Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Innovations in CRISPR technology

Alexandra K Brooks and Thomas Gaj

CRISPR-Cas9 is a versatile tool for genome engineering that has revolutionized biotechnology and is poised to impact medicine. Recent advances in the identification of unique CRISPR systems, as well as the re-engineering of the Cas9 protein for expanded function, has enabled the diversification of the CRISPR genome engineering toolbox. In this review, we highlight these innovations and discuss how advances in CRISPR technology can lead to breakthroughs in the field of gene therapy.

Address
Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Corresponding author: Gaj, Thomas (gaj@illinois.edu)

Current Opinion in Biotechnology 2018, **52**:95–101

This review comes from a themed issue on **Tissue, cell and pathway engineering**

Edited by **David Schaffer** and **Stanislav Y Shvartsman**

https://doi.org/10.1016/j.copbio.2018.03.007

Introduction

The RNA-guided Cas9 endonuclease [1] — adopted from type II clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) systems, where it evolved to help provide adaptive immunity to prokaryotes — is driving a revolution in biotechnology and biomedical research. Due in large part to its simplicity, versatility and efficiency compared to other gene-modifying technologies, CRISPR-Cas9 has spurred advances in drug discovery [2], synthetic biology [3], and gene therapy [4] at an astonishing pace that has captivated the scientific community. The Cas9 nuclease can be deployed to a specific DNA site via RNA-DNA base complementarity using an engineered single guide RNA (sgRNA) to induce a DNA double-strand break (DSB) that stimulates genome editing [1,5–8]. The only requirement for Cas9-mediated DNA recognition and cleavage is the presence of a short protospacer adjacent motif (PAM) — which can vary in its identity according to the Cas9 variant — immediately 3′ of the targeted DNA site [1].

Critically, DSBs induced by Cas9 are corrected by one of several DNA repair pathways [9], including non-homologous end-joining (NHEJ) — which enables the introduction of random base insertions and/or deletions (indels) that can disrupt gene function — or homology directed repair (HDR) — a process that can drive the insertion of genetic material from a homologous donor template following its co-delivery into cells alongside Cas9 and a sgRNA. Given the range of DNA editing outcomes possible using CRISPR-Cas9 technology, as well as the ability for the Cas9 protein to serve as a scaffold for initiating processes such as transcriptional activation [10,11] or repression [12], there is substantial interest to both expand on and refine the capabilities of Cas9 and push forward the boundaries of genome engineering.

Here, we review recent innovations in CRISPR technology that are helping to drive a new wave of advances in genome engineering. We highlight how tapping into the wealth of CRISPR systems that exist in nature has enabled the diversification of the CRISPR genome editing toolbox and how molecular engineering has enhanced the capabilities of the Cas9 protein. Additionally, we discuss how these innovations have impacted the field of gene therapy.

Harnessing CRISPR diversity to expand the genome editing repertoire

The Cas9 nuclease from *Streptococcus pyogenes* (SpCas9) is the most well-characterized and commonly employed Cas9 homologue for gene editing. However, owing to its large size, the SpCas9 gene cannot be packaged into a single adeno-associated virus (AAV) particle alongside its sgRNA and the regulatory elements needed to drive its expression. This has emerged as a considerable limitation for this first-generation CRISPR technology, as AAV vectors are a highly promising class of therapeutic gene delivery vehicles [13], evidenced by the recent approval by the U.S. Food and Drug Administration for an AAV-based therapy for Leber's congenital amaurosis, a form of inherited blindness.

The characterization of alternate Cas9 orthologues from other type II CRISPR-Cas systems, however, has provided an opportunity to address this shortcoming. For example, smaller Cas9 variants — including ones from *Campylobacter jejuni* (CjCas9) [14] and *Staphylococcus aureus* (SaCas9) [15] — have been reported to edit DNA with efficiencies similar to SpCas9. The CjCas9 nuclease gene is ~1.1 kilobases smaller than SpCas9 and has been delivered to the mouse retina alongside an sgRNA using an all-in-one AAV vector to facilitate the

