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Stephen H. Tsang *Editor*

Precision Medicine, CRISPR, and Genome Engineering

Moving from Association to Biology and Therapeutics

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Foreword by George M. Church

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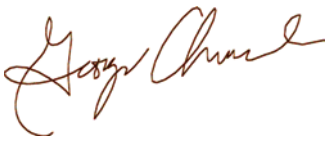
Foreword

All of life is encoded in the four letters of DNA, ATGC. Recent advances in genome engineering now enable us to manipulate, customize, and reprogram our genomes, thus empowering us to rewrite our fate. Transplanting genetically altered cells and organs into humans, helping prevent and cure cancers, and reversing aging would once have been miracles and are suddenly now within our reach.

This comprehensive new text on genome engineering and the CRISPR revolution comes at a perfect time in the evolution of the field. The growth in new publications, techniques, and discoveries is exponential, and keeping pace with the ever-expanding applications of genome engineering can prove difficult.

This book provides a general introduction to the field and to the mechanics of CRISPR, the most recent advance in genome engineering. The text subsequently presents CRISPR's applications in a variety of model systems, from cells to agriculture to animal models. It concludes with a presentation of the future applications and ethical considerations of genome engineering. Suitable for novices and experts alike, this book will serve as a gateway for making original research publications in the field more accessible by providing a broad overview of genome engineering applications in a variety of contexts.

We are at a unique time in history where we have the unprecedented ability to play an active role in our own evolution. This book will equip readers with the knowledge to determine in which direction we should take it.



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Stephen H. Tsang For years, Stephen H. Tsang has been culturing embryonic stem (ES) cells and created the first mouse model for a recessive form of retinitis pigmentosa by applying homologous recombination to ES cell technology. Two elements define his laboratory. First, by leveraging his genetics clinical practice, in which over 1000 retinal patients are cared for, he brings an array of clinical resources to his research, including stem cells and live imaging data. Second, he and his students are recognized authorities in a broad array of state-of-the-art technologies. Most recently, he was invited as a Moderator for the Gene Editing/Rewriting the Genome session during the 65th American Society of Human Genetics Annual Meeting.

George M. Church, professor at Harvard and MIT, coauthor of 425 papers, 95 patent publications, and the book *Regenesis*, developed methods used for the first genome sequence (1994) and millionfold cost reductions since (via NGS and nanopores), plus barcoding, DNA assembly from chips, genome editing, writing, and recoding. He co-initiated the BRAIN Initiative (2011) and Genome Projects (1984, 2005) to provide and interpret the world's only open-access personal precision medicine datasets.

Part I
Introduction to the CRISPR Revolution

Chapter 1
Viral Vectors, Engineered Cells and the CRISPR Revolution

James E. DiCarlo, Anurag Deeconda, and Stephen H. Tsang

Abstract Over the past few decades the ability to edit human cells has revolutionized modern biology and medicine. With advances in genome editing methodologies, gene delivery and cell-based therapeutics targeted at treatment of genetic disease have become a reality that will become more and more essential in clinical practice. Modifying specific mutations in eukaryotic cells using CRISPR-Cas systems derived from prokaryotic immune systems has allowed for precision in correcting various disease mutations. Furthermore, delivery of genetic payloads by employing viral tropism has become a crucial and effective mechanism for delivering genes and gene editing systems into cells. Lastly, cells modified *ex vivo* have tremendous potential and have shown effective in studying and treating a myriad of diseases. This chapter seeks to highlight and review important progress in the realm of the editing of human cells using CRISPR-Cas systems, the use of viruses as vectors for gene therapy, and the application of engineered cells to study and treat disease.

Keywords CRISPR/Cas • Ophthalmology • Genome Surgery • Gene Therapy

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1.1 Introduction

Precise engineering of human cells using genetic tools has revolutionized biology. Frederick Griffith's observation of the transforming principle in *Pneumococci* almost 100 years ago was a foundational step that laid the ground work for the entire field of gene therapy and genome engineering [1]. At their core, these areas rely on the on the transforming principle. Avery et al. later discovered that DNA was responsible for the transforming principle, which allowed the field of modern molecular biology to take another groundbreaking step forward [2]. Experiments in DNA transfer into mammalian cells by Szybalska and Szybalski showed that genes could be transferred between cell lines to modify their phenotype [3]. Decades later, building on primary gene transfer experiments, genome editing tools such as CRISPR-Cas systems are revolutionizing how we modify human cells [4].

Coupled with the development of genome editing tools, controlled delivery of foreign DNA into human cells has been an ongoing challenge in biomedicine. Viruses represent an important and powerful tool that scientists have levied for foreign DNA delivery. In fact, one of the first viral gene therapy experiments occurred in nonhuman cell lines in 1964. Temin et al. showed that Rous sarcoma viral mutations could be passed on in chicken cells [5, 6]. The observation that viral sources could induce introduction of heritable DNA laid the foundation for viruses to become a crucial vector of genetic modification of eukaryotic cells. This chapter will cover important milestones in the use of three commonly used groups of viral vectors that have been successfully used to modify human cells in the laboratory and in patients: retroviral vectors, adenoviral vectors, and adeno-associated viral vectors. As of 2016, these three vectors make up more than half of all vectors used in gene therapy (Fig. 1.1).

