ANNUAL REVIEWS

Annual Review of Cancer Biology

Modeling Cancer in the CRISPR Era

Andrea Ventura[1] and Lukas E. Dow[2]

[1]Department of Cancer Biology and Genetics, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA; email: venturaa@mskcc.org

[2]Department of Medicine, Hematology and Medical Oncology, Weill Cornell Medicine, New York, NY 10021, USA; email: lud2005@med.cornell.edu

Annu. Rev. Cancer Biol. 2018. 2:111–31

First published as a Review in Advance on November 15, 2017

The *Annual Review of Cancer Biology* is online at cancerbio.annualreviews.org

https://doi.org/10.1146/annurev-cancerbio-030617-050455

Keywords

CRISPR, Cas9, in vivo, cancer, genome editing

Abstract

In just a few short years, CRISPR/Cas9 genome editing has fundamentally changed basic, agricultural, and biomedical research, but no field has felt a more profound impact than cancer research. The ability to quickly and precisely manipulate the genome has opened the floodgates for a new and more elaborate understanding of how genes and gene regulation influence disease. Here we review how the development and implementation of CRISPR-based technology is redefining the way we study cancer, and ultimately how it may be used to improve treatment outcomes.

INTRODUCTION

Well, there's a lot going on. If you haven't heard of CRISPR, we've got some catching up to do. Literally, CRISPR stands for clustered regularly interspaced short palindromic repeats, a key component of bacterial adaptive immunity. But, as it is better known, "crisper" has caused a revolution in genetic research, ignited fierce competition in biotech and pharmaceutical development, and captured the imagination of the general public in a way few basic science discoveries ever do. For oncology research, CRISPR has been a phenomenon, providing an opportunity to interrogate the cancer genome with unparalleled speed and precision. In this review, we describe the rapid development and application of CRISPR tools over the past five years and explore how innovations in genome editing tools are driving new discoveries in cancer biology, defining the factors that influence oncogenesis, and leading to new opportunities in diagnosis and treatment.

BC: BEFORE CRISPR

Since the development of recombinant DNA technology, targeted manipulation of genes in mammalian cells has been the primary approach for interrogating cell and molecular function. In cancer biology, this proved key to define the functions of oncogenes and tumor suppressors. Initially, genetic manipulation efforts focused on the enforced expression of exogenous complementary DNAs (cDNAs) through plasmid transfection and viral delivery; around the turn of the century, the tool kit expanded to allow the suppression of endogenous gene products through antisense and RNA interference (RNAi) technologies. For many years, it has also been possible to manipulate the epigenome of cells with drugs such as demethylating agents; however, because of the lack of specificity, such approaches are more of a sledgehammer than a scalpel when it comes to dissecting gene function. In the five to ten years leading up to the dawn of the CRISPR era, we witnessed the development and optimization of gene editing tools such as zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs), that promised to change the way we study gene function. While the impact of ZFNs and TALENs was less than originally touted, it is hard to know how these technologies could have evolved, as they were quickly overshadowed by CRISPR/Cas9.

A BRIEF HISTORY OF CRISPR

Prior to the publication of three seminal papers in early 2013 (Cong et al. 2013, Jinek et al. 2013, Mali et al. 2013), for most people, CRISPR was the place in the refrigerator where fruit and vegetables went to die. While CRISPR was a focus of intense research for those studying bacterial adaptive immunity (Marraffini & Sontheimer 2010), few in cancer research had heard of it, and fewer still saw its potential.

The history of CRISPR actually dates back 30 years to 1987, when Nakata and colleagues identified "highly homologous sequences of 29 nucleotides . . . arranged as direct repeats" (Ishino et al. 1987, p. 5432). In this work, the authors noted that the "the biological significance of these sequences is not known" (p. 5432): the perfect cliffhanger for the sequel that was to come 25 years later. By 2012, CRISPR and CRISPR-associated (Cas) proteins had been well characterized, including the generation of databases and algorithms for finding and cataloging CRISPR sequences (Grissa et al. 2007). The biggest leap forward came from the discovery that the RNA component of the CRISPR system could be engineered or programmed to target alternate DNA sequences (Jinek et al. 2012). It did not take long for multiple labs to realize the potential of this system for genome editing in mammals, and soon after, three groups, led by George Church, Jennifer Doudna, and Feng Zhang, described the first iterations of CRISPR/Cas9-based genome editing in human cells (Cong et al. 2013, Jinek et al. 2013, Mali et al. 2013). The rest, as they say, is history.

CRISPR 101

In bacteria and archaea, CRISPR-based adaptive immunity is a complex system, involving multiple Cas proteins and RNA complexes, and many different subtypes have evolved in different species. In this review, we focus predominantly on the minimal type II CRISPR system, as described by Church, Doudna, and Zhang. This consists of a single (or synthetic) guide RNA (sgRNA) and a protein, Cas9. When expressed together in cells or combined from purified individual components, the resulting ribonucleoprotein (RNP) complex is sufficient to induce targeted DNA alterations in almost any cell type (or cell-free system).

Cas9 is a large DNA endonuclease that induces double-strand breaks (DSBs) through two adjacent catalytic domains. It is targeted to specific regions of the genome by association with an sgRNA that recognizes DNA sequences through Watson-Crick DNA-RNA base pairing (**Figure 1**). Docking and DNA cleavage proceed in a stepwise manner, whereby Cas9-sgRNA complexes first survey the genome for small nucleotide consensus sequences or protospacer adjacent motifs (PAMs), which act as the primary point of contact for the protein. For Cas9 derived from *Streptococcus pyogenes* (SpCas9), currently the most commonly used in genome editing experiments, the PAM sequence is NGG and—with lower efficiency—NAG. PAM recognition destabilizes the DNA helix adjacent to the PAM, enabling unwinding and recognition of the target sequence by the bound RNA molecule. Single-molecule imaging and binding assays suggest that Cas9-sgRNA complexes are capable of associating tightly with genomic regions that contain 8–9 base pairs (bp) of complementary seed sequence proximal to a consensus PAM, while lack of complementarity immediately adjacent to the PAM promotes rapid dissociation (Singh et al. 2016, Sternberg et al. 2014). Given sufficient homology and interaction energy between the Cas9-sgRNA complexes and DNA, the Cas9 nuclease domains mediate PAM-dependent, double-strand DNA cleavage that is thought to generate a blunt cut. The resulting DSB is either repaired seamlessly by homology directed repair (HDR) (in which case it is likely recut by Cas9) or by error-prone nonhomologous end joining (NHEJ) machinery, which often results in the generation of small indels at the cut site that prevent recleavage by the Cas9-sgRNA complex. Providing a single-stranded or double-stranded DNA template for repair can promote integration of specific alterations or larger transgenic insertions.

From a practical standpoint, the beauty of CRISPR/Cas9 is its simplicity. The straightforward rules that govern DNA recognition enable users to easily design their own CRISPR reagents and, perhaps more importantly, to alter target specificity by changing just a 17- to 20-bp DNA recognition sequence within a larger sgRNA scaffold (Jinek et al. 2012). Such is its ease of use, that within a year of initial publication, hundreds of papers described effective CRISPR-based genome editing in diverse organisms, from yeast to humans (Dow 2015). Indeed, the broad utility and the effectiveness of CRISPR has led to a number of exciting applications outside cancer research, including the modification of plants for improved agriculture (Shan et al. 2013, Woo et al. 2015), the generation of gene drives to eliminate disease carriers in insect populations (Gantz et al. 2015, Hammond et al. 2016), and possible clinical treatments for monogenic disorders such as muscular dystrophy (Bengtsson et al. 2017, Long et al. 2016, Nelson et al. 2016, Tabebordbar et al. 2016).

In addition to *S. pyogenes*, numerous other bacteria and archaea harbor Cas enzymes that are suitable for gene editing in mammalian cells. Among the many that have been tested (Ran et al. 2015), a few have risen as the go-to alternative systems for genome modification. These are Cas9 from *Staphylococcus aureus* (SaCas9) (Ran et al. 2015), Cas12a (or Cpf1) from *Acidaminococcus* and *Lachnospiraceae* (Zetsche et al. 2015), and the RNA-editing Cas13a (C2c2) enzyme from *Leptotrichia shahii* (Abudayyeh et al. 2016). In addition, both SpCas9 and SaCas9 have been extensively modified through molecular evolution to define new enzymes with altered PAM specificity (Kleinstiver et al.