disruption of *Vegfa* and *Hif1a* [14], two genes involved in macular degeneration, a leading causes of blindness in adults. Similarly, SaCas9 has been used to correct a specific splice-site mutation in a mouse model of congenital muscular dystrophy type 1 [16•], a neuromuscular disorder characterized by muscle weakness and muscle wasting, and drive the excision of a mutant exon from the dystrophin gene in a mouse model of Duchenne muscular dystrophy (DMD) [17–19], a fatal disorder resulting in muscle degeneration and early death. In addition, a dual vector delivery approach enabled SaCas9 to correct a mutation via HDR in the ornithine transcarbamylase (OTC) gene in the liver of mice with OTC deficiency, a rare X-linked metabolic disorder that affects the urea cycle and leads to increased levels of ammonia in the blood [20]. More recently, SaCas9 was reported to have facilitated the disruption of a mutant version of the superoxide dismutase gene in spinal cord motor neurons in a mouse model of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease that leads to progressive muscle atrophy [21]. This therapeutic genome editing strategy slowed muscle deterioration and improved survival, indicating that CRISPR technology can be harnessed to treat central nervous system disorders caused by dominant gene mutations. Moving forward, AAV vectors engineered to possess cell [22] or tissue-targeting [23] capabilities could be united with optimized CRISPR-based cargo for highly specific DNA editing, similar to that achieved in a recent study demonstrating the delivery of SaCas9 to a specific population of cortical neurons via an AAV vector evolved to facilitate retrograde transport to projection neurons [22].

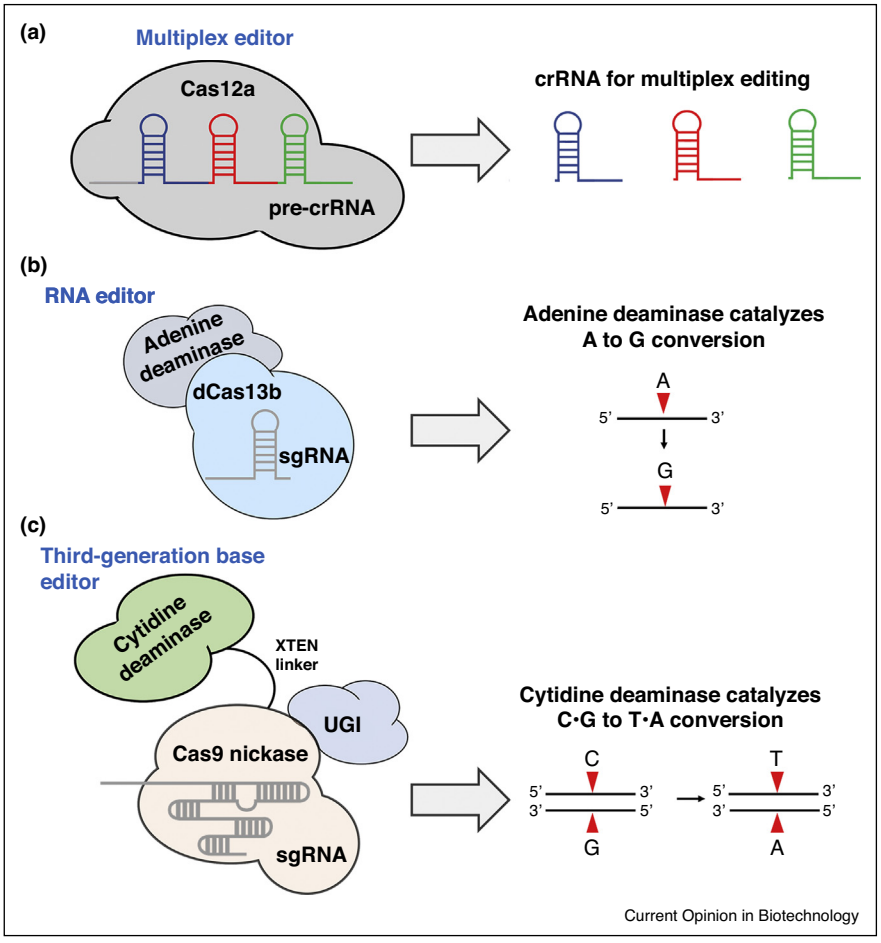
In addition to smaller Cas9 orthologues, the characterization of alternate CRISPR systems has led to the identification of proteins with unique properties compared to other type II CRISPR effectors, such as Cas12a (formally known as Cpf1) (Figure 1a) [24]. Unlike Cas9, Cas12a can process its pre-CRISPR array and thus can be used to generate multiple CRISPR RNA (crRNA) from a single transcript [24]. This feature has been exploited to facilitate efficient multiplex genome editing in the mouse brain, exemplified by the knockdown of three neuronal genes (specifically, Mecp2, Nlgn3, and Drd1) following AAV-mediated delivery of Cas12a and an engineered pre-crRNA array [25]. This unique processing capability has also been extended to gene regulation, as the fusion of a catalytically dead version of the Cas12a protein to a transcriptional repressor domain [26] or a tripartite transcriptional activator protein [27] has been used to direct combinational control of gene expression [28]. Additionally, unlike many Cas9 orthologues that require G nucleotides in their PAMs, Cas12a recognizes a T-rich PAM sequence [24], and thus offers new opportunities for DNA targeting, a characteristic that has enabled the germline correction of a mutant dystrophin gene in zygotes from DMD mice [29]. In the future, metagenomic analysis of uncultivated microbes, as opposed to previously isolated bacteria, could lead to the identification of even more diverse Cas9 functions, illustrated by the recent discovery of several previously unknown CRISPR systems from bacteria and archaea [30].