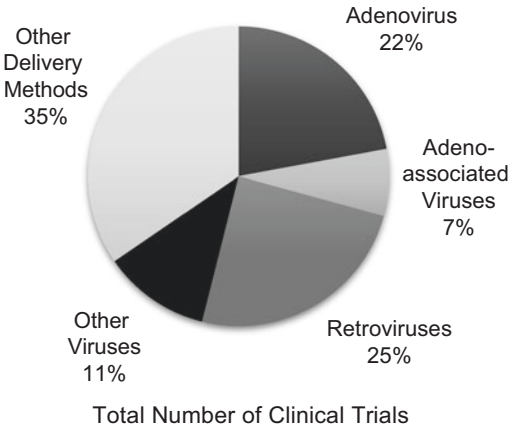

Fig. 1.1 Delivery methods used in gene therapy clinical trials as of August 2016. Viral vectors make up almost 65% of vectors used in gene therapy clinical trials, with a majority being composed of either adenoviral, adeno-associated viral, or retroviral vectors

Cellular manipulation has produced engineered cells with great therapeutic potential. Notably, the use of chimeric antigen receptor T-cells (CAR T-cells) and induced pluripotent stem cells (iPSCs) has been of keen interest in bridging the gap between genome editing *in vitro* using mouse models and eventually treatment of inherited human diseases, with promising efforts made in models of β-thalassemia and Duchenne muscular dystrophy. The use of CRISPR/Cas9 gene editing in conjunction with these methods has resulted in much more efficient correction of genetic abnormalities and restoration of function *in vivo.*

1.2 CRISPR-Cas Genome Manipulation

1.2.1 A Brief Overview of Genome Modification Using Endonucleases

Genomic incorporation of foreign DNA can occur by several means, most of which take advantage of protein recombination machinery, such as recombinases or integrases. Frequently, endogenous homologous recombination systems in eukaryotic genomes have been utilized by scientists to incorporate foreign DNA flanked by homologous sequences to the genomic locus of interest [7, 8]. Homologous recombination (HR) in eukaryotic cells is greatly stimulated after the introduction of a double-stranded break (DSB) in the host genome [7, 9]. If homologous recombination does not occur, an error prone process called non-homlogous end joining (NHEJ) can occur, resulting in mutations at the cut site [7]. Figure 1.2b diagrams the process of either non-homologous end joining or homologous recombination using a DNA donor, which could be supplied exogenously. A common method for introducing DSBs in host genomes is the use of site-specific endonucleases. These enzymes cleave DNA at sequence-specific regions [8]. The first implementation of site-specific endonucleases for eukaryotic genome modification was in mouse and plant cells using the meganuclease I-SceI, which has an 18-base pair recognition sequence [10, 11]. These meganucleases stimulated genome incorporation of foreign DNA by several orders of magnitude in mouse cells, putting a spotlight on an endonuclease approach for stimulating HR [11]. The downside of the I-SceI fixed 18-base pair recognition sequence moved scientists and engineers to design or discover reprogrammable site-specific endonucleases [8, 12].

Zinc-finger proteins were appealing first choices for the generation of engineerable endonucleases as these proteins contain specific nucleotide binding motifs that could be rearranged and then fine-tuned via selection for specific binding to a desired DNA sequence. When fused with an endonuclease domain, such as the FokI endonuclease, these proteins became some of the first engineered endonucleases, termed zinc finger nucleases (ZFNs) [13–15]. The average ZFN has an 18-base pair recognition sequence, which is constricted to the nucleotide triplets that zinc finger DNA binding motifs recognize via individual nucleotide binding domains.