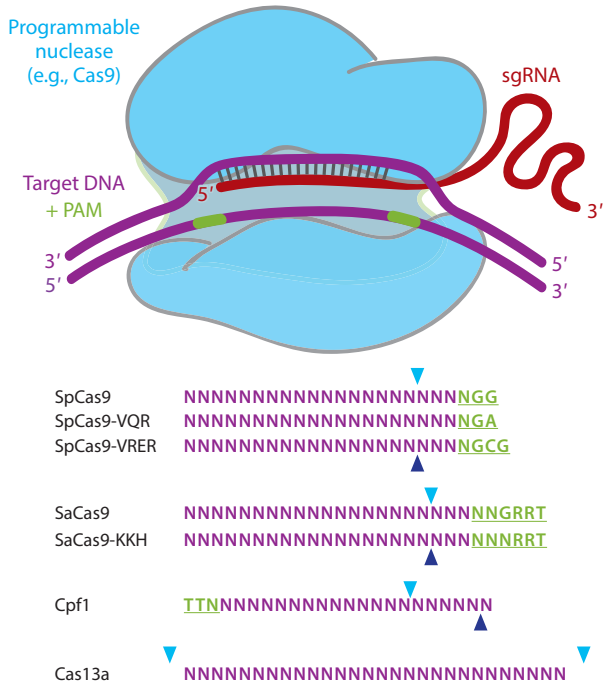

Figure 1

CRISPR/Cas9 targeting. Single-stranded guide RNAs (sgRNAs) bind Cas proteins and direct them to target sites on DNA or RNA. Recognition initiates at the protospacer adjacent motif (PAM) and proceeds by Watson-Crick DNA-RNA base pairing through the protospacer (target sequence). Each Cas protein (only some examples are shown) has different requirements for the PAM sequence and some variation in the optimal length of the protospacer [e.g., 20 bp for Cas9 derived from *Streptococcus pyogenes* (SpCas9) and 21 bp for Cas9 derived from *Staphylococcus aureus* (SaCas9)]. Engineered variants of SpCas9 (VQR and VRER) and SaCas9 (KKH) have distinct PAM requirements. Target recognition results in endonuclease cleavage within the protospacer at positions indicated by the blue arrows. Dark blue indicates cleavage on the complementary strand. Unlike Cas9, which produces blunt ends, Cpf1 cleavage produces a staggered cut, similar to restriction endonucleases. Cas13a targets RNA molecules and cleaves both targets 5′ and 3′ of the protospacer target site.

2015a,b), expanding the number of genomic regions that can be targeted with CRISPR systems (**Figure 1**).

The widespread adoption of CRISPR tools has created a virtuous cycle of innovation, optimization, and refinement, facilitated by the willingness of the research community to quickly share new CRISPR tools at not-for-profit repositories like Addgene. This shift in technology is reflected in a rapidly growing literature. There is clearly too much CRISPR data to comprehensively review all of the technologies and applications. Instead, we will discuss how CRISPR/Cas9 tools have changed the landscape of cancer research, both in the dish and in the animal, with a particular focus on those aspects of gene regulation and oncogenic transformation that have been challenging or impossible to study with previously existing tools.

PRECISION GENETICS IN THE DISH

For decades, the culture and manipulation of patient-derived cancer cell lines has been the workhorse of cancer research. During this time, defining the impact of specific genes or genetic

alterations has been largely restricted to measuring the effect of suppressing a target (through antisense or RNAi), overexpressing a cDNA, or assessing correlations over many genetically profiled lines. While we have learned a great deal about cancer genetics and individual genes from such approaches, none truly recapitulates the events that occur during tumorigenesis. A few labs took on the laborious task of creating isogenic lines by traditional gene-targeting methods (Sur et al. 2009, Yun et al. 2009), but these were rare and restricted to specific cell lines and a small number of genomic targets. Enter CRISPR/Cas9. Off-the-shelf CRISPR tools suddenly made it easy to create individual or multiplexed gene knockout lines (Ran et al. 2013).

While mutational disruption of genes is straightforward in most cases, the introduction of specific gene alterations or large transgenic insertions has been less adaptable than originally hoped. Initial studies reported high-efficiency (15% of clones) creation of targeted knock-in alleles in mouse zygotes or embryonic stem cells (ESCs) (Andersson-Rolf et al. 2017, Yang et al. 2013), but subsequent efforts to manipulate distinct loci in mouse zygotes or to generate isogenic lines by HDR in somatic cells have proven less efficient. This prompted efforts to improve HDR-mediated gene editing in vitro. Many of these strategies have exploited the distinct differences in DNA repair mechanisms that are active in different stages of the cell cycle by synchronizing cells in G2/M phase (Lin et al. 2014, Yang et al. 2016) or by limiting Cas9 activity to this phase of the cell cycle (Gutschner et al. 2016), where the enzymes required for HDR are most highly expressed. Others have taken a pharmacologic approach to directly block NHEJ repair and thereby bias toward the use of HDR for DNA repair events (Chu et al. 2015, Maruyama et al. 2015, Yu et al. 2015). In a different approach, Corn and colleagues showed that the use of asymmetric and strand-specific donor DNA templates had a significant impact on the rate of HDR (Richardson et al. 2016). Each of these methods shows promise, but none has fully solved the problem. What works for one cell type may not work well for another, and what works at one locus may not be true elsewhere. Thus, although CRISPR-based HDR-mediated genome editing is certainly more effective than conventional gene-targeting methods, there is room for improvement.

As an alternate strategy, several groups working in parallel developed fusion proteins that tethered Cas9 to the cytidine deaminases, AID or APOBEC (**Figure 2**). Depending on the configuration and positioning of the deaminase, these hybrid proteins enable targeted base substitutions within a narrow (5–10 bp) window (Komor et al. 2016, Nishida et al. 2016) or more broadly (up to 100 bp) across the target region (Hess et al. 2016, Ma et al. 2016). Early work indicates that such approaches can be used to recreate or repair disease-associated mutations (Komor et al. 2016) or to accelerate gene evolution to prospectively identify drug resistance mechanisms (Hess et al. 2016, Ma et al. 2016). We are in the early days of Cas9-mediated base editing, but the possibilities are exciting and time will tell which systems will be embraced by the research community.

Despite these challenges, there are many successful examples of using CRISPR to create targeted DNA edits, alone or in combination with NHEJ-mediated gene disruption. Several labs have demonstrated the ability to create isogenic induced pluripotent stem cell lines for disease modeling or to perform disease allele correction in primary patient-derived cells (Dever et al. 2016, Howden et al. 2015, H.L. Li et al. 2015, Paquet et al. 2016, Schwank et al. 2013, Soldner et al. 2016), including large chromosomal aberrations (Park et al. 2015). These initial studies have focused on single genetic aberrations, but CRISPR/Cas9 technology is particularly well suited to engineer and study the complex genetic configurations observed in human cancers. Two elegant studies (Drost et al. 2015, Matano et al. 2015) reported the sequential genome editing of wild-type human colon organoids to recapitulate the proposed Vogelgram of stepwise accumulated mutations in colorectal cancer (Fearon & Vogelstein 1990). For this, the authors induced both loss-of-function indels and HDR-mediated oncogenic point mutations that could be isolated through the targeted withdrawal of growth factors or the use of pathway inhibitors. Sato and colleagues also showed

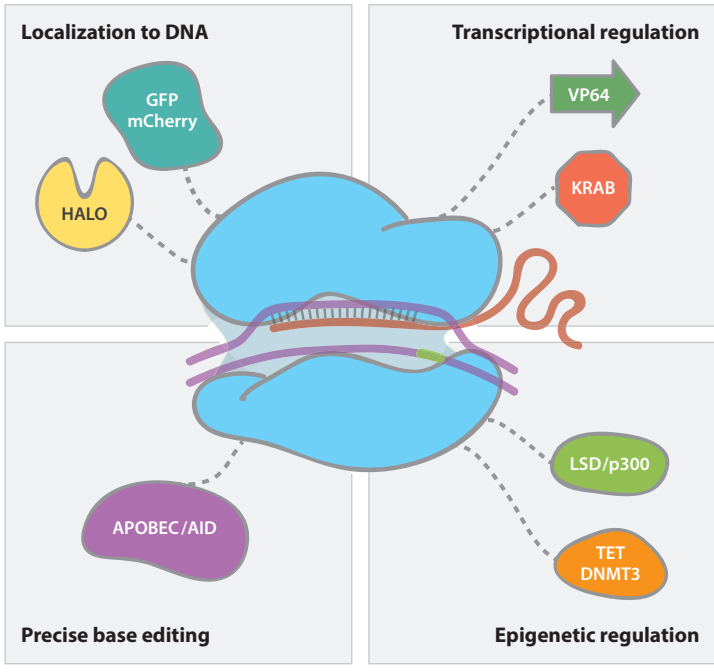

Figure 2

Nonendonuclease functions of CRISPR/Cas9 complexes. High-fidelity target recognition by CRISPR/Cas complexes provides an opportunity to manipulate and measure gene regulation in different ways. Gene expression can be immediately controlled by directly or indirectly tethering Cas9 to transcriptional activation (VP64) or repression (KRAB) domains. Local DNA methylation or histone modifications can be altered by associating Cas9 with various epigenetic regulators (e.g., TET or DNMT3). Fusion with cytidine deaminases AID or APOBEC allows the induction of specific single nucleotide bases changes, while linking Cas9 with fluorescent reporters or biochemical tags provides a means to localize specific DNA sequences within living cells or perform specific biochemical assays.

that CRISPR could be adapted to engineer large knock-in, lineage-tracing cassettes to primary human tumor organoids, providing an unprecedented level of genetic flexibility (Shimokawa et al. 2017). While the efficient selection approaches described in these studies may not be applicable to all situations, this work is a powerful illustration of the capabilities of CRISPR for interrogating the cancer genome in primary human cells.

