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CRISPR/Cas-based devices for mammalian synthetic biology

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Since its first demonstration for mammalian gene editing, CRISPR/Cas technology has been widely adopted in research, industry, and medicine. Beyond indel mutations induced by Cas9 activity, recent advances in CRISPR/Cas have enabled DNA or RNA base editing. In addition, multiple orthogonal methods for the spatiotemporal regulation of CRISPR/Cas activity and repurposed Cas proteins for the visualization and relocation of specific genomic loci in living cells have been described. By harnessing the versatility of CRISPR/Cas-based devices and gene circuits, synthetic biologists are developing memory devices for lineage tracing and technologies for unbiased, high-throughput interrogation of combinatorial gene perturbations. We envision that such approaches will enable researchers to gain deeper insights into the translation of genotypes to phenotypes in healthy and diseased states.

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Introduction

CRISPR/Cas technology has revolutionized the editing and controlling of genomes and has quickly become the method of choice over other sequence-specific nucleases for targeted gene editing in mammalian cells [1]. Beyond generating indel mutations, the CRISPR/Cas toolbox has rapidly expanded over the last few years. One approach has involved searches in metagenomes for novel Cas proteins with distinct substrates (e.g. protospacer adjacent motif [PAM] or protospacer flanking site [PFS] requirements) and modes of regulation (e.g. accessory proteins). Another major effort has involved engineering Cas9 from *Streptococcus pyogenes* (SpCas9) so that it has novel functionalities and can be regulated by orthogonal ligand-mediated or light-mediated systems. The ever-expanding palette of novel and engineered Cas proteins not only enables increasingly powerful mammalian genome editing but also provides synthetic biologists with the tools to create gene circuits with unprecedented control over cellular phenotype and function.

Synthetic biology aims to engineer cells with gene circuits that confer new biological functions. CRISPR/Cas-based devices are useful in gene circuits because they can be easily repurposed to perturb or act on any arbitrary nucleic acid sequence of interest by designing complementary gRNA. The use of CRISPR/Cas-based devices for gene circuits is still in its infancy but is gaining popularity especially for encoding biological memory and for performing lineage tracing and forward genetic screens. We discuss recently described CRISPR/Cas-based devices and emerging CRISPR/Cas-based synthetic biology applications.

CRISPR/Cas-based devices

Cas13: RNA-editing Cas proteins

RNA-guided RNA endonucleases are recently identified Cas proteins that have been adapted for mammalian cell engineering. Searches for novel Cas proteins were performed by looking for open reading frames encoding large proteins with a putative nuclease domain near a CRISPR array or Cas1 proteins [2], common features of CRISPR loci in bacterial and archaeal species. This approach led to the discovery of a family of type VI CRISPR/Cas systems with HEPN RNase domains containing RNA-guided RNA endonucleases, collectively named Cas13 proteins. Cas13a-d [3,4••,5], the four members of the Cas13 family discovered so far, were shown to be able to achieve RNA tracking or knockdown in mammalian cells. Each Cas13 protein has distinct properties, such as its manner of regulation and its size. For example, Cas13b can be repressed and activated, respectively, by accessory proteins Csx27 and Csx28 [6]. Cas13d (~900 amino acids [a.a.]) is smaller than other Cas13 proteins (1100–1300 a.a.) and is activated by the accessory protein WYL1 [7]. We envision that these RNA-editing CRISPR-Cas systems will enable genes to be regulated without making irreversible changes in DNA sequence or the epigenetic landscape.