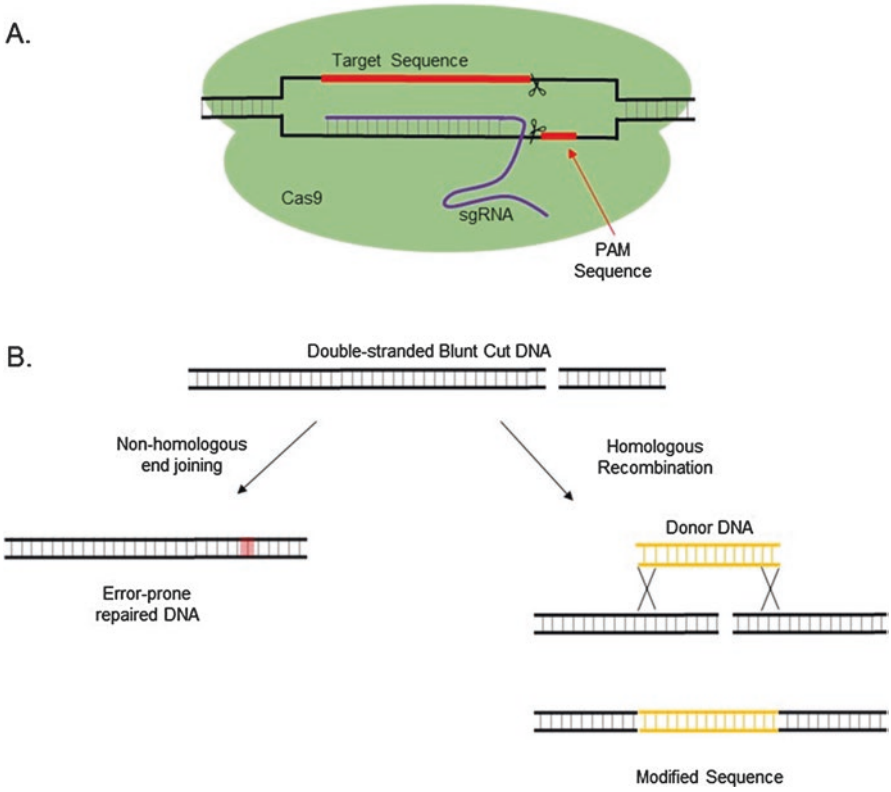

Fig. 1.2 Schematic of CRISPR-Cas9 DNA cleavage and DNA repair. (**a**) Cas9 complexes with the sgRNA to direct cleavage to region specified by the target sequence encoded on the sgRNA. (**b**) Repair of the double-stranded cut can occur via non-homologous end joining or homologous recombination. In the case of homologous recombination, if a donor DNA is supplied exogenously the region can be modified in a targeted fashion

These endonucleases are highly efficient and have been used in the modification of human cells as well as numerous other eukaryotic systems [16, 17]. However, rapid design and selection of ZFNs with novel binding sites that do not cleave off-target regions can still be a challenge [12, 18].

Inspired by the success of ZFNs, many groups turned their attention to DNA binding transcription activator-like (TAL) proteins of *Xanthomonas*, a plant pathogen that uses these domains for virulence factors in the nuclease of their host plant [19, 20]. TAL proteins, like zinc fingers, have a motif code for binding to DNA nucleotides, this time with a repeat motif recognizing one nucleotide instead of three, as in the case of zinc finger proteins [19, 20]. Fusion of an endonuclease, such as FokI, to these TAL proteins created TAL endonucleases or TALENs that offered another tool for engineering genomes [21, 22]. These proteins, while efficient at stimulating HR, have a downside of being large and contain repetitive regions, which can be problematic on the DNA level due to mutagenic recombination events [12, 23].

1.2.2 CRISPR-Cas Systems

While both ZFNs and TALENs are highly used in biomedicine, their individual disadvantages have led researchers to continue searching for easily engineerable and improved reprogrammable DNA cleaving enzymes. In the late 1980s, Ishino et al. noticed a group of repeated nucleotides in the E. coli genome while studying an unrelated enzyme [24, 25]. Later, groups found similar repeats in the genomes of other bacteria and archaea, leading to the name: clustered, regularly interspaced short palindromic repeats (CRISPR) [26–28]. The proteins often proximal to these repeat regions were given the name CRISPR-associated (Cas) proteins. Later groups would discover that CRISPR and Cas proteins were part of an immune system to protect from bacteriophage invaders [27, 29].

An important breakthrough in the study of the type II CRISPR-Cas system of *Streptococcus pyogenes* occurred in 2012 when the biochemical processing of DNA by this prokaryotic immune system was revealed [30]. In this three-component system, an endonuclease guided by two RNA molecules generates a DSB at a site determined by one of the RNA molecules. Cas9, the RNA-guided endonuclease, interacts with a CRISPR RNA (crRNA), which determines the location of cleavage, as well as a trans-activating RNA (tracRNA) to generate a protein-RNA complex capable of DNA cleavage [4, 30]. In the same paper, Jinek et al. fused the two RNA molecules to create a chimeric RNA called a single guide RNA (sgRNA), which was able to guide Cas9 to the desired cleavage site efficiently [30]. Within the sgRNA there is a ~20 base pair region that is important for sequence recognition with the cleavage site. This region must also be upstream of a canonical NGG triplet called a protospacer associated motif (PAM) in order for Cas9 to generate a blunt DSB [30–32]. Figure 1.2a represents Cas9 complexed with a sgRNA and the target DNA. In addition to this type II CRISPR-Cas system, the molecular mechanisms of the four other types of CRISPR-Cas systems have been elucidated to varying degrees [33].

1.2.3 CRISPR Tools in Biology

The *S. pyogenes* CRISPR-Cas9 system was quickly adapted for use in human cells, which showed tremendous success for genomic introduction of foreign DNA [34, 35]. Application in human cells showed *S. pyogenes* Cas9 (SpCas9) could be used to correct pathogenic mutations across a variety of diseases, from Fanconi anemia to mutations involved in retinitis pigmentosa in patient-derived cells [36–38]. Delivery of CRISPR-Cas systems and foreign DNA to various cell lines using viral approaches will be discussed later in this chapter. A creative use of SpCas9's targeted cleavage is its use to eradicate proviruses within human cells, such as HIV and Herpes Simplex-1 [39, 40]. Similarly, Yang et al. used

Cas9 to remove all porcine endogenous retroviruses from a porcine epithelial cell line, with the ultimate future goal of safe porcine-to-human xenotransplantation [41, 42].