DESIGN, REFINE, AND REPEAT

Refinement and optimization of CRISPR systems continue at breakneck speed. From a mutagenesis perspective, the focus is on potency and specificity. The availability of genome-scale screening data has gone a long way to defining sequence features that mediate efficient CRISPR cutting across different cell types, and there are now numerous online tools that predict sgRNA potency. But potency is only one part of the equation when considering the efficiency of gene disruption. Because DNA repair following Cas9-mediated cleavage is somewhat random, even the most effective sgRNA designed to disrupt a protein-coding gene will induce loss-of-function frameshift mutations only two-thirds of the time; the remaining are in-frame insertions or deletions that may or may not disrupt protein function. While individual sgRNAs may show bias in the types of repair events (van Overbeek et al. 2016), the chance of generating biallelic loss-of-function

events in the absence of selective pressure is, on average, less than 50%. This is particularly problematic when using pooled libraries for dropout screens, where the investigators are interested in finding sgRNAs that are depleted rather than enriched. To overcome this limitation, Vakoc and colleagues described an approach that targets functional or evolutionarily conserved protein domains, under the assumption that such regions are essential for protein function (Shi et al. 2015). They showed that distinct sgRNAs that target the same (essential) gene can have dramatically different outcomes in polyclonal populations, but those focused on critical domains reproducibly drive loss-of-function phenotypes. We expect that researchers will adopt approaches like this in the development of future sgRNA prediction tools and large-scale libraries to further improve the generation of gene knockouts using CRISPR/Cas9.

The final, but equally important, part of the problem is specificity. Computational prediction and experimental evidence (Fu et al. 2013, 2014; Kleinstiver et al. 2016b; Perez et al. 2017; Tsai et al. 2015) highlight the promiscuity of some sgRNA sequences throughout the genome, which can induce localized indels at off-target sites or even drive unintended chromosomal rearrangements (Perez et al. 2017, Weber et al. 2015). Improved computational pipelines provide an option to eliminate troublesome sgRNAs and are particularly useful when there are many possible sgRNAs to choose from. However, in cancer research, target mutations are often dictated by their prevalence in human disease, and it is important to model specific hot spot mutations, regardless of whether a high-confidence sgRNA is available. To more broadly increase CRISPR specificity, two groups have independently developed high-fidelity Cas9 variants through rational design and screening mutagenesis (Kleinstiver et al. 2016a, Slaymaker et al. 2016). Slaymaker and colleagues iteratively tested mutations at positively charged residues within the DNA binding groove, with the goal of weakening nonspecific interactions between Cas9 and the target, while Kleinstiver and colleagues focused on disrupting the interaction of Cas9 with the phosphate backbone of the target DNA strand. Both developed Cas9 variants showing improved specificity against a range of selected targets, although off-target activity was not completely eliminated at all sites. Furthermore, in some of the reported cases (Kleinstiver et al. 2016a, Slaymaker et al. 2016), and in our experience, improved specificity came at the cost of reduced potency against the intended genomic target. While in some settings such a trade-off can be acceptable (for example, when individual clones can be easily isolated), it is not acceptable when highly efficient on-target editing is needed—for example, with pooled dropout screens or in vivo gene therapy approaches. Despite these current limitations, we expect future optimization and refinement of these high-fidelity variants will provide powerful gene editing resources.

THE SCREEN IS DEAD, LONG LIVE THE SCREEN

At the dawn of this century, following the publication of the first draft of the human genome, oncology research was transformed by the idea of functionalizing the cancer genome using focused and genome-wide genetic screens. The technology du jour enabling such a large-scale challenge was RNAi, and it led to the identification of hundreds of disease-associated alleles and potential drug targets through negative selection and synthetic lethality screens. However, due to the pervasive presence of off-target effects associated with RNAi, as well inherent limitations of using cancer cell lines, many of these findings have translated poorly in vivo. As a consequence, the field was hungry for a new technology, and CRISPR fit the bill. The modular cloning pipeline and simple nature of sgRNA design lent itself perfectly to library production, and the first genome-wide CRISPR screens proved highly successful (Shalem et al. 2014, Wang et al. 2014). Unlike short hairpin RNA–driven gene knockdown, which induces different levels of gene silencing for each specific sequence, CRISPR provided a binary readout (mutant versus not mutant), produced consistent

results between distinct sgRNAs, and displayed high signal-to-noise ratio. Over the past few years, CRISPR screens have been used to identify new tumor-suppressor genes (Chen et al. 2015, Katigbak et al. 2016), define genes and pathways that lead to drug resistance (Kurata et al. 2016, Shalem et al. 2014), direct druggable targets (Munoz et al. 2016, Shi et al. 2015, Steinhart et al. 2017), and more recently, explore the factors that mediate immune cell function (Jaitin et al. 2016, Parnas et al. 2015). CRISPR screening can be so effective that four groups recently described the ability to combine pooled screens with single-cell transcriptomics (Adamson et al. 2016, Datlinger et al. 2017, Dixit et al. 2016, Jaitin et al. 2016), allowing complex phenotypic screens that go beyond the routine readouts of cell proliferation, death, and reporter gene expression.

CRISPR has not only improved screening capabilities, it has opened new doors for genetic analysis. Particularly useful is the possibility to generate arbitrary genomic deletions by using pairs of sgRNAs flanking the genomic feature under investigation. Combined with strategies to generate large libraries of sgRNA pairs (Vidigal & Ventura 2015), CRISPR provides an unprecedented opportunity to interrogate both noncoding RNAs and nontranscribed regions of the genome. For example, Zhu et al. (2016) developed a paired sgRNA library to induce targeted deletion of long noncoding RNAs (lncRNAs) and, through this, implicated nine distinct lncRNAs in regulating proliferation in cancer cell lines. In addition, multiple studies have adapted CRISPR libraries to decode the functional regions of enhancer elements. Because of the vast sequence space of the noncoding genome and the number of possible CRISPR target sites, these types of screens require some biological rationale to focus the libraries. For instance, Canver et al. (2015) used an sgRNA tiling approach across DNase hypersensitive sites implicated in regulation of fetal hemoglobin from GWAS studies. Agami and colleagues narrowed their search using p53 or estrogen receptor α ChIP-seq (chromatin immunoprecipitation sequencing) data, in combination with known chromatin marks, and predicted binding motifs (Korkmaz et al. 2016), while Sanjana et al. (2016) focused a tiled library around three genes previously implicated in BRAF inhibitor resistance in an exploratory search for noncoding elements that could impact therapy response. On the flip side, the nature of CRISPR-mediated mutagenesis also provides an unexpected advantage; the somewhat random nature of DNA repair following Cas9 cleavage at specific sites creates its own library of genomic lesions. Targeted deep sequencing of indel frequencies in specific loci can provide base pair resolution of functional elements that regulate gene expression (Canver et al. 2015).

In sum, CRISPR is proving to be a game changer for functional genomics. The ability to consistently identify essential genes across multiple independent experiments with genome-scale libraries leads many to believe that current CRISPR tools are capable of true saturating genetic screens. Have we entered a world without false negatives? The answer right now is most likely "no," but if we have learned anything from the CRISPR revolution, it is to never say never.

TEACHING THE NEW DOG OLD TRICKS

Cancer is more than just a collection of mutations. Cancer cells show widespread changes to transcriptional and epigenetic networks that are critical for the tumorigenic phenotype. However, interrogating these effects is not always easy. Driving enforced expression of particular genes relies almost exclusively on the introduction of cDNA constructs, and in the era before CRISPR, there were few ways, if any, to target epigenetic mediators. This radically changed with the realization that Cas9-sgRNA complexes could act as homing beacons to specific genomic regions and with the subsequent emergence of many new CRISPR-derived technologies to activate and inhibit endogenous gene transcription, control the chromatin landscape, and localize DNA mutagens for precise base editing (**Figure 2**).