CRISPR proteins capable of engaging with RNA in an RNA-dependent manner — known as Cas13 effectors — have also been discovered. To date, four Cas13 protein families have been uncovered — Cas13a (originally known as C2c2) [31], Cas13b [32], Cas13c [33,34••], and Cas13d [35,36]. Several of these proteins have thus far been employed to enable nucleic acid detection [37] and the knockdown of RNA transcripts in mammalian cells [34••,36,38]. Given that Cas13 proteins rely on a programmable crRNA to bind RNA, they can also be harnessed as scaffolds to direct RNA editing (Figure 1b), demonstrated by the fusion of a deactivated Cas13b protein to a catalytic domain from the ADAR family of enzymes (adenosine deaminase acting on RNA) for targeted adenosine-to-inosine editing in a therapeutically relevant RNA transcript [34••]. Though this technology is currently restricted to adenosine-to-inosine conversions, the fusion of Cas13 to other catalytic domains with expanded RNA editing capabilities could broaden the repertoire of CRISPR-mediated RNA editing.

Using molecular engineering to enhance the CRISPR toolbox

While identifying new CRISPR systems from known or uncultivated microbes is an effective approach for diversifying the genome editing repertoire, entirely new functionalities can also be engineered onto existing CRISPR proteins. One powerful example of this is the advent of base-editors (BEs), a technology that enables the conversion of one base to another in the absence of a DNA break (Figure 1c). Given the multitude of disorders caused by single-base substitutions, technologies that can specifically convert a mutant base to its wild-type counterpart hold tremendous therapeutic potential. By fusing a Cas9 nickase to a cytidine deaminase or an engineered adenosine deaminase, C•G and A•T base pairs (bps) can be converted to T•A [39••,40,41] and G•C [42•], respectively (BEs containing a cytidine deaminase are typically fused to a uracil glycosylase inhibitor to prevent uracil excision and other processes that can decrease base editing efficiency). BEs provide an especially elegant approach for introducing nonsense mutations to silence gene expression [43,44]. This concept has been leveraged to silence gene expression in the liver of mice, as adenoviral vector-mediated delivery of a BE enabled the knockdown of Pcsk9 [45] and Angptl3 [46], two proteins associated with coronary artery disease. Realizing the considerable therapeutic potential of BEs, however, will require further optimization to ensure their compatibility with AAV vectors, though DNA-free delivery of BEs to certain cell types has been reported [47].

Figure 1



Expanding the CRISPR toolbox. Schematic of the **(a)** Cas12a protein and its ability to process a pre-crRNA array; **(b)** an RNA editor and its ability to mediate the deamination of adenosine to inosine, a nucleobase that is functionally equivalent to guanosine in translation; **(c)** a third-generation base-editor and its ability to catalyze the conversion of a C•G base pair to a T•A base pair. Abbreviations are as follows: UGI, uracil DNA glycosolase inhibitor; XTEN, a flexile peptide linker.

Despite the many successes of CRISPR-Cas9, increasing its effectiveness and specificity remains a top priority, particularly given its potential for gene therapy. To this end, Cas9 variants with refined specificity have been created using a variety of protein engineering strategies [48–51]. Most recently, the continuous directed evolution system known as PACE (phage-assisted continuous evolution) [52] was harnessed to lessen one of the major restrictions associated with the SpCas9 nuclease — the requirement for a PAM sequence — by facilitating the creation of protein variants with the widest range of PAM preferences reported to date [53]. Most impressively, these SpCas9 variants also displayed markedly improved targeting specificity [53]. This work, in combination with a separate study demonstrating that engineered Cas9 proteins can facilitate the mutation-specific disruption of the rhodopsin gene for treatment of retinitis pigmentosa [54], lends support to the idea that personalized Cas9 variants could be created for certain therapeutic applications.