Currently, there is a veritable arms race to identify new CRISPR-Cas systems that could be used to engineer cells, with the same or improved genome engineering efficiencies as the systems currently in use [4, 43, 44]. Recently, Burstein et al. utilized metagenomic approaches to mine novel CRISPR-Cas systems from unculturable microbes [43]. This group identified and characterized two new systems, CRISPR-CasX and CRISPR-CasY, both of which are smaller than the CRISPR-Cas9 system, a benefit to gene targeting as size is a consideration in most gene delivery vectors [43]. Additionally, Kleinstiver et al. showed that by decreasing nonspecific interactions of SpCas9 with DNA, the off-targeting cleavage of SpCas9-HF (High Fidelity) was removed for 8/8 sgRNAs analyzed as compared to wild type SpCas9, which had off-targeting cleavage with 7/8 sgRNAs [45]. Other CRISPR-Cas systems such as the Cpf1 CRISPR system have been elucidated and used in human cells with good results, broadening the CRISPR toolbox for genome engineering [44]. Generation and identification of Cas9 proteins that contained altered PAM specificities have also expanded the diversity of CRISPR-Cas tools [46]. In addition to engineering cells for therapeutic applications, CRISPR-Cas systems have been used to make libraries of gene knockouts more efficiently than previous approaches such as small hairpin RNA knockdowns [47].

CRISPR-Cas systems have also been modified for a diverse group of applications. As off-target cleavage is a concern for wild-type Cas9, SpCas9 has been rationally engineered by several groups based on crystal structure data to increase its specificity and decrease the likelihood of off-target cleavage [32, 45, 48–50]. Another notable modification was the generation of a catalytically attenuated version of SpCas9, termed the SpCas9 nickase (SpCas9D10A). The SpCasD10A nickase can be used in pairs to increase the specificity of cleavage of a particular locus only if it is flanked by both sgRNA encoded sites [51]. Furthermore, a catalytically inactivated SpCas9 has been used for both targeted transcriptional repression and also as a chassis for fusion of genetic effector proteins such as activators, deaminases, and epigenetic modifiers [51–56].

1.3 Gene Therapy Using Viruses

1.3.1 Retroviral Vectors

Retroviruses are positive-sense RNA viruses that require reverse transcriptase to convert their RNA genome into DNA, and in turn integrate the DNA genome into the host genome [57, 58]. The canonical genome of a retrovirus contains four genes. The *pol* gene encodes a reverse transcriptase (which reverse transcribes the RNA genome to DNA), a RNase H (used to process RNA), and an integrase gene (which integrates the viral genome into the host genome) [59]. The *gag* gene encodes the

structural polyprotein, and the *env* gene encodes envelope proteins essential in binding to host cells and determining viral tropism [59]. Lastly, the *pro* gene encodes a protease that is required for maturation of the viral particle via proteolysis of immature polyproteins to functional components [59]. The first retroviral vectors used to transduce human cells were based on Moloney murine leukemia virus (MLV) and were capable of only transducing dividing cells efficiently [60]. Additionally, these vectors were refined to only integrate transgenes of interest and not viral genes, which was a crucial step as specific gene integration is essential for precise gene therapy and genome modification [60]. Further work in retroviral gene delivery led to the development of viral vectors derived from human immunodeficiency virus (HIV) and had the advantage of expanding viral tropism to non-dividing cells [61]. As HIV is in the genus of *Lentivirus* of the Retrovirus family, vectors based on HIV components are often referred to as lentiviral vectors and have an ability to transduce non-diving cells [59].

1.3.2 Modifications and Implementation of Retroviral Vectors

While integration of transgenes can be seen as a benefit in the sense of permanent modification of the host genome, it can also be deleterious if specific integration into safe-loci is not achieved. Early clinical trials aimed at correction of X-Linked Severe Combined Immunodeficiency (X-SCID) resulted in several patients developing T cell acute lymphoblastic leukemia due to vector insertion and activation of proto-oncogenes [62, 63]. Hence, mapping the insertion profile for a retroviral vector or the development of integrase-deficient lentiviral vectors (IDLVs) via mutations in the integrase gene are solutions to the potential danger of damaging integration [56, 61, 63]. Additional modifications of lentiviral envelope proteins allowed for broadening the cell tropism via a method termed pseudotyping [64]. For example, by employing envelope glycoprotein from rabies virus, a lentiviral vector can be pseudotyped to transduce neuronal cells [64]. Pseudotyped IDLVs have allowed for efficient targeting of numerous cell types. Additionally, lentiviruses and other retroviruses have had their tropism modified toward specific cell types using antibodies and small peptide ligands that bind to the target cell [65–67].

IDLVs have been used as a method to deliver gene editing nucleases such as zinc-finger nucleases, transcription-activator like nucleases (TALENs), and CRISPR-Cas systems [56, 68, 69]. These programmable nucleases are used to cleave specific genomic regions and stimulate homologous recombination between the target locus and donor DNA. However, packaging gene editing components into IDLVs can be a challenge. In the case of zinc-finger nucleases, originally three distinct vectors were required, one for each zinc-finger nuclease (each of which cleaves one strand of genomic DNA) and a third for the donor DNA [69, 70]. More recently, fusing of each zinc-finger component to viral proteins has allowed for the generation of efficient singular IDLVs containing each zinc-finger nuclease and the donor DNA [69, 71]. For TALENs, the challenge has been that the repetitive nature of the DNA encoding

the nuclease can recombine during the reverse transcription of the viral genome [72]. However, using a similar approach as the zinc-finger nucleases, Cai et al. fused TALEN proteins to viral proteins to allow incorporation into a single vector [69]. Additionally, IDLVs have been modified further to inactivate the reverse transcriptase, allowing for vectors that can deliver TALEN mRNA into host genomes, avoiding the possibility of recombination by viral machinery [73]. CRISPR-Cas systems have also been delivered by IDLVs and integrating lentiviral vectors for a variety of experiments, ranging from library-on-library screening of CRISPR-Cas cleavage efficiency across the genome to removal of proviral DNA such as HIV-1 and Hepatitis B [68, 74–77]. In addition to nuclease delivery, an interesting application of IDLVs has been used to deliver nucleic acid modifying enzymes such as deaminases and epigenetic modifying enzymes such as histone deacetylases [56, 78]. Such *in situ* histone and DNA modification allows for genotypic or epigenetic change without the introduction of foreign DNA.