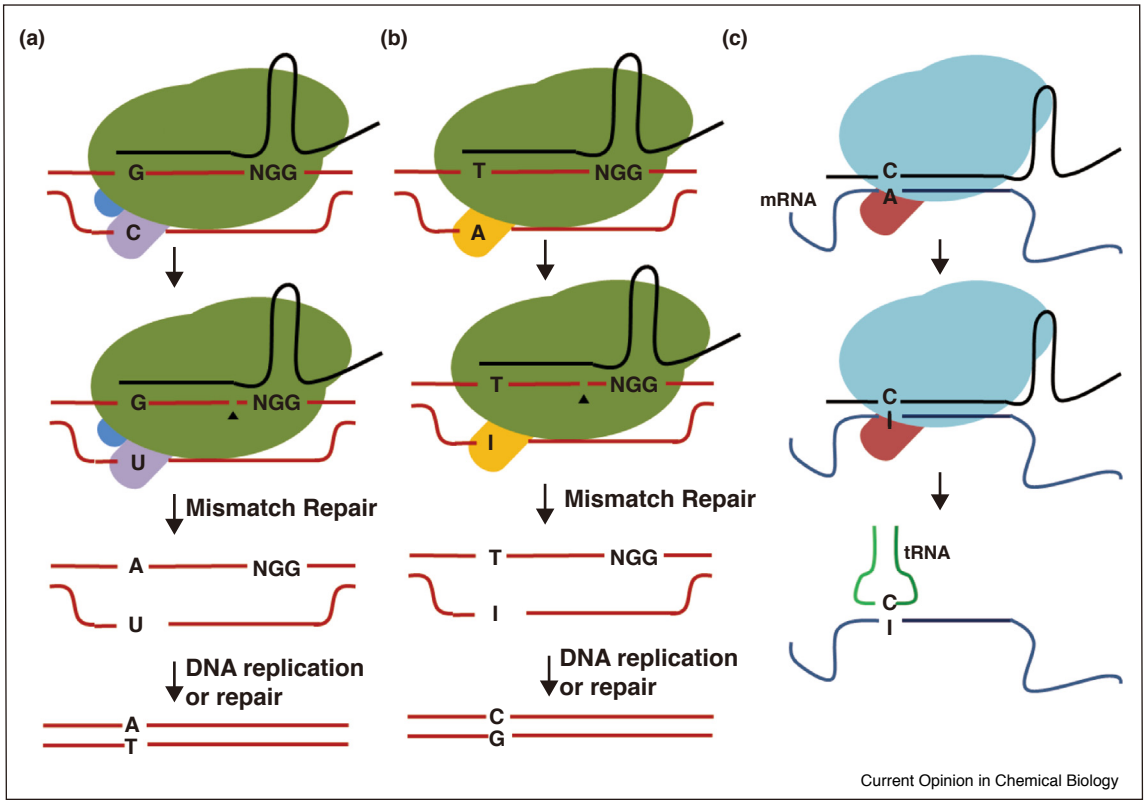
DNA/RNA base editors

Cas9-based gene editing in mammalian cells has mainly relied on achieving indel mutations after non-homologous end joining (NHEJ) following a double-strand break by

Cas9-mediated cleavage. While this is precise enough to make gene knockouts within open reading frames, the consequence of NHEJ at the single-nucleotide level is unpredictable. This limits the precision of Cas9-mediated gene editing in gene circuits, where nucleotide-level modifications are used to encode information or perform computations. Double-strand breaks by Cas9 can also be repaired by homology-directed repair (HDR), which uses an exogenously provided repair template to replace the sequence surrounding the double-strand break. HDR can achieve precise single nucleotide substitution by providing a repair template with the desired sequence change. However, the efficiency of HDR in making desired sequence changes is very low (0.1–5%) [8].

To overcome these limitations, recent advances have enabled base substitution without indel formation. Base editor 3 (BE3) [9] and Adenine base editor (ABE) [10••] enable cytosine to thymine, and adenine to guanine substitutions, respectively (Figure 1a,b). Both editors are fusion proteins of the SpCas9 nickase (nCas9; Cas9 D10A) and a base-editing enzyme (rAPOBEC1 for BE3, engineered *Escherichia coli* TadA for ABE). These enzymes share the common feature of nicking the DNA strand to which the gRNA binds and editing bases on the other strand. This creates a mismatch: one strand is nicked and the other is base edited. Intracellular mismatch repair machinery preferentially removes the nicked strand to resolve the mismatch according to the sequence of the intact strand. Thus, the mismatch is preferentially resolved with the base-edited sequence. Subsequent optimization of the base-editing enzymes involved the rational design of the Cas9 protein and appending an additional uracil glycosylase inhibitor that inhibits base excision repair and removes the edited uracil. These efforts have further improved precision by narrowing the window of editable bases from ~5 nucleotides to 2–3 nucleotides and increasing the efficiency of editing to a maximum of ~60% [11,12]. nCas9-based base editors have also been equipped with different cytidine deaminases, including PmCDA1 from sea lamprey [13] and APOBEC3A engineered to have a single-nucleotide editable window [14].