In addition to re-engineering the Cas9 protein for enhanced or specialized functions, the incorporation of ligand-inducible regulatory domains has proven an effective approach for controlling its activity [55]. As a recent example, fusing the Cas9 protein to a destabilized and rapidly degraded version of the FKBP12 protein led to the establishment of a Cas9 variant that edited DNA only in the presence of a synthetic ligand that bound to FKBP12 and shielded the Cas9 protein from proteasomal destruction, enabling ligand-dependent Cas9-mediated tumor reduction in mice [56•]. Similarly, CRISPR activity can be modulated by embedding an aptazyme — a fusion of an aptamer domain and a self-cleaving ribozyme — within the sgRNA [57].

Optimizing the design of the sgRNA has also emerged as an effective approach for increasing on-target CRISPR editing [58,59]. Given that the nucleotides preceding the PAM are particularly important for conferring specificity

[60], a recent report demonstrated that altering the crRNA (one of the two components recognized by the Cas9 protein) to contain DNA nucleotides at more accommodating positions enabled efficient DNA editing with reduced tolerance for nucleotide mismatches compared to the native crRNA [61]. Though it remains unknown if these hybrid crRNA can stimulate the modification of therapeutically relevant genes *in vivo* at an efficiency similar to chemically modified sgRNA [62–65], they nonetheless represent a potentially more cost effective option for certain genome editing applications.

Overcoming the shortcomings of CRISPR genome editing

Though CRISPR-based tools are effective for editing and regulating genes, they nonetheless possess limitations in certain situations. One such example is the relative inefficiency of homology-directed genome editing in non-dividing or post-mitotic cells, such as neurons or cardiomyocytes. Homology-independent approaches, however, have been developed to help overcome this challenge. One such strategy, dubbed HITI for homology-independent targeted integration, relies on the use of a donor template-encoding plasmid or AAV vector that is linearized by Cas9 and subsequently captured by the Cas9-induced DNA break [66••]. This method has facilitated the site-specific insertion of a therapeutic transgene into the rat retina for treatment of retinitis pigmentosa [66••] and is poised to help establish gene therapies for a range of disorders. In addition to homology-independent approaches, the well-documented ability of AAV vectors to stimulate homologous recombination (potentially due to its single-stranded genome or inverted-terminal repeats) [67,68] has been harnessed to tag endogenous genes with a fluorescent reporter in neurons in the cerebral cortex and hippocampus of adult mice via HDR [69]. While the applicability of this approach requires further investigation, this and another study [19] suggest that HDR can be achieved at appreciable rates in post-mitotic cells.

Finally, while many therapeutic efforts involving CRISPR-Cas9 employ viral vectors to mediate its delivery *in vivo*, the persistent expression of Cas9 from nucleic acids could potentially lead to off-target effects [70]. To this end, methods for delivering Cas9 protein — which is degraded soon after its internalization by the cells and thus induces fewer off-target effects [71] — have been developed. For example, injection of gold nanoparticle-encapsulated Cas9 ribonucleoprotein (RNP, recombinant Cas9 protein complexed with an sgRNA), a donor template for HDR and a synthetic polymer that enhances endosomal escape facilitated the correction of the dystrophin gene in a mouse model of DMD [72]. Additionally, cationic lipid-mediated delivery of RNP has enabled the disruption of a dominant deafness-associated allele for the treatment of hearing loss in mouse model of the disorder [73]. Thus, there is increasing evidence supporting the therapeutic potential of Cas9 protein delivery.

Future directions and conclusions

The identification of new, diverse CRISPR systems and the use of molecular engineering to refine and enhance the function of the Cas9 protein is enriching biotechnology and fueling advances in the field of gene therapy. However, a new wave of pioneering innovations in CRISPR technology is already beginning to take shape. In particular, recent work has demonstrated that CRISPR-based transcriptional effectors can be engineered for increased compatibility with AAV vectors for *in vivo* gene activation [74]. Such transcriptional and epigenetic modifiers [74–76,77••] hold tremendous therapeutic potential, as shown by recent work demonstrating that CRISPR-mediated demethylation of a repeat expansion in the 5′ untranslated region of the FMR1 gene could reverse fragile X syndrome abnormalities in patient-derived neurons [78]. Genome-scale CRISPR functional screens [79,80], which have already driven tremendous advances in drug discovery and basic science, are also poised to continue impacting biotechnology. A recent study has demonstrated that AAV vectors can facilitate *in vivo* CRISPR screens in the brain [81], establishing the potential of this technology to dissect gene function in more biologically relevant microenvironments. Given the many innovations that have been realized and are on the horizon, this golden age of genome engineering is poised to continue.

