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Here to stay: Writing lasting epigenetic memories

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In this issue of *Cell*, Nuñez et al. develop CRISPRoff, a programmable epigenetic memory writer capable of establishing specific gene silencing programs that are stably maintained across cell division and differentiation. The singular dCas9 fusion offers a simple, reliable, and general tool for genome-wide screens, multiplexed editing, and potential therapeutics.

Forgetting your significant other's birthday could be a recipe for disaster. Luckily, there are systems in check to keep you in their good graces—calendar alerts, that one dedicated friend, or the horrified look on their face when you forgot last year. Cells are very much the same—there is information they need to keep, very faithfully, for very long periods of time. Epigenetic mechanisms involving stable and heritable changes to chromatin establish epigenetic memories that keep cell identity expression patterns in check (Bonasio et al., 2010). By leveraging programmable DNA-targeting elements, most notably CRISPR-Cas9, researchers have been developing increasingly powerful and precise methods to rewrite epigenetic information in cells to dissect complex epigenetic pathways and enact heritable control over gene expression (Thakore et al., 2016). In a new advance in this issue of *Cell*, Nuñez et al. (2021) report the development of CRISPRoff, a dCas9-based epigenome editor that can install repressive chromatin modifications at specific loci and establish hyper-stable epigenetic memory programs in mammalian cells (Nuñez et al., 2021).

While targeted epigenetic modifiers are nothing new, the goal of readily creating long-lived epigenetic states has been elusive. Foundational single-fusion technologies like the repressive CRISPRi or activating CRISPRa are useful for creating temporary transcriptional perturbations in mammalian cells (Gilbert et al., 2014); however, their effects generally wane soon after the reagent dissipates. To overcome transient silencing, recent attempts have used multiple CRISPR effector fusions to target the deposition of orthogonal and synergistic repressive epigenetic marks to the same loci (Amabile et al., 2016; O'Geen et al., 2019). While these methods enabled more permanent gene regulatory alterations, their reliance on multiple CRISPR epigenome editors limits their utility in applications lacking efficient CRISPR delivery modalities and thus may reduce their efficacy and generalizability.

In a singular optimized fusion, CRISPRoff combines a robust *de novo* DNA methyltransferase apparatus (DNMT3a and DNMT3L) and a repressive KRAB domain that recruits histone-modifying repressive factors. Following a transient exposure, CRISPRoff proved sufficient to establish gene silencing in 80%–90% of a cell population (Figure 1A). In some experiments, this repression persisted for hundreds of generations, long after CRISPRoff's expression had dissipated. CRISPRoff also showed a significantly expanded targeting window relative to CRISPRi, thus allowing for more guide RNA-targeting flexibility (Figure 1C). Capitalizing on the reversibility of epigenetic regulation, Nuñez et al. (2021) also created a reciprocal activator, CRISPRon, consisting of a DNA-demethylating TET1 enzyme and the tripartite VPR (VP64-p65-RTa) transactivation domain (Figure 1A). Transient exposure to CRISPRon was sufficient to revert the repressive states induced by CRISPRoff, making CRISPRon/off a bidirectional writer/eraser pair for tuning and erasing hyper-stable epigenetic states at will.

Armed with a potent, programmable, and singular tool, Nuñez et al. (2021)