Transcriptional Regulation

As discussed above, Cas9 proteins have two critical functions: (*a*) to bind the sgRNA that determines genomic targeting and (*b*) to cleave the target DNA. The realization that disrupting the nuclease activity of Cas9 (through specific mutations in the two endonuclease domains) does not alter binding to the genomic target led to a range of applications beyond DNA cleavage (reviewed in detail by Dominguez et al. (2016). For example, fusions of nuclease dead Cas9 (dCas9) to VP64 via transcriptional activation (CRISPRa) or to KRAB via transcriptional interference (CRISPRi) domains was used to control expression of endogenous genes (Gilbert et al. 2013). Similarly, localizing the Cas9-sgRNA complex to gene bodies was shown to be sufficient to disrupt transcriptional elongation (Qi et al. 2013). Since targeting specificity in this system, like with regular Cas9, is driven only by the sgRNAs, multiple labs quickly produced large libraries for functional genetic screens (Gilbert et al. 2014, Konermann et al. 2015). On the face of it, such libraries closely resemble RNAi and cDNA or open reading frame (ORF) libraries that have been used extensively for genetic screens; however, CRISPR-mediated tools have some important differences. First, CRISPRa libraries are easier to generate than ORF-based systems due to the small, consistent size of the sgRNAs. Second, CRISPRi and CRISPRa act directly on the endogenous gene, preserving other regulatory elements such as 5′ and 3′ untranslated regions. The direct control of transcription is also a key feature of CRISPRi, as it provides a means to suppress expression of not only protein-coding genes, but also miRNAs (Chang et al. 2016, Zhao et al. 2014) and lncRNAs (Liu et al. 2017), which had proven challenging with RNAi-based tools. Of course, CRISPRa and CRISPRi are not without drawbacks: Both systems depend on appropriate positioning of the sgRNA-Cas9 complex at the transcription start site (Radzisheuskaya et al. 2016). Given the complex regulation of some genes by multiple or lineage-dependent transcription factors, what works in one cell type may not work in another. Defining these rules will require a lot more data.

Epigenome Regulation

The development of dCas9 has also enabled targeted epigenetic editing. For instance, specific dCas9 fusions can enable the targeted histone demethylation (dCas9-LSD1) (Kearns et al. 2015) and acetylation (dCas9-p300) (Hilton et al. 2015), as well as the methylation (dCas9-DNMT3A), and demethylation (dCas9-TET) of DNA (Liu et al. 2016, Vojta et al. 2016) (**Figure 2**). To date, most of these studies have provided proof-of-concept without major biological insights, but the approach clearly has wide-ranging applications. For example, targeted methylation of CTCF binding sites can disrupt DNA looping and alter gene expression in neighboring regions (Liu et al. 2016). More standard CRISPR tools have also been adapted to manipulate gene expression by repositioning regulatory elements. In an elegant set of experiments, pairs of sgRNAs were to invert or delete genomic regions that dictate topologically associated domains (TADs) and show that disruption of three-dimensional chromatin interactions has a significant impact on developmental patterning (Lupianez et al. 2015). Although we have yet to see application of CRISPR-based epigenome editing in cancer cells, these tools will clearly improve our understanding of the role of epigenetic alterations in tumorigenesis and therapy response.

IT'S ALIVE!: IN VIVO APPLICATIONS OF CRISPR-Cas SYSTEMS

For more than three decades, genetically engineered mice have been used to study the development of autochthonous tumors in a physiologic context (Van Dyke & Jacks 2002). Until recently, pronuclear injection of transgenes in the mouse zygote and gene targeting by homologous recombination in embryonic stem cells were the most commonly used strategies to generate transgenic,

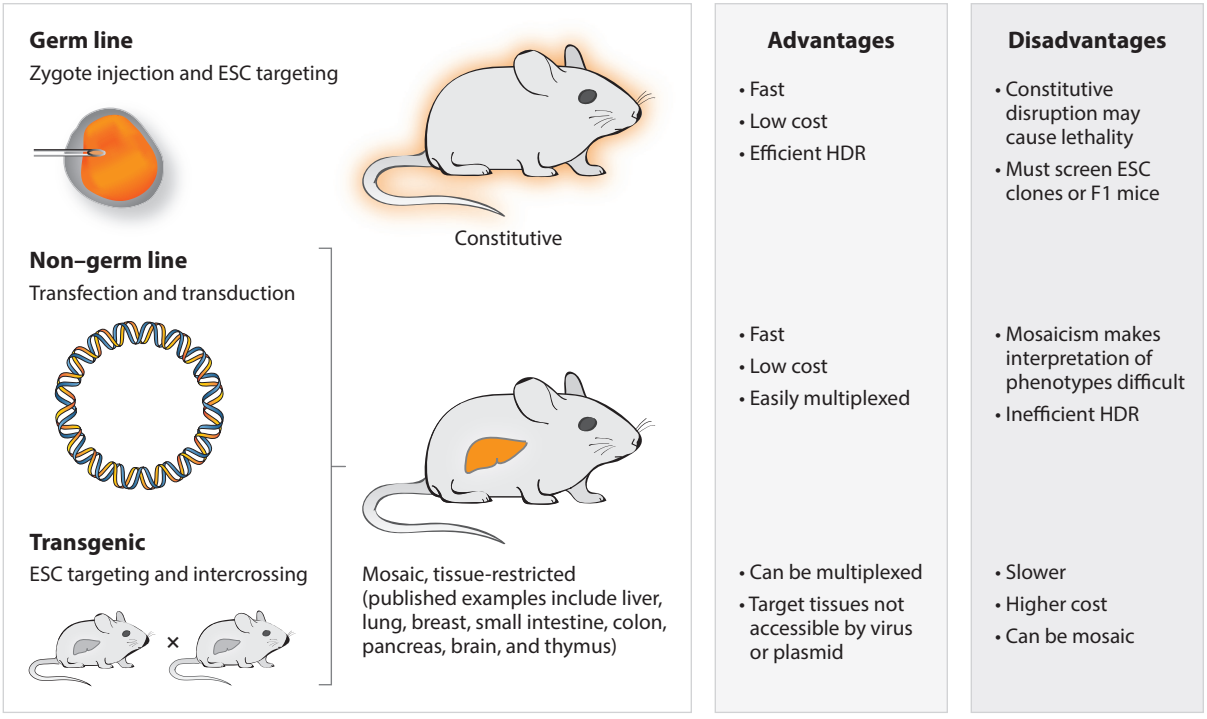

Figure 3

In vivo genome editing with CRISPR. CRISPR-based modifications can be engineered in the germ line of mice, such that each cell carries the mutation or is introduced into somatic tissues of the adult animal. Somatic genome editing can be achieved by delivering CRISPR vectors (as naked DNA or viruses) to target tissues or by generating transgenic mice that carry all or some of the required CRISPR components. While each strategy has strengths and weaknesses (some examples are listed on the right), cancer modeling usually requires the generation of mosaic or tissue-restricted genomic alterations that are only achievable by somatic CRISPR strategies. Abbreviations: ESC, embryonic stem cell; F1, first filial generation; HDR, homology directed repair.

knockout, and knock-in strains (Bouabe & Okkenhaug 2013, Capecchi 2005). Although these traditional approaches have been substantially improved over the years and remain essential tools for cancer researchers, their intrinsic limitations (they are time consuming, costly, and technically complex) make them less useful to systematically interrogate the increasing number of cancer-associated mutations that are being identified in human cancers.

The development of CRISPR technology has greatly simplified the generation of targeted mutations in model organisms and expanded the options available to cancer researchers, allowing for the first-time experiments that until a few years ago would have been either impossible or prohibitively complex. Here, we focus on studies using the laboratory mouse, but many of these ideas can be applied to other model organisms (Guo et al. 2014, Jao et al. 2013, Ma et al. 2014, Niu et al. 2014, Wan et al. 2015). Broadly speaking, in vivo applications of the CRISPR/Cas system fall into two categories: germ line genome editing and somatic genome editing (**Figure 3**), each with its unique advantages and limitations.

GERM LINE GENOME EDITING: REALITY VERSUS HYPE

Germ line genome editing allows the generation of animals harboring the desired genetic modification in every cell. A quick way of doing this is by injecting sgRNA targeting the desired genes

and the mRNA encoding Cas9—or by delivering the preassembled RNP—into pronuclear stage, one-cell embryos, which are then implanted in the uterus of a pseudopregnant female (Wang et al. 2013). Using this strategy, Wang and colleagues reported biallelic inactivation of the target gene in up to 95% of newborn mice.

This strategy is not limited to generating inactivating mutations. Much more specific alterations can be seamlessly generated by providing an appropriate donor template and taking advantage of HDR-mediated genome editing. For example, co-injection of a short DNA oligonucleotide carrying the desired genetic modification can be used to introduce point mutations (Wang et al. 2013), loxP sites, and short epitope tags (Yang et al. 2013), while larger fragments such as fluorescent proteins can be added to a gene of interest by injecting plasmid DNA as a donor template (Yang et al. 2013).

Although editing one-cell embryos has proven generally effective and allows for scarless editing of the genome—no selection marker is needed—the initial parent (F0) generation needs to be screened for founders with the desired genotype, with no guarantee of success. In addition, the process's initially reported high success rate has not proven universal, especially with respect to HDR-mediated gene editing, and several groups have shown high rates of somatic mosaicism for the desired genetic modification in F0 animals (Oliver et al. 2015, Yang et al. 2013, Yen et al. 2014).