Figure 1



Engineered Cas proteins for base substitution. **(a)** BE3 edits cytosine to thymine. Cas9 nickase (green, nCas9) is equipped with cytidine deaminase (rAPOBEC1, violet). Cytidine deaminase changes cytosine to uracil. The nicked but unedited strand is replaced with the base-edited strand by mismatch repair. Uracil is prevented from removal by base excision repair by uracil glycosylase inhibitor (UGI, blue). **(b)** ABE edits adenine to guanine. It works similarly to BE3 except that nCas9 is equipped with an ABE engineered from *E. coli* TadA. **(c)** mRNA editing by a Cas13-based RNA base editor. Initial mismatch between mRNA and gRNA exposes the base for editing. Nuclease-dead Cas13 is equipped with adenine deaminase ADAR2, which converts adenine to inosine, which is treated as guanine by tRNA.

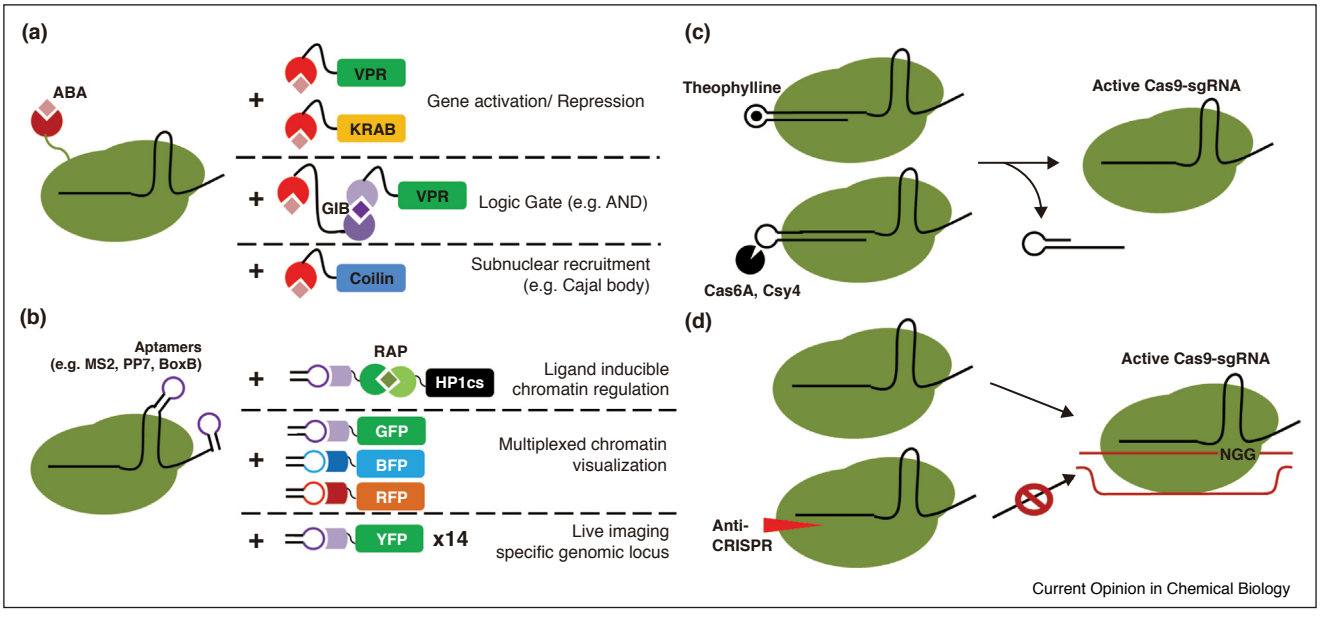
A similar approach was used to fuse nuclease-deficient RNA-binding Cas13b with the ADAR2 adenosine deaminase domain to make specific RNA base substitutions from adenine to inosine (which is treated as guanine during translation) for editing pathogenic mutations [4••] (Figure 1c). RNA editing has potential advantages over DNA editing in terms of safety because it does not make any heritable, irreversible changes in the genome.

