Gilbert et al. 2014
PHOTO-INDUCED ACTIVATION	VP64 with interacting CRY2 and CINBN	Polstein & Gersbach 2015
CHEMICAL-INDUCED ACTIVATION	VP64 with interacting FKBP and FRB	Zetsche et al. 2015
NONCODING DNA/RNA ACTIVATION	SAM	Konnerman et al. 2015
FLUORESCENT GENOMIC IMAGING	EGFP	Chen et al. 2013
PROTEIN INTERACTION ANALYSIS	Antibody tag	Fujita & Fujii 2014

Table 1. *Recent practical applications of CRISPRi/a. hiPSCs = human induced pluripotent stem cells. Effector protein domain abbreviations: KRAB = Krüppel associated box, DNMT3A = de novo methyltransferase 3A, EFGP = enhanced green fluorescent protein, CRY2 = Cryptochrome Circadian Clock 2, CINBN = N fragment of calcium and integrin binding protein 1, SAM = synergistic activation mediator, FKBP = FK506 binding protein, FRB = FKBP rapamycin binding domain*

Importantly, CRISPRi/a mediated gene editing show increased efficiency and minimal off-target effectscompared to traditional Cas9 editing (Dominguez et al. 2016). These advantages thus offer more accurate engineering power that could lead to safer human therapeutic development. Here we explore how next-generation gene editing systems have developed in the context of CRISPR-based protocols.

Transcriptional Repression with CRISPRi

In CRISPRi, the most basic gene regulator is a deactivated Cas9 (dCas9) with catalytically dead RuvC and HNH endonuclease domains (**Fig. 1a**) (Jinek et al. 2012). dCas9 retains its genomic homing capacity and seeks out the target site, but can no longer make a double-strand break (Thakore et al. 2016). Once bound, dCas9 inhibits downstream transcriptional initiation and elongation by dissociating RNA polymerase and transcription factors from the target site, thereby silencing the gene. Alternatively, a Kruppel associated box (KRAB) effector can be fused to dCas9 to induce heterochromatin formation for a similar effect (**Fig. 1b**).

Although CRISPRi works similarly to RNA interference (RNAi), the two silencing methods remain distinct. Both techniques act as dimming knobs to gene expression compared to traditional on/off CRISPR editing. This knockdown effect can impact the intensity of phenotypic outcome when compared with traditional CRISPR knockout.

However, while CRISPRi and RNAi both transiently silence genes, their mechanism of action varies. RNAi inhibits protein translation by targeting messenger RNA whereas CRISPRi inhibits transcription from DNA.

CRISPRi is a powerful alternative to RNAi as the latter is often associated with inefficiency, off-target effects and oversaturation of natural microRNA pathways (Thakore et al. 2016). However, CRISPRi has limitations of its own. The method requires a defined target sequence and can only silence sequences adjacent to a PAM site and in proximity to a transcriptional start site (TSS), whereas RNAi can in principle target any mRNA sequence without restriction (Wang et al. 2016).

Milestones in CRISPRi research

In 2013, Qi and authors demonstrated the efficacy of CRISPRi in prokaryotes and eukaryotes. They showed that dCas9 could cause reversible genome scale knockdown with up to 99.7% efficiency in bacteria, but only approximately 50% in mammalian cells (Qi et al. 2013). Gilbert et al. tackled this problem by fusing dCas9 with effector domains to enhance its silencing efficiency. The authors fused dCas9 to KRAB directly, although others have shown that dCas9 can also be linked to effectors using scaffold RNAs (Zalatan et al. 2015). For Gilbert et al., KRAB-induced heterochromatin formation led to a 93% reduction in gene expression (Gilbert et al. 2013). Researchers subsequently demonstrated that dCas9-KRAB can target both proximal and distant regulatory promoters and enhancers, which further expands how investigators can use CRISPRi to fine-tune gene silencing (Thakore et al. 2016). However, whether the use of a KRAB effector is required for strong endogenous gene repression is still unclear.