An appealing way to overcome these limitations is to edit murine ESCs rather than one-cell embryos. With this approach, many ESC clones can be screened to identify those harboring the desired genetic change, and selected clones can then be extensively characterized in vitro or subjected to additional rounds of mutagenesis before being used to generate the edited animals (**Figure 3**).

A significant advantage of germ line genome editing over conventional gene-targeting strategies is the ability to multiplex, that is, performing simultaneous and biallelic editing of multiple loci (Ma et al. 2014, Wang et al. 2013, Yang et al. 2013, Yin et al. 2015, Zhou et al. 2014). Although the risk of also hitting off-target loci increases with the number of gRNAs injected, this strategy has been successfully used to inactivate entire gene families in mice in a single experiment (Wang et al. 2013).

IN VIVO SOMATIC GENOME EDITING

CRISPR has certainly simplified and accelerated the generation of genetically engineered animal models, but it is the field of somatic mutagenesis that has been most profoundly revolutionized by the development of programmable endonucleases. The ability to generate specific somatic mutations in vivo in only a subset of cells of an organism is the Holy Grail for cancer researchers, enabling researchers to model the stochastic nature of cancer initiation and progression in the most physiologic context possible.

In its simplest implementation, in vivo somatic genome editing consists in delivering a programmable endonuclease (typically spCas9) and the desired gRNAs to the tissue of choice. This strategy can be used to somatically inactivate classic tumor-suppressor genes, but it can also be adapted to identify genes essential for cancer progression and potential therapeutic targets.

Somatic Gene Inactivation to Model Cancer

Multiple strategies have been developed to deliver the Cas9/gRNA complexes to somatic cells in mice and to model cancer (**Figure 3**). The liver appears to be the easiest tissue to reach, and efficient gene editing and generation of liver cancers can been achieved by hydrodynamic tail vein injection of naked plasmid DNA encoding Cas9 and the gRNA or by tail vein injection of recombinant adenoviral vectors (Wang et al. 2015, Xue et al. 2014). Delivery of naked DNA also works well in the pancreas (Maresch et al. 2016) and in the brain, where medulloblastomas and

gliomas have been engineered by in utero electroporation or in situ transfection of Cas9/gRNA-expressing plasmids, respectively (Zuckermann et al. 2015). More recently, somatic editing of postmitotic neurons has been achieved by direct intracranial injection of Cas9 RNPs (Staahl et al. 2017), although this method has not yet been used to model brain tumors.

The lung is another success story for in vivo somatic genome editing, showcasing the power of combining this technology with the wide array of already available genetically engineered mouse strains to recapitulate the genetic complexity of human cancers. Pioneering this strategy, the Jacks laboratory used a lentiviral vector encoding Cas9/gRNA and the Cre recombinase to infect the lungs of $Kras^{LSL\text{-}G12D}$;$p53^{fl/fl}$ mice and to test the consequence of CRISPR-mediated disruption of three tumor-suppressor genes (*Nkx2-1*, *Apc*, and *Pten*) on the pathogenesis of lung adenocarcinomas (Sanchez-Rivera et al. 2014). Subsequently, other researchers developed similar strategies to study cooperating genetic events in mouse models of pancreatic (Chiou et al. 2015, Maresch et al. 2016) and breast cancer (Annunziato et al. 2016).

As these examples illustrate, in vivo gene editing is ideal for modeling tumor development in mice by introducing specific mutations somatically; however, the efficiency and simplicity of CRISPR also provides an appealing tool for high-throughput in vivo studies in which hundreds or thousands of mutations can be studied in parallel. The ability to screen directly in whole organisms for novel synthetic lethal interactions and cancer dependencies—overcoming the intrinsic limitation of cell-based studies—could prove revolutionary, and it is likely that we will soon see more drug target screens performed directly in vivo, within a functional tumor microenvironment.

Generation of Chromosomal Rearrangements

The reach of in vivo somatic genome editing extends beyond simple gene inactivation, enabling experiments previously unthinkable. Perhaps the most striking example is the in vivo somatic engineering of chromosomal rearrangements, a class of cancer-associated mutations that had proven difficult to model using conventional gene-targeting strategies (Maddalo & Ventura 2016). By simultaneously expressing two distinct gRNAs targeting the desired breakpoints, researchers can induce specific chromosomal rearrangements with low but detectable frequency. Using this strategy, large deletions, inversions, duplications, and even reciprocal translocations can be generated in cells and, more importantly, in vivo (Blasco et al. 2014, Choi & Meyerson 2014, Ghezraoui et al. 2014, Kraft et al. 2015, Lekomtsev et al. 2016, Lupianez et al. 2015, Maddalo et al. 2014, Park et al. 2015, Spraggon et al. 2017, Vanoli et al. 2017).

First applied to generate a mouse model of non-small-cell lung cancer driven by the *EML4-ALK* chromosomal inversion (Blasco et al. 2014, Maddalo et al. 2014), this strategy has been successfully adapted to model chromosomal rearrangements in the liver (Y. Li et al. 2015), the brain (Cook et al. 2017), and the intestine (Han et al. 2017). These initial studies have focused on modeling gene fusions or on generating rearrangements directly involving tumor-suppressor genes, but we expect that CRISPR-based in vivo somatic chromosomal engineering will have an even greater impact in characterizing the oncogenic potential of chromosomal aberrations that only indirectly affect the expression of cancer genes, for example, by altering the structure and extension of TADs and promoting aberrant gene-enhancer interactions (de Wit et al. 2015, Guo et al. 2015).

THE LURE OF HOMOLOGY DIRECTED REPAIR–MEDIATED SOMATIC GENOME EDITING

As discussed in previous sections, if a suitable donor template is provided, programmable endonucleases can be used to enhance the efficiency of gene targeting by HDR, allowing scarless

generation of virtually any genetic modifications. Translating this to the context of adult animals, however, has proven challenging. In adult mice, the liver seems to be the most tractable organ, where delivery of CRISPR-Cas9 and a donor template has been used to mutate four phosphorylation sites in the β-catenin gene (Xue et al. 2014) or to repair the phenylalanine hydroxylase gene in a mouse model of hereditary tyrosinemia (Yin et al. 2014). The efficiency of HDR in this context, however, is rather low. For example, in the tyrosinemia experiment, the mutant *Fah* gene was repaired in less than 0.5% of hepatocytes, and phenotypic correction was possible only because the repaired hepatocytes had a strong selective advantage.

CRISPR-mediated HDR has also been used to generate an oncogenic mutation in *Kras* in the lung of adult mice, but the efficiency of the process seems even lower in this context (Platt et al. 2014). The technical challenge of simultaneously delivering not only the Cas enzyme and the gRNA, but also a sufficient amount of HDR template, could in part explain this discrepancy in efficiency between ex vivo and in vivo CRISPR-mediated HDR, but it is probably more important that HDR is largely restricted to cells in the S/G2 phase of the cell cycle, which are only a small fraction of most adult tissues. Devising strategies to improve the efficiency of HDR in vivo is an area of intense investigation, and the success or failure of these efforts will have a major impact on cancer modeling and gene therapy. However, even if in vivo gene editing by HDR proves unfeasible, alternative strategies that do not rely on HDR, such as base editing with AID or APOBEC tethered to dCas9 (discussed above), could soon enable the precise editing of individual nucleotides in vivo.

IN VIVO SOMATIC GENOME EDITING MADE EASIER

The ability to generate site-specific mutations and chromosomal rearrangements in a mosaic fashion in adult animals is inarguably as close as it gets with respect to modeling cancer initiation and progression. Key to the widespread application of this strategy, however, is the availability of methods to efficiently deliver the Cas/gRNA complex to the tissue of interest. In practice, despite remarkable successes in the liver, lung, pancreas, breast, and brain, not every tissue can be transduced with recombinant viruses, and the relatively large size of Cas9 and other programmable endonucleases limits the investigator's choice to delivery systems that accept large payloads. Furthermore, ectopic expression of Cas enzymes in immunocompetent mice can elicit a significant immune response (Wang et al. 2015) that might complicate the interpretation of such experiments.

An elegant way to overcome these limitations is to have mice endogenously expressing the programmable endonuclease (Chiou et al. 2015, Dow et al. 2015, Platt et al. 2014). At the time of this writing, mice harboring Cre-inducible (Chiou et al. 2015, Platt et al. 2014) and doxycycline-inducible (Dow et al. 2015) SpCas9 alleles have been generated and used to model cancers in the lung, brain, pancreas and intestine. Additional mouse strains expressing programmable endonucleases recognizing different PAMs, and even specialized Cas enzymes for base editing and transcriptional regulation, will likely soon be available to the scientific community. In addition to avoiding the anti-Cas immune response and enabling the use of a wider range of viral and nonviral delivery systems for the sgRNAs, these strains address legitimate biosafety concerns raised by the use of viral vectors containing Cas9 and sgRNAs.