Acknowledgements

We apologize to those authors whose excellent work was omitted due to space constraints. This work was supported by the Judith & Jean Pape Adams Charitable Foundation.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: **A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity**. *Science* 2012, **337**:816-821.
2. Fellmann C, Gowen BG, Lin PC, Doudna JA, Corn JE: **Cornerstones of CRISPR-Cas in drug discovery and therapy**. *Nat Rev Drug Discov* 2017, **16**:89-100.
3. Jusiak B, Cleto S, Perez-Pinera P, Lu TK: **Engineering synthetic gene circuits in living cells with CRISPR technology**. *Trends Biotechnol* 2016, **34**:535-547.
4. Maeder ML, Gersbach CA: **Genome-editing technologies for gene and cell therapy**. *Mol Ther* 2016, **24**:430-446.
5. Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J: **RNA-programmed genome editing in human cells**. *Elife* 2013, **2**: e00471.
6. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA *et al.*: **Multiplex genome engineering using CRISPR/Cas systems**. *Science* 2013, **339**:819-823.

7. Cho SW, Kim S, Kim JM, Kim JS: **Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease**. *Nat Biotechnol* 2013, **31**:230-232.

8. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM: **RNA-guided human genome engineering via Cas9**. *Science* 2013, **339**:823-826.

9. Ceccaldi R, Rondinelli B, D'Andrea AD: **Repair pathway choices and consequences at the double-strand break**. *Trends Cell Biol* 2016, **26**:52-64.

10. Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, Thakore PI, Glass KA, Ousterout DG, Leong KW *et al.*: **RNA-guided gene activation by CRISPR-Cas9-based transcription factors**. *Nat Methods* 2013, **10**:973-976.

11. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK: **CRISPR RNA-guided activation of endogenous human genes**. *Nat Methods* 2013, **10**:977-979.

12. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA *et al.*: **CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes**. *Cell* 2013, **154**:442-451.

13. Kotterman MA, Schaffer DV: **Engineering adeno-associated viruses for clinical gene therapy**. *Nat Rev Genet* 2014, **15**:445-451.

14. Kim E, Koo T, Park SW, Kim D, Kim K, Cho HY, Song DW, Lee KJ, Jung MH, Kim S *et al.*: **In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni**. *Nat Commun* 2017, **8**:14500.

15. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS *et al.*: **In vivo genome editing using *Staphylococcus aureus* Cas9**. *Nature* 2015, **520**:186-191.

16. • Kemaladewi DU, Maino E, Hyatt E, Hou H, Ding M, Place KM, Zhu X, Bassi P, Baghestani Z, Deshwar AG *et al.*: **Correction of a splicing defect in a mouse model of congenital muscular dystrophy type 1A using a homology-directed-repair-independent mechanism**. *Nat Med* 2017, **23**:984-989.
Gene correction via homology directed repair (HDR) is difficult to achieve in non-dividing cells. This report describes an approach to correct splice-site mutations using non-homologous end-joining and demonstrates its ability to correct a pathogeinc mutation in a mouse model of congenital muscular dystrophy type 1A.

17. Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Castellanos Rivera RM, Madhavan S, Pan X, Ran FA, Yan WX *et al.*: **In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy**. *Science* 2016, **351**:403-407.

18. Tabebordbar M, Zhu K, Cheng JKW, Chew WL, Widrick JJ, Yan WX, Maesner C, Wu EY, Xiao R, Ran FA *et al.*: **In vivo gene editing in dystrophic mouse muscle and muscle stem cells**. *Science* 2016, **351**:407-411.

19. Bengtsson NE, Hall JK, Odom GL, Phelps MP, Andrus CR, Hawkins RD, Hauschka SD, Chamberlain JR, Chamberlain JS: **Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy**. *Nat Commun* 2017, **8**:14454.

20. Yang Y, Wang L, Bell P, McMenamin D, He Z, White J, Yu H, Xu C, Morizono H, Musunuru K *et al.*: **A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice**. *Nat Biotechnol* 2016, **34**:334-338.

21. Gaj T, Ojala DS, Ekman FK, Byrne LC, Limsirichai P, Schaffer DV: **In vivo genome editing improves motor function and extends survival in a mouse model of ALS**. *Sci Adv* 2017, **3**:eaar3952.

22. Tervo DG, Hwang BY, Viswanathan S, Gaj T, Lavzin M, Ritola KD, Lindo S, Michael S, Kuleshova E, Ojala D *et al.*: **A designer AAV variant permits efficient retrograde access to projection neurons**. *Neuron* 2016, **92**:372-382.