Other studies have revealed the versatility of dCas9 in multiplexed gene function analysis. For example, CRISPRi was used to simultaneously knock down multiple gene targets in the bacterial species *Staphylococcus aureus* (Dong et al. 2017). Rock and colleagues screened multiple dCas9 variants to knockdown operons in *Mycobacterium tuberculosis* (Rock et al. 2017) and Liu et al. disrupted hundreds of essential genes required for the growth of *Staphylococcus pneumonia*, suggesting novel approaches for eradicating bacteria (Liu et al. 2017a). A medical application of CRISPRi in battling viruses was highlighted in 2016 by Park and authors who used the technique to identify key genes required for HIV infection (Park et al. 2016).

In addition to prokaryotic editing, CRISPRi has been used in several eukaryotic studies. dCas9 was used to create the largest eukaryotic gene circuit to date to regulate yeast cell growth (Gander et al. 2017). Adding functional methylating effectors to dCas9 has been shown to edit DNA methylation in postmitotic mouse neurons and facilitate mice fibroblast differentiation (Liu et al. 2016). CRISPRi has now been used to study genetic interactions and chromatin mapping in human embryonic kidney cells (Du et al. 2017). This approach has also shed light on the early stages of human embryo development by identifying novel functional long noncoding RNAs in human induced pluripotent stem cells (hiPSCs) (Liu et al. 2017b).

Moreover, researchers have demonstrated the biomedical potential of the dCas9-KRAB fusion. Using this technique, Farhang et al. repressed inflammatory cytokine receptor expression in adipose-derived stem cells (Farhang et al. 2017). The cells targeted by dCas9-KRAB were less affected by inflammation during tissue transplantation without permanently disrupting their genome. Repressing gene expression with CRISPRi might therefore be used to develop stem cell therapies with more potent and safer clinical application.

Gene Activation with CRISPRa

In contrast to CRISPRi gene repression, CRISPRa constructs are effective genetic activators as they mimic natural transcription factors in cells (Thakore et al. 2016). CRISPRa has high sgRNA-DNA mismatch sensitivity which increases specificity. Zhang et al. were first to show that the viral activation domain VP16 linked to a zinc finger binding domain could recruit RNA polymerase machinery to the target locus (Zhang et al. 2000). Gilbert et al. expanded on this proof-of-concept by fusing VP64 and p65AD activators to dCas9 (**Fig. 1c**). These activators attracted remodeling factors to alter histone conformation and DNA accessibility, resulting in a 25-fold and 12-fold increase in gene activation with dCas9-VP64 and dCas9-p65AD, respectively (Gilbert et al. 2013).