Regulated CRISPR/Cas activity

A wide range of strategies for regulating CRISPR/Cas activity has been developed to achieve controlled gene editing as well as to build artificial gene circuits. For example, CRISPR/Cas activity can be modulated by using ligand-inducible transcription factors to titrate the expression of Cas proteins and gRNA [15,16]. We also developed an RNA Pol II-driven sgRNA expression platform to enable multiplexed sgRNA expression under the control of endogenous promoters [17]. Recent studies have used chemical-induced dimerization (CID) [18] by abscisic acid (ABA) or gibberellin (GIB) to recruit desired effector proteins to dCas9. This is done by fusing one of the two proteins that dimerize upon ligand treatment to dCas9 and fusing the other protein to the effector of interest [19] (Figure 2a). Multiple orthogonal systems for CID have been used to create logic gates, such as AND gates and OR gates, with transcriptional activation or repression as outputs [19]. In addition, CID by ABA has been used to recruit dCas9 (along with its target genomic DNA) to specific subnuclear locations [20•]. Light has also been used to regulate CRISPR/Cas activity, including blue [21] and far red light [22]. Because far red light has less phototoxicity and better penetration into opaque bodies (e.g. cells within living tissues) than blue light, far-red-light-inducible Cas9 will be a valuable tool for the *in vivo* regulation of CRISPR/Cas activity.

gRNA for SpCas9 has also been engineered to regulate and expand the functionalities of CRISPR/Cas systems. For example, gRNAs were appended to a functional RNA domain, such as an aptamer, to recruit effector proteins to the Cas9-gRNA complex (Figure 2b). Several groups have appended a protein-binding RNA aptamer sequence (such as an MS2 loop) to recruit an effector protein of interest fused to an RNA-aptamer-binding partner. This approach enabled ligand-inducible epigenetic regulation [23] and multiplexed-imaging [24] or live-imaging [25] of specific endogenous loci of interest within the chromatin of an intact nucleus. gRNA was also engineered to regulate its own activity with a cleavable 5′ extension that blocks the spacer sequence of gRNA by reverse complementarity (Figure 2c). A ligand-inducible, self-cleaving aptazyme [26] or a Csy4 or Cas6A [27] ribonuclease target sequence placed in between the spacer sequence and the 5′ blocking extension sequence resulted in the liberation of functional gRNA only upon ligand (e.g. theophylline or guanine) treatment or ribonuclease expression.

Figure 2



Novel regulation of Cas proteins. **(a)** CID enables ligand-mediated recruitment of the effector protein of interest. **(b)** The RNA aptamer functionalizes Cas protein for diverse tasks. **(c)** gRNA engineering for ligand-activated or nuclease-activated CRISPR/Cas activity. gRNA has a 5′ extension that forms a hairpin with the gRNA spacer sequence, thereby blocking the target DNA from binding. Cleavage of the 5′ extension by ligand or ribonuclease liberates active gRNA for CRISPR/Cas activity. **(d)** Acr protein inhibits CRISPR/Cas by multiple mechanisms. One of the Acr proteins, AcrIIA4, acts as a DNA mimic, interfering with target DNA recognition.

Anti-CRISPR (Acr) can be used to effectively switch off CRISPR/Cas activity (Figure 2d). Acrs, which evolved in bacteriophages, are proteins that enable the phages to evade bacterial immune surveillance by binding and inhibiting CRISPR/Cas [28,29••,30]. Inhibitors of Cas9, Cas12, and the type 1 CRISPR/Cas system have been discovered [31,32]. A blue-light-inducible Acr has been used to inhibit SpCas9 [33]. An Acr under the control of cell type-specific microRNA has been adapted for gene editing specifically in hepatocytes or myocytes [34•].