Although somewhat less physiologic than direct in vivo somatic genome editing, ex vivo editing of isolated stem/progenitor cells or of murine organoids followed by orthotopic implantation into syngeneic immunocompetent animals offers another appealing alternative to model cancer in mice. This approach has been used to model hematologic malignancies (Heckl et al. 2014), gliomas (Cook et al. 2017), and colorectal cancers (O'Rourke et al. 2017, Roper et al. 2017) and

offers the significant advantage of being more easily scalable to interrogate a large number of mutations in parallel.

FROM BACTERIA, TO BENCH, TO BEDSIDE

The biotechnology and pharmaceutical sector has been whipped into a frenzy over the potential therapeutic opportunities of precise gene editing. Numerous new companies have emerged with CRISPR therapies as a primary focus, and most large pharmaceutical entities have CRISPR in their drug development pipeline. From a translational perspective, CRISPR offers an exciting alternative to traditional gene therapy approaches. Whereas most previous strategies involved the introduction of a wild-type gene via viral transduction, CRISPR could allow the correction of disease-causing mutations in situ or in patient-derived cells for autologous transplant. While effective delivery is still a significant hurdle, where this approach shows real and immediate promise is in the management of (often life-threatening) monogenic disorders that can be ameliorated by restoring partial function to the affected tissue. For instance, numerous groups provided proof-of-concept evidence that correction of mutations the *Dystrophin* gene, which cause muscular dystrophy, can induce at least partial phenotypic reversal in mice (Bengtsson et al. 2017, Long et al. 2016, Nelson et al. 2016, Tabebordbar et al. 2016). In cystic fibrosis, it is feasible that defects in multiple affected organs (lungs and intestine) could be treated using in situ or through ex vivo autologous editing (Schwank et al. 2013).

There are many more preclinical examples of preclinical disease gene correction, although few that target cancer. This is for obvious reasons, as just a few escaping cells can lead to tumor relapse. Similarly, it is likely that any therapy that directly targets the genome of cancers would be prone to resistance, and cells need only mutate the precise recognition site(s) of the sgRNA. Yet, as CRISPR has repeatedly reminded us over the past five years, anything is possible. If in situ delivery were significantly optimized, CRISPR therapies might provide clinical benefit for patients with familial cancer-prone syndromes, such as familial adenomatous polyposis or Peutz-Jacob disease. Still, given the vast number of cells in the target issues that would need to be altered, any such approaches are unlikely to be curative. One unique and recently reported use of CRISPR for targeting cancer cells takes advantage of tumor-specific sequence motifs created following oncogenic gene fusions to deliver suicide genes to tumor cells.

Perhaps the most promising use of CRISPR for cancer therapeutics is in the production of chimeric antigen receptor (CAR)-T cells. CAR-T cells are effector lymphocytes engineered to express a receptor for target antigens present on tumor cells, such as CD19 for B cell malignancies (Kochenderfer & Rosenberg 2013). CAR-T therapies have shown immense promise in clinical trials and will likely soon gain approval from the US Food and Drug Administration. However, almost all efforts to date have been developed using viral or transposon-mediated insertion of the CAR, which can result in variegated transgene expression. Sadelain and colleagues recently showed that using CRISPR to precisely insert a CAR into the *TRAC* locus provides a more uniform expression of the CAR, as well as overall increased and sustained antitumor activity in animal models (Eyquem et al. 2017).

CRISPR may also find a somewhat unconventional home in clinical cancer treatment as a diagnostic tool. For instance, Pardee et al. (2016) used CRISPR tools into enable sequence-specific detection of distinct viral strains. Others have shown that the Cas13a enzyme (formerly known as C2c2), through its RNA endonuclease activity, can be adapted to detect very small amounts of cellular transcripts for sensitive detection of specific RNA species (East-Seletsky et al. 2016, Gootenberg et al. 2017). The unique feature of Cas13a is that sequence recognition initiates a feed-forward cascade of nuclease activity that can be harnessed to develop detection tests with

attomolar sensitivity. Where this could have a major impact on cancer diagnosis and treatment is in the detection of very low levels of circulating or excreted (e.g., sputum, feces) tumor-specific DNA. Early detection of new and relapse tumors can have a significant influence on the ultimate clinical outcome for patients, and there are major efforts underway to follow tumor response and relapse through cell-free DNA (Abbosh et al. 2017, Jamal-Hanjani et al. 2017). Thus, ultimately, CRISPR technologies could play a key role in screening, diagnosis, treatment, and follow-up for cancer patients.

THE FUTURE IS NOW

The speed with which the genome editing field has progressed over the past few years is mind boggling: Just between 2013 and the first half of 2017, well over 5,000 papers with the keyword CRISPR in the title or abstract have been indexed in Pubmed. Indeed, it is virtually impossible to write a comprehensive review on such a rapidly moving field, not only because space constraints restrict the depth to which some applications can be covered, but also because, at the current pace, by the time this review goes to press, countless additional developments and improvements will surely have been made.

Although every aspect of biomedical research is benefitting from this technological revolution, no field of inquiry is impacted more profoundly than cancer research. The ability to interrogate the cancer genome with unprecedented precision and speed, combined with the exponential increase in sequencing power and computational analysis, is radically changing the way we understand, study, and treat human cancers. Here, we have provided a taste of what we feel are not only the most promising and innovative applications of genome editing to cancer research, but also the limitations that must be overcome. We hope we have succeeded in sharing our enthusiasm for this field and our conviction that the future of cancer research is now!

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

LITERATURE CITED

Abbosh C, Birkbak NJ, Wilson GA, Jamal-Hanjani M, Constantin T, et al. 2017. Phylogenetic ctDNA analysis depicts early stage lung cancer evolution. *Nature* 545:446–51

Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, et al. 2016. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* 353:aaf5573

Adamson B, Norman TM, Jost M, Cho MY, Nunez JK, et al. 2016. A multiplexed single-cell CRISPR screening platform enables systematic dissection of the unfolded protein response. *Cell* 167:1867–82.e21

Andersson-Rolf A, Mustata RC, Merenda A, Kim J, Perera S, et al. 2017. One-step generation of conditional and reversible gene knockouts. *Nat. Methods* 14:287–89

Annunziato S, Kas SM, Nethe M, Yucel H, Del Bravo J, et al. 2016. Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland. *Genes Dev.* 30:1470–80

Bengtsson NE, Hall JK, Odom GL, Phelps MP, Andrus CR, et al. 2017. Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. *Nat. Commun.* 8:14454

Blasco RB, Karaca E, Ambrogio C, Cheong T-C, Karayol E, et al. 2014. Simple and rapid in vivo generation of chromosomal rearrangements using CRISPR/Cas9 technology. *Cell Rep.* 9:1219–27

Bouabe H, Okkenhaug K. 2013. Gene targeting in mice: a review. *Methods Mol. Biol.* 1064:315–36

Canver MC, Smith EC, Sher F, Pinello L, Sanjana NE, et al. 2015. BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. *Nature* 527:192–97

Capecchi MR. 2005. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat. Rev. Genet.* 6:507–12

Chang H, Yi B, Ma R, Zhang X, Zhao H, Xi Y. 2016. CRISPR/cas9, a novel genomic tool to knock down microRNA *in vitro* and *in vivo*. *Sci. Rep*. 6:22312

Chen S, Sanjana NE, Zheng K, Shalem O, Lee K, et al. 2015. Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell* 160:1246–60

Chiou SH, Winters IP, Wang J, Naranjo S, Dudgeon C, et al. 2015. Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing. *Genes Dev*. 29:1576–85

Choi PS, Meyerson M. 2014. Targeted genomic rearrangements using CRISPR/Cas technology. *Nat. Commun.* 5:3728

Chu VT, Weber T, Wefers B, Wurst W, Sander S, et al. 2015. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat. Biotechnol.* 33:543–48

Cong L, Ran FA, Cox D, Lin S, Barretto R, et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339:819–23

Cook PJ, Thomas R, Kannan R, de Leon ES, Drilon A, et al. 2017. Somatic chromosomal engineering identifies BCAN-NTRK1 as a potent glioma driver and therapeutic target. *Nat. Commun.* 8:15987

Datlinger P, Rendeiro AF, Schmidl C, Krausgruber T, Traxler P, et al. 2017. Pooled CRISPR screening with single-cell transcriptome readout. *Nat. Methods* 14:297–301

de Wit E, Vos ES, Holwerda SJ, Valdes-Quezada C, Verstegen MJ, et al. 2015. CTCF binding polarity determines chromatin looping. *Mol. Cell* 60:676–84

Dever DP, Bak RO, Reinisch A, Camarena J, Washington G, et al. 2016. CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. *Nature* 539:384–89

Dixit A, Parnas O, Li B, Chen J, Fulco CP, et al. 2016. Perturb-seq: dissecting molecular circuits with scalable single-cell RNA profiling of pooled genetic screens. *Cell* 167:1853–66.e17

Dominguez AA, Lim WA, Qi LS. 2016. Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nat. Rev. Mol. Cell Biol.* 17:5–15