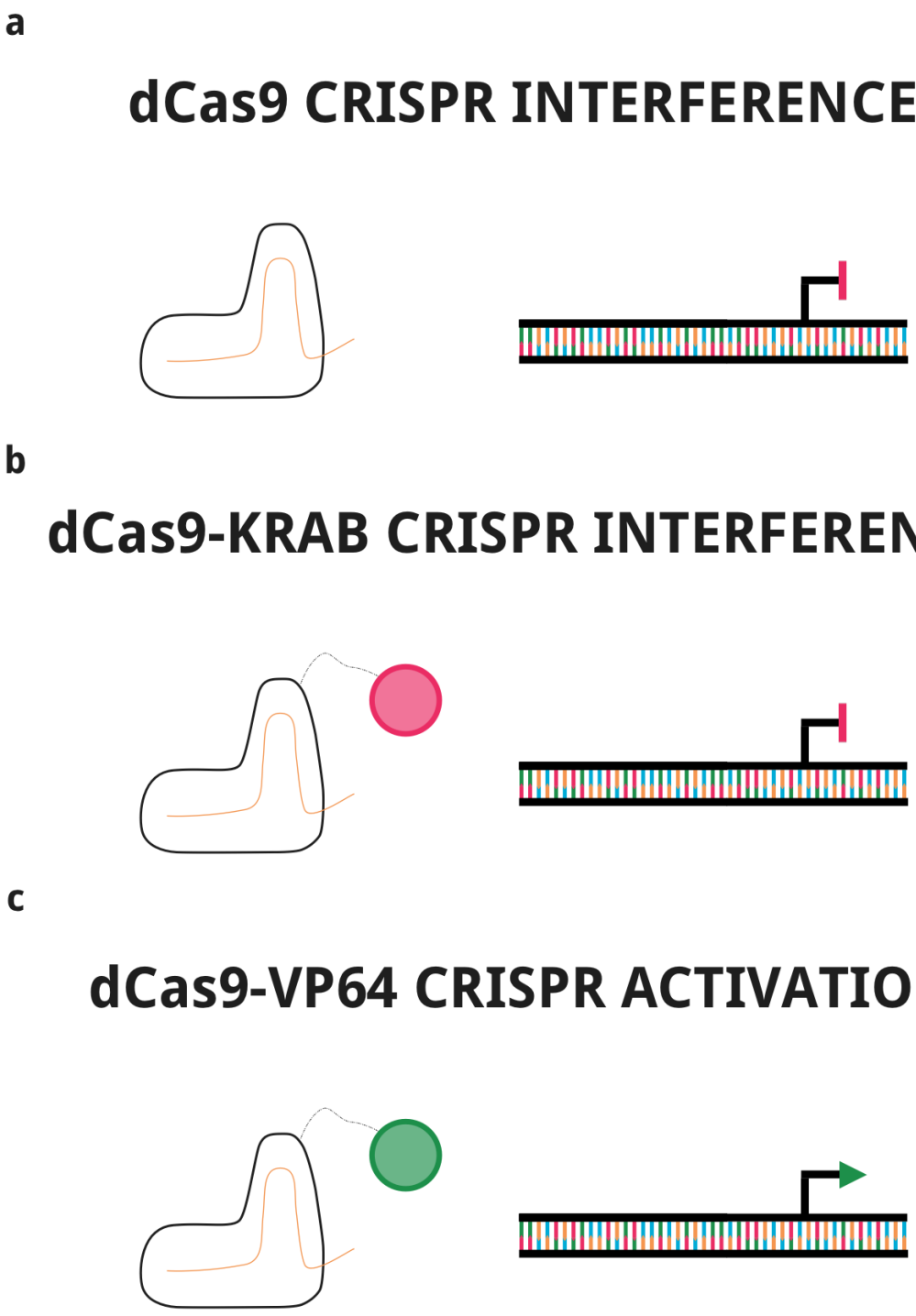

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Figure 1. *Options for transcriptional regulation with CRISPRi/a. a) Transcriptional CRISPR interference (CRISPRi) with nuclease domain deactivated Cas9 (dCas9). The sgRNA directs the dCas9 to bind but not cut at the target site. dCas9 inhibits transcription initiation/elongation by blocking RNA polymerase activity. b) Alternatively, effector domains like KRAB can cause downstream transcriptional repression by modifying epigenetic DNA tags. c) Transcriptional CRISPR activation (CRISPRa) via dCas9 attached to an activator (e.g. VP64). Figure adapted from Wang et al. 2016.*

A single activator is often insufficient for increasing the expression of coding and noncoding DNA in mammalian cells. Researchers have therefore expanded on the dCas9-VP64 model and produced a creative range of next-generation activators (**Table 1**). One example of this is the synergistic activation mediator (SAM) system designed by Konnerman et al. Their activator model contains multiple effectors (MPC, p65 and HSF1) fused to a dCas9-V64-RNA scaffold assembly and was used effectively to upregulate a range of long noncoding RNA sequences (Konnerman et al. 2015).