CRISPR/Cas-based synthetic gene circuits

Synthetic gene circuits for memory

CRISPR/Cas-mediated indel formation leaves irreversible and inherited changes in the DNA sequence. This property has been repurposed to create a memory of cellular events by linking CRISPR/Cas activity with the specific event of interest. Synthetic gene circuits for biological memory will be an invaluable tool for obtaining insight into the signals that regulate cellular decision making.

Lineage tracing circuits have been built with CRISPR/Cas-based memory in mammalian cells. The CRISPR/Cas-based lineage tracing method involves gRNA target sequences that are engineered to be edited multiple times by gRNA. Thus, with carefully tuned CRISPR/Cas activity, gRNA target sequences slowly accumulate indel mutations over generations. Mutated DNA can be sequenced with Next Generation Sequencing (NGS) and deconvoluted as a lineage tree (Figure 3a). There are several designs for the DNA sequence targeted by the gRNA that can accumulate mutations for lineage tracing. These designs include tandem arrays of 'barcodes' [35] and multiple copies of integrated transgenes in the genome [36]. A challenge with these approaches is the limited capacity of the lineage-tracing system to record information over time, as editable target sites are lost with progressive editing. We and others have addressed this problem by adapting the gRNA gene itself as the memory unit. To do this, we engineered self-targeting gRNA (stgRNA) that can repeatedly edit itself so that mutations accumulate over time [37•,38] (Figure 3b). These lineage-tracing methods can be integrated with single-cell RNA sequencing by expressing the gRNA target sequence as mRNA to simultaneously profile the lineage tree and determine the identity of the cell types that make up the lineage tree, as has been done to study zebrafish development [36,39]. *In situ* lineage tracing was also demonstrated in cultured human embryonic stem cells. In these cells, tandem arrays of gRNA target sequences led to truncation of the gRNA target sequence to form a memory of CRISPR/Cas activity, which could be measured by loss of signal with single-molecule FISH [40].