1.3.3 Translational and Clinical Progress Using Retroviral Vectors

Vectors based on retroviruses made an impact on treating human disease, and their use may increase as vectors become increasingly safe. A landmark study exemplified the success of a self-inactivating (SIN) γ-retrovirus vector to treat X-SCID [79]. This vector was an improvement on the previous generation Moloney murine leukemia virus vectors used to treat X-SCID and was shown to be less mutagenic due to a long terminal repeat (LTR) U3 enhancer deletion and the human elongation factor 1-α short promoter used to control the delivered gene [79–81]. Using this vector, the group showed that 8/9 treated patients exhibited improved immune function, with one patient dying due to a preexisting infection caused by an adenovirus [79]. Other recent trials have shown success in the use of integrating lentiviral vectors to reduce the autoimmune complications and microthrombocytopenia associated with Wiskott-Aldrich syndrome [82, 83]. One of the most promising uses of retroviral vectors is the modification of patient T-cells to target malignant cell populations by employing antigen receptors that bind to antigens specific to cancer cells. This method, called chimeric antigen receptor T-cell therapy, or CAR T-cell therapy, will be described in detail later in this chapter [84].

1.4 Adenoviral Vectors

Adenoviruses are non-enveloped double-stranded DNA viruses with ~35 kilobase pair genomes and are somewhat larger than many viruses commonly used in gene therapy [85]. In the adenoviral genome, several genes exist to regulate expression of

viral and host factors. These genes are often removed or manipulated to both make room for transgenes as well as decrease the ability of the virus to replicate after transduction into the host cell [86–88]. While there are over 50 serotypes of adenovirus, the most commonly used and best understood is serotype 5, often referred to as Human adenovirus serotype 5 (HAdV-5) [89]. These viruses, unlike retroviruses, have no endogenous integration machinery and do not incorporate into host genomes at high frequency, instead remaining as episomal elements [89]. Their episomal nature means that they have a much lower mutagenic potential than retroviruses. Naturally, the HAdV-5 vector has an affinity for transduction in hepatocytes, which is a benefit for delivery of transgenes to the liver, but a downside if other cell targets are desired [89, 90]. Additionally, adenoviral vectors have been shown to be highly immunogenic, due to natural exposure to adenoviral particles that most humans experience early on in life [89, 90].

1.4.1 Modifications and Implementation of Adenoviral Vectors

One of the first applications of HdAdV-5 for gene therapy was by Jaffe et al. who deleted the E1 and E3 viral genes to inhibit viral replication and make room for the human α1-antitrypsin gene and a β-galactosidase gene (as a marker of viral transduction). After intraportal injection into rats, the group found that α1-antitrypsin was detectable in serum for up to 4 weeks, demonstrating the power of modified adenoviral vectors for gene therapy [86, 91]. Shortly after, the same group showed the efficacious use of the HAdV-5 vector without E1/E3 genes to transfer human Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) genes into the respiratory epithelium of rats, demonstrating the potential for such vectors to treat Cystic Fibrosis [92].

Further removal of essential viral genes has produced vectors with transgenes flanked by inverted terminal repeats (ITRs, necessary for packaging the genome into the vector) referred to as gutless adenoviral vectors, with the viral genes needed for production supplied by the cell line used to manufacture the virus [93, 94]. "Gutless" vectors have been used to introduce DNA into human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) by homologous recombination [88, 95, 96]. For example, such vectors have been used to repair genes involved in laminopathy, muscular dystrophy, and hemophilia B [96–98]. Given the large genome size of adenoviruses, these vectors are ideal delivery systems for genes that are too large for other viral vectors.

In addition to solely delivering DNA to replace or complement ineffective/mutated genes, groups have also delivered nucleases and recombinases that stimulate recombination between the donor DNA and the host genome [89, 99–101]. As discussed with lentiviral vectors, zinc-finger nucleases, TALENs, and CRISPR-Cas systems have been similarly delivered with adenoviral vectors [100, 101]. For instance, Perez et al. used zinc-finger nucleases encoded in a viral vector to dis-

rupt the CCR5 locus of CD4+ T cells with high frequency, making them resistant to HIV-1 infection [102]. However unlike lentiviral vectors, TALEN genes have been shown to be packaged stably with a lower spontaneous recombination frequency [23]. Also, multiple nucleases can be encoded in one efficacious vector due to the large genome size of adenoviral vectors [103]. Moreover, as in the lentiviral vectors discussed earlier, adenoviral vectors have modified to change cell type tropism. By modification of capsid components, the preference for liver tropism for adenoviral vectors has been decreased as well as re-targeted to other cell types, such as muscle cells [104, 105].