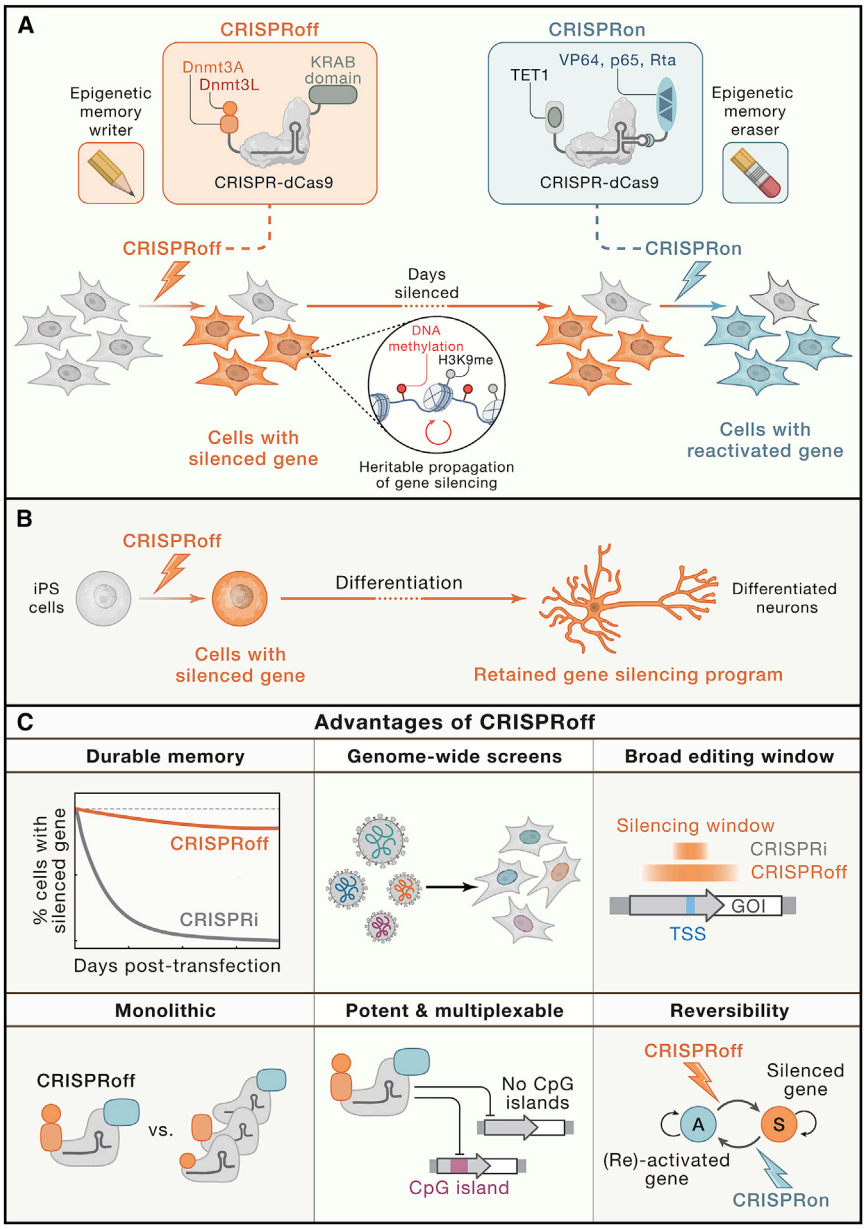

Figure 1. CRISPRoff: A programmable epigenome memory writer consisting of a single dead Cas9 fusion that can establish ultra-stable and heritable gene silencing

(A) Nuñez et al. (2021) develop CRISPRoff, a single dCas9 fusion to KRAB, Dnmt3A, and Dnmt3L effector domains. Transient CRISPRoff expression in human cells initiates highly specific, repressive histone (H3K9) and DNA methylation and highly stable gene silencing, in some cases, persisting for over 450 cell divisions. CRISPRoff gene silencing can be reversed by expression of CRISPRon, a dCas9 fusion to the TET1 DNA demethylase in combination with various transcriptional activators (VP64, p65, RTa).

(B) CRISPRoff gene silencing programs were shown to be stably maintained through the differentiation of induced pluripotent stem cells (iPSCs) to neurons. Epigenetic repression of a neurological disease relevant gene in iPSCs prevented its induction in the differentiated neurons.

(C) The CRISPRoff and CRISPRon technologies hold a number of features that are advantageous for applications, including genome-wide screens, multiplex engineering, enhancer silencing, and exploration of basic mechanisms of epigenetic inheritance.

deployed CRISPRoff in a pooled, genome-wide knockdown screen in human cells, demonstrating its broadly superior silencing relative to its CRISPRi analog and verifying its epigenetic functionality as DNA methylation dependent (Figure 1C). A surprising finding was the amenability of most genes to heritable gene silencing, including those lacking canonical CpG islands or with a low density of CpG sites. This result challenges the notion that higher-density CpG islands are the primary, fundamental functional unit of cytosine methylation, thus raising the exciting prospect that lower-density CpG regions or smaller clusters of CpGs may play an important role in gene regulation (Deaton and Bird, 2011). Future expansions on this work may help researchers interrogate DNA methylation marks with ever increasing granularity, possibly allowing for rigorous mapping of genome-wide CpG functionality.

Bringing many of its unique attributes to bear, Nuñez et al. (2021) showed that genes targeted by CRISPRoff in induced pluripotent stem cells stably maintain silencing not only through cell division but throughout the entire course of a neuronal differentiation experiment (Figure 1B). This result lines up a wealth of possibilities for using CRISPRoff in studies dissecting lineage specification and enacting precise genetic control in the processes of development and differentiation. Moreover, as memory elements are fundamental building blocks of synthetic biological systems that seek to control mammalian cell function, this work may provide inspiration for creating epigenetic writer/eraser pairs that allow sophisticated, dynamic control over gene expression in synthetic circuits (Park et al., 2019).

CRISPRoff's therapeutic potential is also notable. Targeted epigenetic therapy may not be far off—recently, two proof-of-principle studies in mice demonstrated targeted epigenetic repression strategies as potential therapies for chronic pain and neurodegenerative disorders (Moreno et al., 2021; Wegmann et al., 2021). In contrast to conventional CRISPR genome editing, CRISPRoff-induced durable gene silencing would circumvent exposing cells to a reagent known to induce off-target mutations, chromosomal rearrangements, and genomic instability, making it a potentially safer therapeutic agent. Furthermore, epigenetic silencing avoids the error-prone DNA double-strand break repair upon which conventional CRISPR editing relies, a process that could result in

unpredictable gain-of-function mutations at on-target sites. In turn, CRISPRoff has the potential to create purer gene-suppressing outcomes, resulting in the uniformity and predictability characteristic of a reliable therapeutic approach.

Accordingly, Nuñez et al. (2021) used whole-genome bisulfite sequencing to evaluate CRISPRoff's specificity profile, finding that while CRISPRoff seems to induce a modest increase in genome-wide DNA methylation, this effect was small compared to the natural variance in DNA methylation between isogenic cells within a population. Importantly, this analysis also demonstrated that repressive domains propagate from the site of initiation but were generally confined to the intended loci and do not extend into neighboring genes. Correspondingly, RNA sequencing analysis showed minimal off-target alterations in gene expression profiles, although single-cell transcriptomic data may be needed to rule out the possibility of rare "epi-off-targets" that may evade detection in population-level studies. Taken together, these results demonstrate that cells edited with CRISPRoff appear to maintain their epigenome-wide integrity.

Effective, robust, and specific, CRISPRoff offers a generalizable and simple way to install, interrogate, and manipulate epigenetic memory on demand for scientific and therapeutic applications in almost any mammalian cellular context. Therefore, while CRISPRoff can't help you remember those important birthdays yet, it is likely a platform that's here to stay.

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DECLARATION OF INTERESTS

A.S.K. is a co-founder of Fynch Biosciences and K2 Biotechnologies and is a scientific advisor for Senti Biosciences and Chroma Medicine.

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