23. Deverman BE, Pravdo PL, Simpson BP, Kumar SR, Chan KY, Banerjee A, Wu WL, Yang B, Huber N, Pasca SP *et al.*: **Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain**. *Nat Biotechnol* 2016, **34**:204-209.

24. Fonfara I, Richter H, Bratovic M, Le Rhun A, Charpentier E: **The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA**. *Nature* 2016, **532**:517-521.

25. Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS *et al.*: **Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array**. *Nat Biotechnol* 2017, **35**:31-34.

26. Liu Y, Han J, Chen Z, Wu H, Dong H, Nie G: **Engineering cell signaling using tunable CRISPR-Cpf1-based transcription factors**. *Nat Commun* 2017, **8**:2095.

27. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, PRI E, Lin S, Kiani S, Guzman CD, Wiegand DJ *et al.*: **Highly efficient Cas9-mediated transcriptional programming**. *Nat Methods* 2015, **12**:326-328.

28. Tak YE, Kleinstiver BP, Nunez JK, Hsu JY, Horng JE, Gong J, Weissman JS, Joung JK: **Inducible and multiplex gene regulation using CRISPR-Cpf1-based transcription factors**. *Nat Methods* 2017, **14**:1163-1166.

29. Zhang Y, Long C, Li H, McAnally JR, Baskin KK, Shelton JM, Bassel-Duby R, Olson EN: **CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice**. *Sci Adv* 2017, **3**:e1602814.

30. Burstein D, Harrington LB, Strutt SC, Probst AJ, Anantharaman K, Thomas BC, Doudna JA, Banfield JF: **New CRISPR-Cas systems from uncultivated microbes**. *Nature* 2017, **542**:237-241.

31. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DB, Shmakov S, Makarova KS, Semenova E, Minakhin L *et al.*: **C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector**. *Science* 2016, **353**:aaf5573.

32. Smargon AA, Cox DBT, Pyzocha NK, Zheng K, Slaymaker IM, Gootenberg JS, Abudayyeh OA, Essletzbichler P, Shmakov S, Makarova KS *et al.*: **Cas13b is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28**. *Mol Cell* 2017, **65**:618-630 e617.

33. Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI *et al.*: **Diversity and evolution of class 2 CRISPR-Cas systems**. *Nat Rev Microbiol* 2017, **15**:169-182.

34. •• Cox DBT, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, Zhang F: **RNA editing with CRISPR-Cas13**. *Science* 2017, **358**:1019-1027.
This study demonstrates that fusion of a catalytic domain from the adenosine deaminase acting on RNA type 2 family of enzymes to dCas13b enables targeted editing of an RNA transcript in mammalian cells.

35. Yan WX, Chong S, Zhang H, Makarova KS, Koonin EV, Cheng DR, Scott DA: **Cas13d is a compact RNA-targeting type VI CRISPR effector positively modulated by a WYL-domain-containing accessory protein**. *Mol Cell*. in press. https://doi.org/10.1016/j.molcel.2018.02.028.

36. Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD: **Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors**. *Cell*. in press. https://doi.org/10.1016/j.cell.2018.02.033.

37. Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ, Zhang F: **Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6**. *Science* 2018.

38. Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DBT, Kellner MJ, Regev A *et al.*: **RNA targeting with CRISPR-Cas13**. *Nature* 2017, **550**:280-284.

39. •• Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR: **Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage**. *Nature* 2016, **533**:420-424.
This study establishes the concept of base editing. The most effective base-editors described here consisted of a Cas9 nickase fused to a uracil DNA glycosylase inhibitor and a cytidine deaminase.

40. Komor AC, Zhao KT, Packer MS, Gaudelli NM, Waterbury AL, Koblan LW, Kim YB, Badran AH, Liu DR: **Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:**

G-to-T:A base editors with higher efficiency and product purity. *Sci Adv* 2017, **3**:eaao4774.

41. Kim YB, Komor AC, Levy JM, Packer MS, Zhao KT, Liu DR: **Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions**. *Nat Biotechnol* 2017, **35**:371-376.

42. • Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR: **Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage**. *Nature* 2017, **551**:464-471.

This report builds on a 2016 study and describes the creation of base-editing enzymes that can convert A•T base pairs to G•C base pairs, thus expanding the functional capacity of base editing.

43. Kuscu C, Parlak M, Tufan T, Yang J, Szlachta K, Wei X, Mammadov R, Adli M: **CRISPR-STOP: gene silencing through base-editing-induced nonsense mutations**. *Nat Methods* 2017, **14**:710-712.