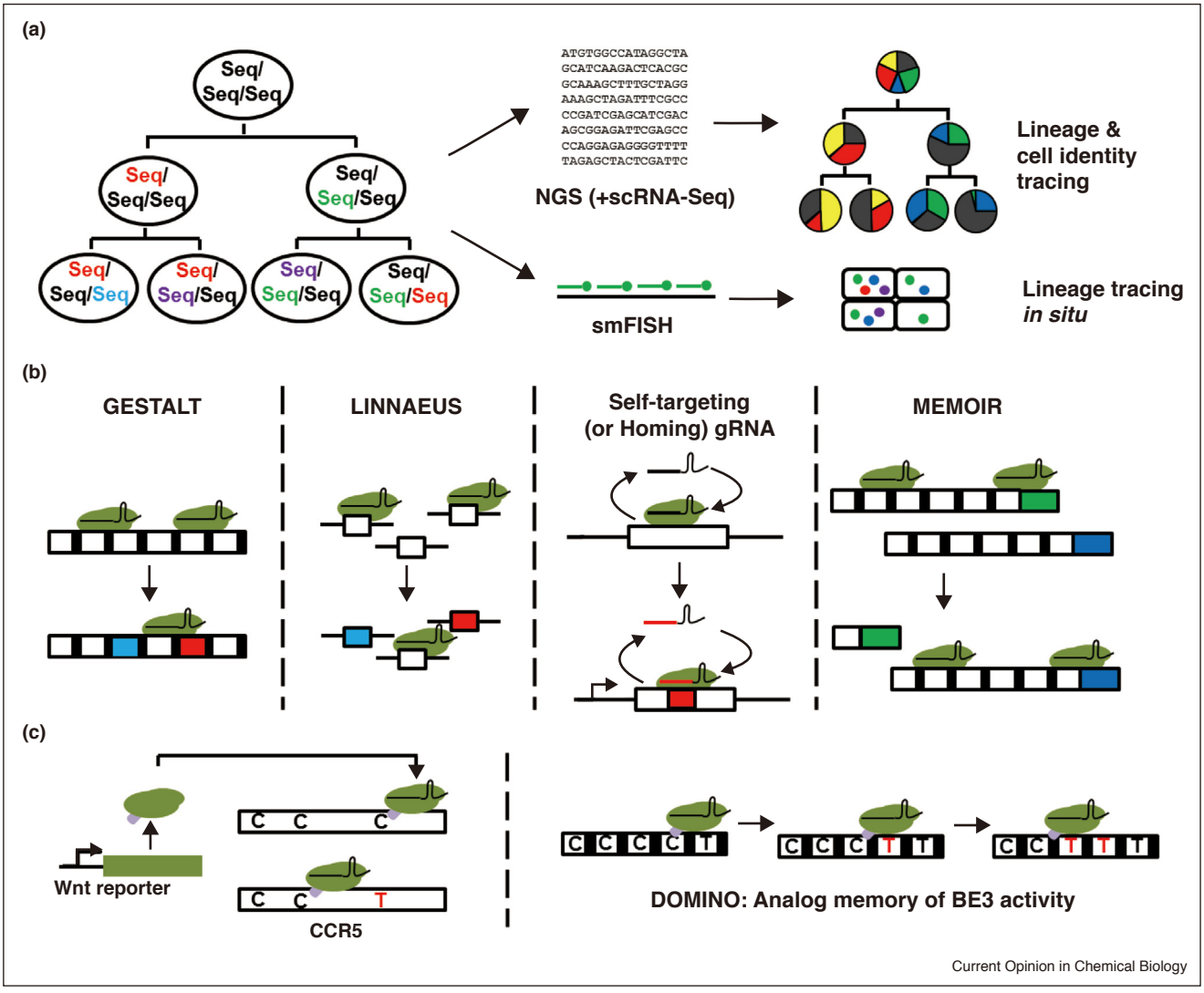
Apart from lineage tracing, CRISPR/Cas has been used to quantify specific stimuli that cells have experienced (Figure 3c). For example, we used stgRNA to form an analog memory of inflammation by linking NF-kB activity to Cas9 expression [38]. Analog memory in living cells was also established via accumulated base editing by BE3. For example, BE3 was used to record Wnt activation in HEK293T cells by linking the Wnt signal to the expression of BE3 for editing the safe harbor gene CCR5 [41]. We have also described a BE3-based computation and memory platform called DNA-based Ordered Memory and Iteration Network Operator (DOMINO) [42], which enables cascades of sequential base-editing for sophisticated, order-dependent computing and memory in living cells.

Visualizing and manipulating cellular behaviors

The versatility of CRISPR/Cas proteins has been exploited to visualize and manipulate cellular behavior. For example, nuclease-dead versions of SpCas9 and Cas13 family proteins were tagged with fluorescent proteins to track the localization of DNA and RNA, respectively, *in situ* [3,24,25,43]. Recent advances enable live imaging to discriminate between two different alleles within the same nucleus with different fluorescent proteins [44]. Beyond the simple visualization of RNA or chromatin, the repositioning of specific genomic DNA sequences has been achieved using CID by ABA. For example, two orthogonal dCas9s from *Staphylococcus aureus* and *S. pyogenes* were engineered to bind to each other by CID upon ABA treatment. This platform was used to bring the two different genomic loci bound by orthogonal dCas9s in proximity by ABA treatment [45]. Also, dCas9 and proteins known to localize in a specific nuclear subcompartment (such as coilin, which localizes to a Cajal body) were engineered to recruit the genomic locus bound by dCas9 to that subcompartment upon ligand (i.e. ABA) treatment [20•].