1.4.2 Translational and Clinical Progress Using Adenoviral Vectors

Adenoviral vectors were some of the first gene therapy tools used in clinical trials. A vector delivering a correct CFTR gene, which had been delivered successfully in rat epithelium, was trialed in humans in a landmark study [92, 106, 107]. While gene transfer did occur, with measurable transcription of transgene mRNA, functional replacement and symptomatic relief was not observed due to transient expression of mRNA and the decreased efficacy upon repeat admissions of the therapy (likely due to immunity associated against the vectors) [108, 109]. Notably, the first human death in a phase I viral gene therapy clinical trial occurred using adenoviral vectors attempting to correct a metabolic deficiency that leads to ammonia buildup. Jesse Gelsinger died shortly after administration of an adenoviral vector carrying an ornithine transcarbamylase gene after a severe reaction to the infusion [110]. While there are still several questions surrounding the exact reasons why Mr. Gelsinger had such a severe reaction, it has been proposed that his high ammonia level pre-infusion could have contributed [111]. Mr. Gelsinger's death was a tragic setback in the field of gene therapy but has highlighted the importance for stringency, informed consent, and quality practices in human gene therapy clinical trials [111, 112].

While the strong immune response that adenoviruses illicit in humans has been a challenge for gene delivery, it has also been used to the advantage of scientists and clinicians. By expressing viral or bacterial antigens proteins using adenoviral vectors, an immune response could be generated against the pathogen. Vaccination using adenoviral expressed antigens of *Mycobacterium tuberculosis* has shown stimulation of CD4+ and of CD8+ populations [113]. Similar approaches using HIV-1 antigens have yet to show significant immune protection [114]. To circumvent this, groups have used adenoviral vectors to express neutralizing antigens to HIV-1 intramuscularly, which have protected humanized mice from HIV-1 infection despite several high-titer exposures [115]. Another use for adenoviral vaccines has been to utilize them to help combat addiction to substances such as

cocaine and nicotine [116]. By covalently linking small molecule analogs of such addictive compounds, De et al. have used adenoviral vectors to illicit humoral immune responses to cocaine and nicotine in mice [116].

Using the potentially cytotoxic effect of viral infection, the use of adenoviral vectors as oncolytic viruses to target and kill cancer cells has shown great potential. To target transduction of adenoviral vectors to cancer cells, fiber modifications have been employed by several groups, for example by modification of the fiber capsid protein of HAdV-5 to contain an RGD-4C integrin binding motif, enhancing binding and transduction of ovarian and prostate cancer cells [117, 118]. In an animal model of ovarian cancer, this oncolytic vector significantly improved survival of treated diseased animals [117]. Additionally, multiple have modified adenoviral vectors such that they will replicate only in cancer cells by using cancer-cell specific promoters, such as prostate serum antigen promoter [119–122].

1.5 Adenoviral-Associated Viral Vectors

Discovered in 1965 in cell cultures co-infected with adenovirus, the adeno-associated virus (AAV) is a small non-enveloped parvo virus that is deficient in replication [123, 124]. The single-stranded AAV genome can integrate into the host genome after complementary strand synthesis or exist as an episomal element post-infection [124–126]. The AAV genome consists of approximately 4.7 kilobase pairs and is relatively refractory to size increases. This genome is composed of two open reading frames (ORF), *rep* and *cap*. Currently 13 AAV serotypes have been identified, many of which have different tissue/organ transduction profiles [127]. The capsid is composed of three subunits, VP1, VP2, and VP3, all of the *cap* ORF are expressed in the capsid with a stoichiometry of 1:1:10, respectively [128, 129]. The *rep* ORF is composed of four proteins that are essential for packaging, transcription, as well [130] integration into the viral genome into the AAVS1 locus on human chromosome 19 due to a Rep binding site at this locus [125, 130, 131]. The last gene encodes the assembly-activating protein (AAP), which is contained within the *cap* ORF in an alternate coding frame. This gene is used to assist in the assembly of VP1, VP2 and VP3 into the mature capsid [129, 130]. The entire genome is flanked by inverted terminal repeat (ITR) sequences, which cap either end of the genome with partially double stranded regions [125, 130].

AAVs utilize several cell surface receptors for host cell entry. The first discovered receptor was the heparin sulfate proteoglycan receptor, followed by discoveries of co-receptors including α5β1 integrin, CD9, the laminin receptor, and the hepatocyte growth factor receptor, all of which contribute to AAV tropism specification depending on the serotype [132–136]. Recently, an essential receptor for AAV host cell incorporation has been discovered via a genetic screen approach in a haploid cell line [137]. After tropism to the nucleus, AAVs stay latent unless a helper virus is present to assist in replication [125].