Another strategy combines dCas9 with a multipeptide array, SunTag, which amplifies activation by chaining together several VP64 effectors. This method was shown to sufficiently recruit GFP molecules to the target sequence and enable long-term imaging of single proteins in living cells (Tanenbaum et al. 2014). A tripartite activator strategy — VP64-p65-Rta (VPR) — caused a 22 to 320-fold increase in endogenous gene activation in human stem cells (Chavez et al. 2015). The VPR model was further tested by Guo et al. to elevate *NANOG*expression in human pluripotent stem cells, demonstrating its potential for high-throughput functional screening (Guo et al. 2017).

CRISPRi/a is just the beginning of alternative genome editing. dCas9 effector constructs have already begun to extend to epigenetic modifiers such as histone methylases, demethylases and acetylases, which could help to understand epigenetic agents in cell identity and disease progression (Kungulovski & Jeltsch 2016). In 2016, Komor et al. described a new strategy, in which dCas9 was fused to a cytosine deaminase effector. This system allowed the authors to generate precise single base switches without introducing double-stranded breaks. Like CRISPRi/a, this editing method avoids harmful indel formation and increases the efficiency of individual DNA bases editing over HDR.

Considerations for CRISPRi/a Libraries

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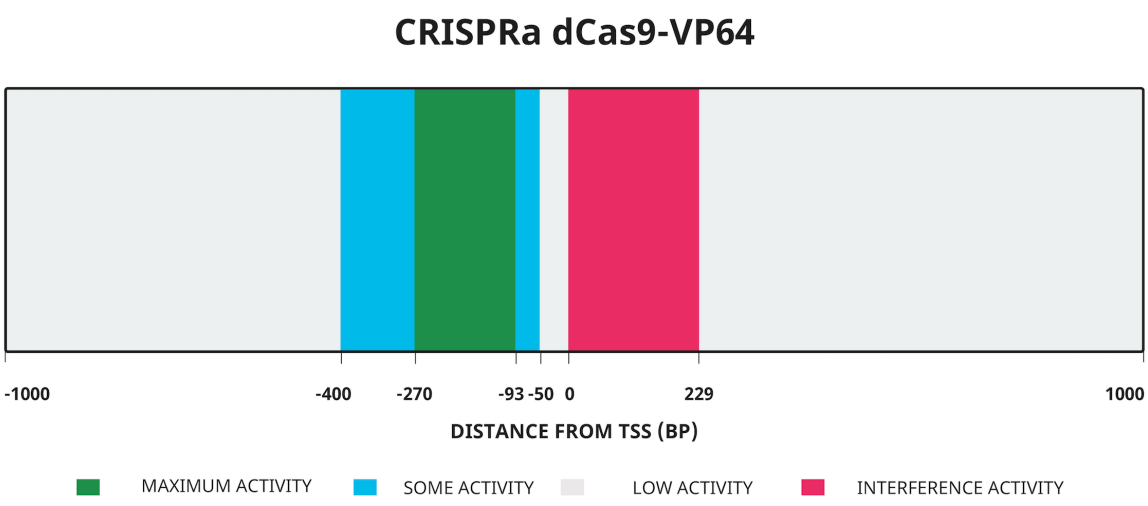

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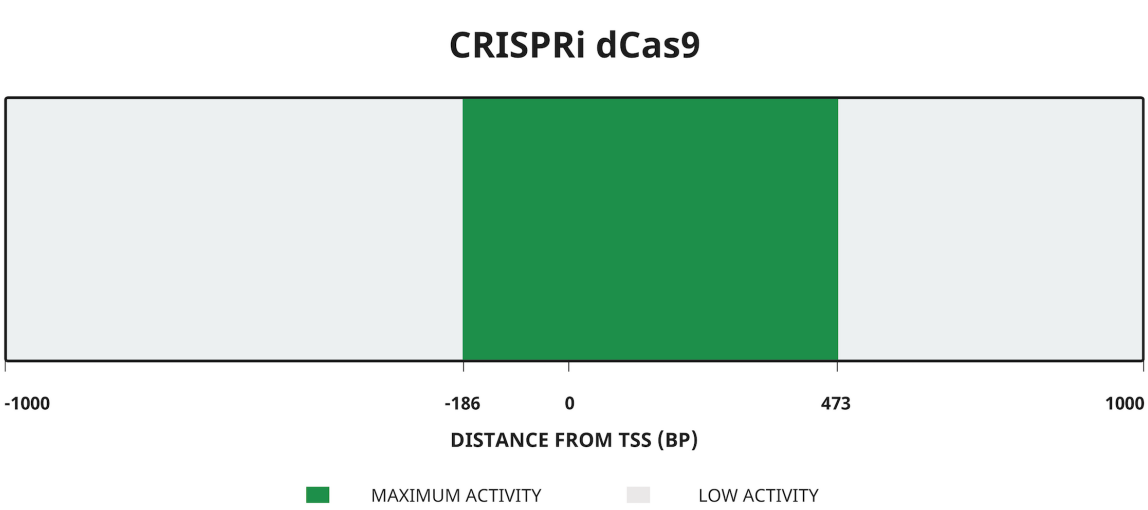