Dow LE. 2015. Modeling disease in vivo with CRISPR/Cas9. *Trends Mol. Med.* 21:609–21

Dow LE, Fisher J, O'Rourke KP, Muley A, Kastenhuber ER, et al. 2015. Inducible in vivo genome editing with CRISPR-Cas9. *Nat. Biotechnol.* 33:390–94

Drost J, van Jaarsveld RH, Ponsioen B, Zimberlin C, van Boxtel R, et al. 2015. Sequential cancer mutations in cultured human intestinal stem cells. *Nature* 521:43–47

East-Seletsky A, O'Connell MR, Knight SC, Burstein D, Cate JH, et al. 2016. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. *Nature* 538:270–73

Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJ, Hamieh M, et al. 2017. Targeting a CAR to the *TRAC* locus with CRISPR/Cas9 enhances tumour rejection. *Nature* 543:113–17

Fearon ER, Vogelstein B. 1990. A genetic model for colorectal tumorigenesis. *Cell* 61:759–67

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, et al. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* 31:822–26

Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* 32:279–84

Gantz VM, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM, et al. 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *PNAS* 112:E6736–43

Ghezraoui H, Piganeau M, Renouf B, Renaud JB, Sallmyr A, et al. 2014. Chromosomal translocations in human cells are generated by canonical nonhomologous end-joining. *Mol. Cell* 55:829–42

Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, et al. 2014. Genome-scale CRISPR-mediated control of gene repression and activation. *Cell* 159:647–61

Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, et al. 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154:442–51

Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, et al. 2017. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* 356:438–42
Grissa I, Vergnaud G, Pourcel C. 2007. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinform.* 8:172
Guo X, Zhang T, Hu Z, Zhang Y, Shi Z, et al. 2014. Efficient RNA/Cas9-mediated genome editing in *Xenopus tropicalis*. *Development* 141:707–14
Guo Y, Xu Q, Canzio D, Shou J, Li J, et al. 2015. CRISPR inversion of CTCF sites alters genome topology and enhancer/promoter function. *Cell* 162:900–10
Gutschner T, Haemmerle M, Genovese G, Draetta GF, Chin L. 2016. Post-translational regulation of Cas9 during G1 enhances homology-directed repair. *Cell Rep.* 14:1555–66
Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C, et al. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nat. Biotechnol.* 34:78–83
Han T, Schatoff EM, Murphy C, Zafra MP, Wilkinson JE, et al. 2017. R-Spondin chromosome rearrangements drive Wnt-dependent tumour initiation and maintenance in the intestine. *Nat. Commun.* 8:15945
Heckl D, Kowalczyk MS, Yudovich D, Belizaire R, Puram RV, et al. 2014. Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. *Nat. Biotechnol.* 32:941–46
Hess GT, Fresard L, Han K, Lee CH, Li A, et al. 2016. Directed evolution using dCas9-targeted somatic hypermutation in mammalian cells. *Nat. Methods* 13:1036–42
Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, et al. 2015. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat. Biotechnol.* 33:510–17
Howden SE, Maufort JP, Duffin BM, Elefanty AG, Stanley EG, Thomson JA. 2015. Simultaneous reprogramming and gene correction of patient fibroblasts. *Stem Cell Rep.* 5:1109–18
Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J. Bacteriol.* 169:5429–33
Jaitin DA, Weiner A, Yofe I, Lara-Astiaso D, Keren-Shaul H, et al. 2016. Dissecting immune circuits by linking CRISPR-pooled screens with single-cell RNA-seq. *Cell* 167:1883–96.e15
Jamal-Hanjani M, Wilson GA, McGranahan N, Birkbak NJ, Watkins TBK, et al. 2017. Tracking the evolution of non-small-cell lung cancer. *N. Engl. J. Med.* 376:2109–21
Jao L-E, Wente SR, Chen W. 2013. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *PNAS* 110:13904–9
Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816–21
Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. 2013. RNA-programmed genome editing in human cells. *eLife* 2:e00471
Katigbak A, Cencic R, Robert F, Senecha P, Scuoppo C, Pelletier J. 2016. A CRISPR/Cas9 functional screen identifies rare tumor suppressors. *Sci. Rep.* 6:38968
Kearns NA, Pham H, Tabak B, Genga RM, Silverstein NJ, et al. 2015. Functional annotation of native enhancers with a Cas9-histone demethylase fusion. *Nat. Methods* 12:401–3
Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, et al. 2016a. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 529:490–95
Kleinstiver BP, Prew MS, Tsai SQ, Nguyen NT, Topkar VV, et al. 2015a. Broadening the targeting range of *Staphylococcus aureus* CRISPR-Cas9 by modifying PAM recognition. *Nat. Biotechnol.* 33:1293–98
Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, et al. 2015b. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 523:481–85
Kleinstiver BP, Tsai SQ, Prew MS, Nguyen NT, Welch MM, et al. 2016b. Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. *Nat. Biotechnol.* 34:869–74
Kochenderfer JN, Rosenberg SA. 2013. Treating B-cell cancer with T cells expressing anti-CD19 chimeric antigen receptors. *Nat. Rev. Clin. Oncol.* 10:267–76
Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533:420–24

Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, et al. 2015. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 517:583–88

Korkmaz G, Lopes R, Ugalde AP, Nevedomskaya E, Han R, et al. 2016. Functional genetic screens for enhancer elements in the human genome using CRISPR-Cas9. *Nat. Biotechnol.* 34:192–98

Kraft K, Geuer S, Will AJ, Chan WL, Paliou C, et al. 2015. Deletions, inversions, duplications: engineering of structural variants using CRISPR/Cas in mice. *Cell Rep*. 34:192–98

Kurata M, Rathe SK, Bailey NJ, Aumann NK, Jones JM, et al. 2016. Using genome-wide CRISPR library screening with library resistant DCK to find new sources of Ara-C drug resistance in AML. *Sci. Rep.* 6:36199

Lekomtsev S, Aligianni S, Lapao A, Burckstummer T. 2016. Efficient generation and reversion of chromosomal translocations using CRISPR/Cas technology. *BMC Genom*. 17:739

Li HL, Fujimoto N, Sasakawa N, Shirai S, Ohkame T, et al. 2015. Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Rep*. 4:143–54

Li Y, Park AI, Mou H, Colpan C, Bizhanova A, et al. 2015. A versatile reporter system for CRISPR-mediated chromosomal rearrangements. *Genome Biol*. 16:111

Lin S, Staahl BT, Alla RK, Doudna JA. 2014. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife* 3:e04766

Liu SJ, Horlbeck MA, Cho SW, Birk HS, Malatesta M, et al. 2017. CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. *Science* 355:aah7111

Liu XS, Wu H, Ji X, Stelzer Y, Wu X, et al. 2016. Editing DNA methylation in the mammalian genome. *Cell* 167:233–47.e17

Long C, Amoasii L, Mireault AA, McAnally JR, Li H, et al. 2016. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science* 351:400–3

Lupianez DG, Kraft K, Heinrich V, Krawitz P, Brancati F, et al. 2015. Disruptions of topological chromatin domains cause pathogenic rewiring of gene-enhancer interactions. *Cell* 161:1012–25

Ma Y, Shen B, Zhang X, Lu Y, Chen W, et al. 2014. Heritable multiplex genetic engineering in rats using CRISPR/Cas9. *PLOS ONE* 9:e89413

Ma Y, Zhang J, Yin W, Zhang Z, Song Y, Chang X. 2016. Targeted AID-mediated mutagenesis (TAM) enables efficient genomic diversification in mammalian cells. *Nat. Methods* 13:1029–35

Maddalo D, Manchado E, Concepcion CP, Bonetti C, Vidigal JA, et al. 2014. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature* 516:423–27

Maddalo D, Ventura A. 2016. Somatic engineering of oncogenic chromosomal rearrangements: a perspective. *Cancer Res*. 76:4918–23

Mali P, Yang L, Esvelt KM, Aach J, Guell M, et al. 2013. RNA-guided human genome engineering via Cas9. *Science* 339:823–26

Maresch R, Mueller S, Veltkamp C, Ollinger R, Friedrich M, et al. 2016. Multiplexed pancreatic genome engineering and cancer induction by transfection-based CRISPR/Cas9 delivery in mice. *Nat. Commun.* 7:10770

Marraffini LA, Sontheimer EJ. 2010. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat. Rev. Genet.* 11:181–90

Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, Ploegh HL. 2015. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat. Biotechnol.* 33:538–42

Matano M, Date S, Shimokawa M, Takano A, Fujii M, et al. 2015. Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nat. Med.* 21:256–62

Munoz DM, Cassiani PJ, Li L, Billy E, Korn JM, et al. 2016. CRISPR screens provide a comprehensive assessment of cancer vulnerabilities but generate false-positive hits for highly amplified genomic regions. *Cancer Discov*. 6:900–13

Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, et al. 2016. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science* 351:403–7

Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, et al. 2016. Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science* 353.aaf8729