1.5.1 Modifications and Implementation of Adeno-associated Viral Vectors

AAV vectors have a long history of use in the field of gene therapy due to their effective tropism in different cell types and lower relative cytotoxicity. With such long history, they have also been modified in various aspects. The removal of the *rep* ORF is a major modification made in recombinant AAV (rAAV) vectors. Without this ORF, the propensity of the viral genome to integrate is decreased and the genome is more likely to exist in the cell as an extrachromosomal episome. This decreases the potential for insertional mutagenesis to the host genome [138]. Additionally, the Rep protein has toxic effects on the host cell and can reduce cell viability post-AAV infection [131]. Another modification made to some AAV vectors is the generation of self-complementary recombinant AAV (scAAV) genomes. By decreasing the genome size in half, the capsid can contain two complementary single stranded copies of the AAV genome. The major advantage of scAAVs is that they are much more efficient at transduction, increasing transduction by more than 140-fold in the original study by McCarty et al. [139]. The small size of AAVs and small packing capacity is an ongoing challenge for AAV vectors used in gene therapies. One solution to large cargoes is to split transgenes between two or more AAV vectors and co-infection, with the transgene transcript combined after transduction into the host cell [140]. Additionally, creating minimal versions of transgenes and regulatory elements have been proposed and attempted as a partial solution to the small DNA capacity of AAV vectors [140]. Gene delivery using AAV vectors (and other gene therapy vectors) falls in two broad categories: gene supplementation and gene replacement. Gene supplementation is useful when adding additional copies of a mutated or missing gene. Gene replacement can be used when the patient's ineffective allele must be inactivated or replaced for normal phenotype to be restored (as is in the case of dominant negative alleles). In the case of gene replacement, the delivery of engineerable nucleases (such as zinc-finger nuclease and CRISPR-Cas systems) to stimulate homologous recombination has been shown to be effective using AAV vectors in animal models [141–144].

The host immune response to AAV vectors is a major obstacle of varying severity depending on the method of delivery. For example, Brockstedt et al. showed that in mice antigen-induced immune reactions in intramuscularly delivered rAAV vectors encoding ovalbumin elicited a much reduced cytotoxic T-cell response to ovalbumin (however the a humoral response was still present) as compared to intraperitoneally, subcutaneously, or intravenously delivered vectors [145]. Additionally, neutralizing antibodies have the ability to inactivate systemically delivered AAV vectors, which can decrease transduction efficiencies in animal models and likely in human trials as well [146–148]. To combat neutralizing antibody effects on transduction into model organisms, Li et al. used *in vitro* directed evolution in the setting of human serum collected from a patient to identify regions in the AAV6 capsid crucial for evasion of neutralizing antibodies [149]. Using their results, this group generated chimeric AAV vectors capable of improved transduction in muscle tissue [149].

Additionally, much effort has been put into understanding the antigenic epitopes of the AAV capsid by many experimental and computational schemes [150]. Targeting AAV vectors to specific tissue types has largely been accomplished by identification of naturally occurring AAV serotypes that efficiently transduce the organ/tissue of interest [124]. The more scientists develop AAV vectors that utilize naturally occurring serotype transduction efficiencies, consider cell surface glycan interactions with AAV capsids, and engineer capsids through *in vitro* diversity generation and functional selection, the more they will be able to generate highly specific and targeted AAVs [124, 151–153].

1.5.2 Translational and Clinical Progress Using Adeno-associated Viral Vectors

With low toxicity, high transduction efficiencies across many tissue types, and facile manipulation, AAV vectors have become one of the most popular vectors for human gene therapy [124, 154]. As with adenoviral vectors, the first target for clinical trials using AAV vectors based on AAV2 was for the delivery of the CFTR gene in patients with cystic fibrosis [155]. Currently there are 173 recorded clinical trials involving AAVs as gene therapy vectors [156]. Recently there have been several successes using AAV vectors for gene therapy with effective therapeutic outcomes. One of these was an scAAV8 vector encoding human clotting factor IX for supplementation delivery to patients with Hemophilia B. Delivery of this vector to ten patients resulted in factor IX levels 1–6% of normal factor IX values. In patients who had a mean of 5.1 ± 1.7% of normal values of factor IX, there were 90% fewer bleeding events [157]. Another recent success involved AAV2 vectors encoding RPE65 to supplement mutated RPE65 genes in patients with Leber's congenital amaurosis as well as in a canine model of the disease. AAV2-RPE65 vector resulted in modest but temporary improvements in retinal sensitivity in patients and canine subjects [158]. Another important AAV vector therapy is the treatment of lipoprotein lipase deficient patients using AAV1-LPL^{S447X}, encoding a gain-of-function lipoprotein lipase which was shown to resolve chylomicronemia in lipoprotein lipase deficient mice. In a 2 year follow-up of a trial using this vector, Gaudet et al. showed that half of the treated patients showed a ≥40% reduction in fasting triglycerides, resulting in a clinical benefit to the patients involved [159]. This vector has been approved in Europe for clinical use, making it the first gene therapy ever approved in Europe or America. The vector, with the proprietary name of Glybera, costs nearly $1 million dollars per treatment, making payment for this treatment of a rare disease a serious consideration for patients and insurance companies [160, 161]. Looking forward, traditional AAV serotypes used for AAV vectors will likely be modified and tailored more specifically for the tissue targets. Recent success with AAV3-based engineered vectors suggests that they may be superior for *in vivo* AAV gene therapy as compared to many traditional AAV

Table 1.1 A summary of viral vector genome size and notable applications

Viral vector	Approximate genome size (kilobase pairs)	Notable applications
Adenovirus	36	In vitro gene delivery (highly immunogenic) [90, 126] Vaccination against pathogens and addictive compounds [87, 113, 116] Destruction of malignant cells [119, 121]
Adeno-associated virus	4.7	In vitro and in vivo gene delivery [140, 155] Relatively lower immunogenicity [145] Broad cell type specificity, depending on serotype [152]
Retrovirus (including lentivirus)	7–12	In vitro and in vivo gene delivery [57, 62, 68, 79] Modification of cells for ex vivo therapy [82]

serotypes used in clinical gene therapy applications (AAV5, AAV8, and AAV9) [162]. Table 1.1 summarizes some of the important features of each viral vector discussed previously.