44. Billon P, Bryant EE, Joseph SA, Nambiar TS, Hayward SB, Rothstein R, Ciccia A: **CRISPR-mediated base editing enables efficient disruption of eukaryotic genes through induction of STOP codons**. *Mol Cell* 2017, **67**:1068-1079 e1064.

45. Chadwick AC, Wang X, Musunuru K: **In vivo base editing of PCSK9 (proprotein convertase subtilisin/kexin type 9) as a therapeutic alternative to genome editing**. *Arterioscler Thromb Vasc Biol* 2017, **37**:1741-1747.

46. Chadwick AC, Evitt NH, Lv W, Musunuru K: **Reduced blood lipid levels with in vivo CRISPR-Cas9 base editing of ANGPTL3**. *Circulation* 2018, **137**:975-977.

47. Rees HA, Komor AC, Yeh WH, Caetano-Lopes J, Warman M, Edge ASB, Liu DR: **Improving the DNA specificity and applicability of base editing through protein engineering and protein delivery**. *Nat Commun* 2017, **8**:15790.

48. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK: **High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects**. *Nature* 2016, **529**:490.

49. Chen JS, Dagdas YS, Kleinstiver BP, Welch MM, Sousa AA, Harrington LB, Sternberg SH, Joung JK, Yildiz A, Doudna JA: **Enhanced proofreading governs CRISPR-Cas9 targeting accuracy**. *Nature* 2017, **550**:407-410.

50. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F: **Rationally engineered Cas9 nucleases with improved specificity**. *Science* 2016, **351**:84-88.

51. Casini A, Olivieri M, Petris G, Montagna C, Reginato G, Maule G, Lorenzin F, Prandi D, Romanel A, Demichelis F *et al.*: **A highly specific SpCas9 variant is identified by in vivo screening in yeast**. *Nat Biotechnol* 2018, **36**:265-271.

52. Esvelt KM, Carlson JC, Liu DR: **A system for the continuous directed evolution of biomolecules**. *Nature* 2011, **472**:499-503.

53. Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z *et al.*: **Evolved Cas9 variants with broad PAM compatibility and high DNA specificity**. *Nature* 2018.

54. Li P, Kleinstiver BP, Leon MY, Prew MS, Navarro-Gomez D, Greenwald SH, Pierce EA, Joung JK, Liu Q: **Allele-specific CRISPR-Cas9 genome editing of the single-base P23H mutation for rhodopsin-associated dominant retinitis pigmentosa**. *CRISPR J* 2018, **1**:55-64.

55. Zhou W, Deiters A: **Conditional control of CRISPR/Cas9 function**. *Angew Chem Int Ed Engl* 2016, **55**:5394-5399.

56. • Senturk S, Shirole NH, Nowak DG, Corbo V, Pal D, Vaughan A, Tuveson DA, Trotman LC, Kinney JB, Sordella R: **Rapid and tunable method to temporally control gene editing based on conditional Cas9 stabilization**. *Nat Commun* 2017, **8**:14370.

This report describes a method for conditionally controlling Cas9 gene-editing activity *in vivo*.

57. Tang W, Hu JH, Liu DR: **Aptazyme-embedded guide RNAs enable ligand-responsive genome editing and transcriptional activation**. *Nat Commun* 2017, **8**:15939.

58. Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK: **Improving CRISPR-Cas nuclease specificity using truncated guide RNAs**. *Nat Biotechnol* 2014, **32**:279-284.

59. Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R *et al.*: **Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9**. *Nat Biotechnol* 2016, **34**:184-191.

60. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O *et al.*: **DNA targeting specificity of RNA-guided Cas9 nucleases**. *Nat Biotechnol* 2013, **31**:827-832.

61. Yin H, Song CQ, Suresh S, Kwan SY, Wu Q, Walsh S, Ding J, Bogorad RL, Zhu LJ, Wolfe SA *et al.*: **Partial DNA-guided Cas9 enables genome editing with reduced off-target activity**. *Nat Chem Biol* 2018, **14**:311-316.

62. Ryan DE, Taussig D, Steinfeld I, Phadnis SM, Lunstad BD, Singh M, Vuong X, Okochi KD, McCaffrey R, Olesiak M *et al.*: **Improving CRISPR-Cas specificity with chemical modifications in single-guide RNAs**. *Nucleic Acids Res* 2018, **46**:792-803.

63. Hendel A, Bak RO, Clark JT, Kennedy AB, Ryan DE, Roy S, Steinfeld I, Lunstad BD, Kaiser RJ, Wilkens AB *et al.*: **Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells**. *Nat Biotechnol* 2015, **33**:985-989.