Niu Y, Shen B, Cui Y, Chen Y, Wang J, et al. 2014. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* 156:836–43

Oliver D, Yuan S, McSwiggin H, Yan W. 2015. Pervasive genotypic mosaicism in founder mice derived from genome editing through pronuclear injection. *PLOS ONE* 10:e0129457

O'Rourke KP, Loizou E, Livshits G, Schatoff EM, Baslan T, et al. 2017. Transplantation of engineered organoids enables rapid generation of metastatic mouse models of colorectal cancer. *Nat. Biotechnol.* 35:577–82

Paquet D, Kwart D, Chen A, Sproul A, Jacob S, et al. 2016. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature* 533:125–29

Pardee K, Green AA, Takahashi MK, Braff D, Lambert G, et al. 2016. Rapid, low-cost detection of Zika virus using programmable biomolecular components. *Cell* 165:1255–66

Park CY, Kim DH, Son JS, Sung JJ, Lee J, et al. 2015. Functional correction of large factor VIII gene chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR-Cas9. *Cell Stem Cell* 17:213–20

Parnas O, Jovanovic M, Eisenhaure TM, Herbst RH, Dixit A, et al. 2015. A genome-wide CRISPR screen in primary immune cells to dissect regulatory networks. *Cell* 162:675–86

Perez AR, Pritykin Y, Vidigal JA, Chhangawala S, Zamparo L, et al. 2017. GuideScan software for improved single and paired CRISPR guide RNA design. *Nat. Biotechnol.* 35:347–49

Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, et al. 2014. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* 159:440–55

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, et al. 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152:1173–83

Radzisheuskaya A, Shlyueva D, Muller I, Helin K. 2016. Optimizing sgRNA position markedly improves the efficiency of CRISPR/dCas9-mediated transcriptional repression. *Nucleic Acids Res.* 44:e141

Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, et al. 2015. In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature* 520:186–91

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8:2281–308

Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. 2016. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat. Biotechnol.* 34:339–44

Roper J, Tammela T, Cetinbas NM, Akkad A, Roghanian A, et al. 2017. In vivo genome editing and organoid transplantation models of colorectal cancer and metastasis. *Nat. Biotechnol.* 35:569–76

Sanchez-Rivera FJ, Papagiannakopoulos T, Romero R, Tammela T, Bauer MR, et al. 2014. Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature* 516:428–31

Sanjana NE, Wright J, Zheng K, Shalem O, Fontanillas P, et al. 2016. High-resolution interrogation of functional elements in the noncoding genome. *Science* 353:1545–49

Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, et al. 2013. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* 13:653–58

Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, et al. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343:84–87

Shan Q, Wang Y, Li J, Zhang Y, Chen K, et al. 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* 31:686–88

Shi J, Wang E, Milazzo JP, Wang Z, Kinney JB, Vakoc CR. 2015. Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. *Nat. Biotechnol.* 33:661–67

Shimokawa M, Ohta Y, Nishikori S, Matano M, Takano A, et al. 2017. Visualization and targeting of $LGR5^{+}$ human colon cancer stem cells. *Nature* 545:187–92

Singh D, Sternberg SH, Fei J, Doudna JA, Ha T. 2016. Real-time observation of DNA recognition and rejection by the RNA-guided endonuclease Cas9. *Nat. Commun.* 7:12778

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

Soldner F, Stelzer Y, Shivalila CS, Abraham BJ, Latourelle JC, et al. 2016. Parkinson-associated risk variant in distal enhancer of α-synuclein modulates target gene expression. *Nature* 533:95–99

Spraggon L, Martelotto LG, Hmeljak J, Hitchman TD, Wang J, et al. 2017. Generation of conditional oncogenic chromosomal translocations using CRISPR-Cas9 genomic editing and homology-directed repair. *J. Pathol.* 242:102–12

Staahl BT, Benekareddy M, Coulon-Bainier C, Banfal AA, Floor SN, et al. 2017. Efficient genome editing in the mouse brain by local delivery of engineered Cas9 ribonucleoprotein complexes. *Nat. Biotechnol.* 35:431–34

Steinhart Z, Pavlovic Z, Chandrashekhar M, Hart T, Wang X, et al. 2017. Genome-wide CRISPR screens reveal a Wnt-FZD5 signaling circuit as a druggable vulnerability of RNF43-mutant pancreatic tumors. *Nat. Med.* 23:60–68

Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA. 2014. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 507:62–67

Sur S, Pagliarini R, Bunz F, Rago C, Diaz LA Jr., et al. 2009. A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. *PNAS* 106:3964–69

Tabebordbar M, Zhu K, Cheng JK, Chew WL, Widrick JJ, et al. 2016. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science* 351:407–11

Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, et al. 2015. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat. Biotechnol.* 33:187–97

Van Dyke T, Jacks T. 2002. Cancer modeling in the modern era: progress and challenges. *Cell* 108:135–44

van Overbeek M, Capurso D, Carter MM, Thompson MS, Frias E, et al. 2016. DNA repair profiling reveals nonrandom outcomes at Cas9-mediated breaks. *Mol. Cell* 63:633–46

Vanoli F, Tomishima M, Feng W, Lamribet K, Babin L, et al. 2017. CRISPR-Cas9-guided oncogenic chromosomal translocations with conditional fusion protein expression in human mesenchymal cells. *PNAS* 114:3696–701

Vidigal JA, Ventura A. 2015. Rapid and efficient one-step generation of paired gRNA CRISPR/*Cas*9 libraries. *Nat. Commun.* 6:8083

Vojta A, Dobrinic P, Tadic V, Bockor L, Korac P, et al. 2016. Repurposing the CRISPR-Cas9 system for targeted DNA methylation. *Nucleic Acids Res.* 44:5615–28

Wan H, Feng C, Teng F, Yang S, Hu B, et al. 2015. One-step generation of *p53* gene biallelic mutant Cynomolgus monkey via the CRISPR/Cas system. *Cell Res.* 25:258–61

Wang D, Mou H, Li S, Li Y, Hough S, et al. 2015. Adenovirus-mediated somatic genome editing of *Pten* by CRISPR/Cas9 in mouse liver in spite of Cas9-specific immune responses. *Hum. Gene. Ther.* 26:432–42

Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, et al. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153:910–18

Wang T, Wei JJ, Sabatini DM, Lander ES. 2014. Genetic screens in human cells using the CRISPR-Cas9 system. *Science* 343:80–84

Weber J, Ollinger R, Friedrich M, Ehmer U, Barenboim M, et al. 2015. CRISPR/Cas9 somatic multiplex-mutagenesis for high-throughput functional cancer genomics in mice. *PNAS* 112:13982–87

Woo JW, Kim J, Kwon SI, Corvalan C, Cho SW, et al. 2015. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat. Biotechnol.* 33:1162–64

Xue W, Chen S, Yin H, Tammela T, Papagiannakopoulos T, et al. 2014. CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature* 514:380–84

Yang D, Scavuzzo MA, Chmielowiec J, Sharp R, Bajic A, Borowiak M. 2016. Enrichment of G2/M cell cycle phase in human pluripotent stem cells enhances HDR-mediated gene repair with customizable endonucleases. *Sci. Rep.* 6:21264

Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154:1370–79

Yen ST, Zhang M, Deng JM, Usman SJ, Smith CN, et al. 2014. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. *Dev. Biol.* 393:3–9

Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, et al. 2014. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat. Biotechnol.* 32:551–53

Yin L, Maddison LA, Li M, Kara N, LaFave MC, et al. 2015. Multiplex conditional mutagenesis using transgenic expression of Cas9 and sgRNAs. *Genetics* 200:431–41

Yu C, Liu Y, Ma T, Liu K, Xu S, et al. 2015. Small molecules enhance CRISPR genome editing in pluripotent stem cells. *Cell Stem Cell* 16:142–47

Yun J, Rago C, Cheong I, Pagliarini R, Angenendt P, et al. 2009. Glucose deprivation contributes to the development of *KRAS* pathway mutations in tumor cells. *Science* 325:1555–59

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, et al. 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163:759–71

Zhao Y, Dai Z, Liang Y, Yin M, Ma K, et al. 2014. Sequence-specific inhibition of microRNA via CRISPR/CRISPRi system. *Sci. Rep.* 4:3943

Zhou J, Shen B, Zhang W, Wang J, Yang J, et al. 2014. One-step generation of different immunodeficient mice with multiple gene modifications by CRISPR/Cas9 mediated genome engineering. *Int. J. Biochem. Cell Biol.* 46:49–55

Zhu S, Li W, Liu J, Chen CH, Liao Q, et al. 2016. Genome-scale deletion screening of human long non-coding RNAs using a paired-guide RNA CRISPR–Cas9 library. *Nat. Biotechnol.* 34:1279–86

Zuckermann M, Hovestadt V, Knobbe-Thomsen CB, Zapatka M, Northcott PA, et al. 2015. Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. *Nat. Commun.* 6:7391