Another powerful application of CRISPR/Cas technology is the mapping of combinatorial gene networks to diverse biological phenotypes. For example, pairwise genetic perturbation screens have been used to discover novel genetic interactions and synergistic drug targets for cancer therapy [46,47,48•,49]. Perturb-Seq and CRISPR droplet (CROP)-Seq integrate single-cell RNA sequencing with CRISPR screens [50,51,52••]. Thus, the readouts for these screens are no longer limited to viability but can include gene signatures, cell differentiation, and changes in cell state. The consequence of gene editing by Cas9 is inherently heterogeneous because indel mutations may generate in-frame mutations that minimally disrupt gene function. This heterogeneity of phenotype by the identical gRNA limits the robustness of CRISPR/Cas screens. Recent approaches have integrated lineage tracing using unique molecular identifiers with CRISPR screens to track the effect of gRNA in individual cells [53]. This method takes into account the heterogeneity of cellular

Figure 3



CRISPR/Cas-based memory devices. **(a–b)** CRISPR/Cas-based lineage tracing (a) Schematics of lineage tracing by CRISPR/Cas. Cells contain sequences (Seq) that are edited over generations. Inherited changes in Seq (depicted as the changes in color of Seq) are analyzed either by NGS or by single molecule FISH (smFISH), to reconstruct the lineage tree. (b) The design of different gRNA target sequences for lineage tracing. Genome editing of synthetic target arrays for lineage tracing (GESTALT) uses tandem arrays of gRNA target sequences. Lineage tracing by nuclease-activated editing of ubiquitous sequences (LINNAEUS) uses target sequences incorporated at multiple loci in the genome. Self-targeting gRNA (stgRNA) is engineered gRNA that targets the genomic locus where the gRNA is being expressed. Continual changes in gRNA sequence are used for lineage tracing. Memory by engineered mutagenesis with optical *in situ* readout (MEMOIR) uses multiple tandem arrays of gRNA target sequences appended with a barcode (colored red or blue). CRISPR/Cas-mediated mutation collapses the gRNA target sequence, resulting in loss of gRNA signal by smFISH. **(c)** Analog memory with BE3. (Left) Wnt signal activates BE3 expression, which in turn makes irreversible changes in the safe harbor gene CCR5 to form a memory of Wnt signaling activity. (Right) DNA-based Ordered Memory and Iteration Network Operator (DOMINO) uses engineered tandem arrays of the gRNA target sequence with a mismatch that is sequentially edited by BE3 to generate a perfectly matched gRNA target sequence. Gradual propagation of the edited bases within this array can form an analog memory of BE3 activity.
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responses to the same gene editing to enhance the sensitivity and robustness of the CRISPR/Cas screens.

Conclusions and outlook

Despite tremendous improvements, more efforts are needed to maximize on-target gene perturbations and minimize off-target ones. Since its advent, mammalian cell engineering with Cas9 has raised concerns of off-target editing. Improving the fidelity of CRISPR/Cas is critical for the reliable performance of CRISPR/Cas effectors within gene circuits and the elimination of any possibility of unpredictable side effects. Also,

relaxing PAM requirements will increase the targetable region of the genome. Directed evolution and rational design based on the structure of Cas9 ribonucleoprotein are ongoing to address these challenges [54,55]. Similar approaches should be taken to improve base editors, so as to achieve an ideal targeting window as narrow as a single nucleotide and 100% efficiency of the on-target base substitution rate for DNA or RNA editing [14].

Successful delivery of CRISPR/Cas components will also be critical for the efficient application of CRISPR/Cas to mammalian synthetic biology. Because Cas nucleases are often very large, viral delivery of CRISPR/Cas components can be challenging. Efforts are under way to discover smaller Cas proteins, such as Cas13d and Cas9 from *Campylobacter jejuni*, which have fewer than 1000 amino acids [56]. Alternatively, a Cas protein and its guide RNA can be assembled together as a single ribonucleoprotein for non-viral delivery [57].

CRISPR/Cas-based devices and gene circuits for mammalian cell engineering are being used for increasingly ambitious tasks, highlighted by regulated CAR-T cells [58,59], gene drives in mammalian cells [60], and even recent claims of a genetically modified baby [61]. Future work in maximizing the fidelity of CRISPR/Cas and delivery will be instrumental for continued advances of this field.

Conflict of interest statement

TKL is a co-founder of Senti Biosciences, Synlogic, Engine Biosciences, Tango Therapeutics, Corvium, BiomX, and Eligo Biosciences. TKL also holds financial interests in nest.bio, Ampliphi, IndieBio, MedicusTek, Quark Biosciences, and Personal Genomics.

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