64. Yin H, Song CQ, Suresh S, Wu Q, Walsh S, Rhym LH, Mintzer E, Bolukbasi MF, Zhu LJ, Kauffman K *et al.*: **Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing**. *Nat Biotechnol* 2017, **35**:1179-1187.

65. Finn JD, Smith AR, Patel MC, Shaw L, Youniss MR, van Heteren J, Dirstine T, Ciullo C, Lescarbeau R, Seitzer J *et al.*: **A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistent in vivo genome editing**. *Cell Rep* 2018, **22**:2227-2235.

66. •• Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, Hatanaka F, Yamamoto M, Araoka T, Li Z *et al.*: **In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration**. *Nature* 2016, **540**:144-149.

HDR-mediated genome editing is challenging to achieve in non-diving cells. This study describes a homology-independent approach for the site-specific insertion of therapeutic transgenes in post-mitotic cells *in vivo*.

67. Russell DW, Hirata RK: **Human gene targeting by viral vectors**. *Nat Genet* 1998, **18**:325-330.

68. Gaj T, Epstein BE, Schaffer DV: **Genome engineering using adeno-associated virus: basic and clinical research applications**. *Mol Ther* 2016, **24**:458-464.

69. Nishiyama J, Mikuni T, Yasuda R: **Virus-mediated genome editing via homology-directed repair in mitotic and postmitotic cells in mammalian brain**. *Neuron* 2017, **96**:755-768 e755.

70. Hendel A, Fine EJ, Bao G, Porteus MH: **Quantifying on- and off-target genome editing**. *Trends Biotechnol* 2015, **33**:132-140.

71. Kim S, Kim D, Cho SW, Kim J, Kim JS: **Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins**. *Genome Res* 2014, **24**:1012-1019.

72. Lee K, Conboy M, Park HM, Jiang F, Kim HJ, Dewitt MA, Mackley VA, Chang K, Rao A, Skinner C *et al.*: **Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair**. *Nat Biomed Eng* 2017, **1**:889-901.

73. Gao X, Tao Y, Lamas V, Huang M, Yeh WH, Pan B, Hu YJ, Hu JH, Thompson DB, Shu Y *et al.*: **Treatment of autosomal dominant hearing loss by in vivo delivery of genome editing agents**. *Nature* 2018, **553**:217-221.

74. Liao HK, Hatanaka F, Araoka T, Reddy P, Wu MZ, Sui Y, Yamauchi T, Sakurai M, O'Keefe DD, Nunez-Delicado E *et al.*: **In vivo target gene activation via CRISPR/Cas9-mediated trans-epigenetic modulation**. *Cell* 2017, **171**:1495-1507 e1415.

75. Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA: **Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers**. *Nat Biotechnol* 2015, **33**:510-517.

76. Liu XS, Wu H, Ji X, Stelzer Y, Wu X, Czauderna S, Shu J, Dadon D, Young RA, Jaenisch R: **Editing DNA methylation in the mammalian genome**. *Cell* 2016, **167**:233-247 e217.

77. •• Morita S, Noguchi H, Horii T, Nakabayashi K, Kimura M, Okamura K, Sakai A, Nakashima H, Hata K, Nakashima K *et al.*: **Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions**. *Nat Biotechnol* 2016, **34**:1060-1065.

This paper uses an engineered CRISPR system to activate the FMR1 gene using epigenome editing in neurons derived from fragile X syndrome induced pluripotent stem cells. This work effectively demonstrates the therapeutic potential of CRISPR-based epigenetic effectors.

78. Liu XS, Wu H, Krzisch M, Wu X, Graef J, Muffat J, Hnisz D, Li CH, Yuan B, Xu C *et al.*: **Rescue of fragile X syndrome neurons by DNA methylation editing of the FMR1 gene**. *Cell* 2018, **172**:979-992 e976.

79. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelson T, Heckl D, Ebert BL, Root DE, Doench JG *et al.*: **Genome-scale CRISPR-Cas9 knockout screening in human cells**. *Science* 2014, **343**:84-87.

80. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H *et al.*: **Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex**. *Nature* 2015, **517**:583-588.

81. Chow RD, Guzman CD, Wang G, Schmidt F, Youngblood MW, Ye L, Errami Y, Dong MB, Martinez MA, Zhang S *et al.*: **AAV-mediated direct in vivo CRISPR screen identifies functional suppressors in glioblastoma**. *Nat Neurosci* 2017, **20**:1329-1341.