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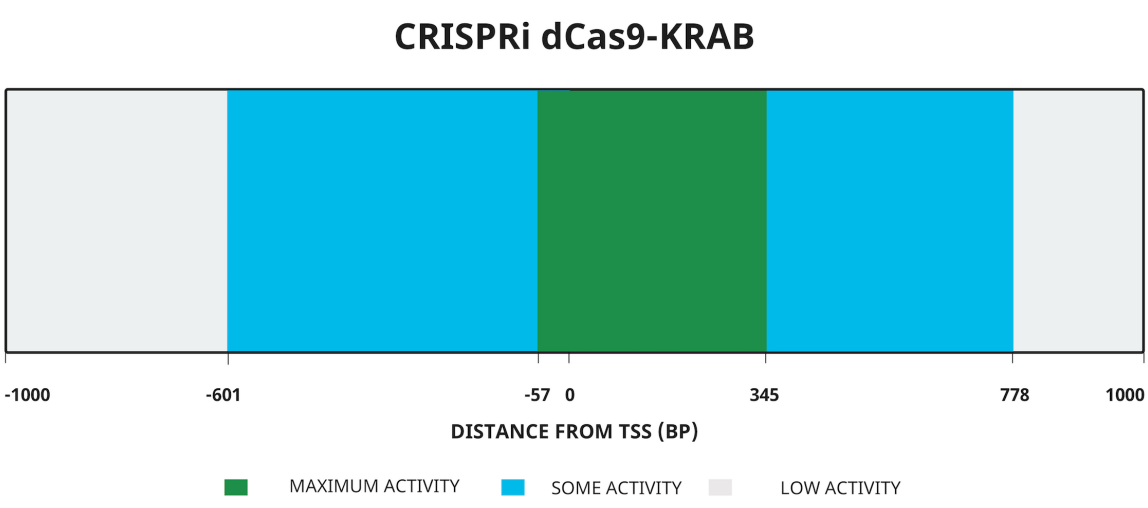

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Figure 2. *Ideal Transcription Start Site (TSS) windows for CRISPRi/a screens. a) Maximum activation for dCas9-VP64 screens is achieved between -270 and -93 from the TSS (green), moderate activation between -400 and -50 (blue), and a repressive effect between 0 and 229 (red). b) Maximum interference is achieved between -186 and 473 for dCas9. c) and -57 to 345 for dCas9-KRAB.*

Several experimental parameters may confound the execution of a CRISPRi/a screen. For instance, the size of a CRISPRi/a construct can affect activation or interference efficiency. Multiple plasmids or transfection reagents may therefore be required to express complex effector platforms (Wang et al. 2016). For example, Qi et al. coexpressed their CRISPRi tools on two separate plasmids: one with dCas9 and the other with target guides. This led to 2-3 fold gene repression (Qi et al. 2013). Furthermore, the window around the TSS offers limited options for CRISPRi/a guide RNA design. In the case of CRISPRi, dCas9 with a fused KRAB effector may widen the region around the TSS where interference is successful versus dCas9 on its own (**Fig. 2**).

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