1.6 *Ex Vivo* CRISPR Therapies

1.6.1 CAR T-Cell Therapy

Adoptive cell immunotherapy, or the transfer of lymphocytes to mediate effector function, is not a novel concept; in 1992, it was shown that a single infusion for CMV-specified CD8 CTLs could be used to treat disseminated CMV infection in post-allogeneic transplant patients [163]. In 2002, CD4 effector cells were shown to be efficiently transferred in HIV and elevated CD4 cell counts, and in 2005 it was shown that vaccine responses could be augmented in patients with myeloma using autologous T cells [164, 165]. It is generally believed that the beginnings of modern human immunooncology began with the approval of '1st Generation' Sipuleucel-T in 2010 and Ipilimumab, a CTLA-4 checkpoint inhibitor, in 2011 for treatment of castrase-resistance prostate cancer. '2nd Generation' agents included programmed cell death protein 1 (PD1) and PD1 ligand 1 (PD-L1) blocking antibodies as well as blinatumomab, a bi-specific antibody, an oncolytic GM-CSF-encoding herpes simplex virus known as talimogene laherparepvec or T-vec for metastatic melanoma, and CAR-T cells in 2014–2015 [166].

As previously mentioned, one of the most promising emerging uses of retroviral vectors to treat human disease, specifically cancer, is known as chimeric antigen receptor or CAR T-cell therapy. It involves the modification of patient T-cells to target malignant cell populations by employing antigen receptors that bind to cancer cell-specific antigens. CARs are fusion proteins that incorporate antigen recognition

variable region antibodies with T-cell activation domains. Unlike TCRs which recognize HLA-presenting peptides and are therefore restricted by the HLA-specific patients, CARs work by recognizing glycoproteins and intact cell-surface proteins and are HLA-independent. Originating from clinical trials of CAR-transduced T cells targeting α-folate receptor on ovarian cancer cells, many subsequent methods have been developed to insert CAR genes into T cells, including gammaretroviruses, lentiviruses, and transposon systems [167].

There are generally three different methods of adoptive cell therapy under investigation and reaching FDA approval: the use of tumor-infiltrating lymphocytes (TILs), chimeric antigen receptor (CAR) and T cell receptor (TCR) engineered T cells. TILs are produced after surgical excision and expansion of cells from a tumor biopsy and have been slow but progressive in development, with a recent phase 3 randomized trial (NCT02278887) underway for treating metastatic melanoma patients. In contrast, gene transfer-based methods that avoid the effects of immune tolerance are produced via peripheral blood lymphocytes and use viral or nonviral methods to engineer the cells and introduce the desired receptors. They involve the transfer of CARs made of antibody-binding domains fused to T cell signaling domains or alternatively TCR α/β heterodimers to promote the re-directing of T cells to target tissues. The first group of CARs was developed in 1991 as a fusion of the extracellular and transmembrane domains of CD8 to the cytoplasmic domain of the TCR ζ chain and shown to be sufficient to replicate TCR signaling; progressively more complicated designs have since been studied [168]. Most CARs currently in use are derived from mouse antibodies and have been shown in clinical trials to elicit both antibody and T cell responses; attempts to resolve this problem have focused on the use of humanized/fully human antibodies obtained from mice transgenic for human-Ig loci.

T cell costimulation experiments revealed the benefit of additional signaling moieties for CD19 CAR-T cell antigen-specific cytokine production and proliferation. Specifically, adding CD28 moieties and CD3 ζ domains to CD19 CAR-T cells enhanced rates of human leukemia cell eradication in mouse models. Other signaling domains, including TNF receptor super-family member 9 (4-IBB), have been shown to have a similar enhancement compared to CD19 alone [169–173]. Other approaches to enhance CD19 CAR-T cell activity include development of an Epstein-Barr virus (EBV) antigen recognized by CD19 CARs, central memory cells for genetic modification, and allogeneic cord blood T cell modification.

In 2016, the imposition of a clinical hold on Juno's JCAR015 in patients with relapsed or refractory B cell acute lymphoblastic leukemia (ALL) due to cerebral edema and death in two patients highlighted the need for skepticism in CAR-T cell therapy and further inquiry into the different modification and manufacturing processes employed by these candidates and the differential side effects that occur as a result. Supported by Phase II data and backed by FDA designations, companies are making the first steps to receiving regulatory approval for candidates to reprogram the immune system using CAR-T therapies; Kite has already filed submission for its KTE-C19 therapy for diffuse large B-cell lymphoma, and Novartis has plans to submit CTL019 for acute lymphoblastic leukemia in early 2017 [166].

The crossover of the CRISPR multiplex editing techniques to CAR-T therapy is a new and exciting area of active investigation. It has been shown that up to five genes can be simultaneously disrupted in mouse embryonic stem cells with high efficiency CRISPR-Cas9; specifically, CAR-T cells with either two or three gene disruptions (TRAC, B2M +/− PD-1) and analysis of *in vivo* and *in vitro* antitumor function. Using CAR-T cells targeting the B-cell antigen CD19, chosen for its expression by nearly all B-cell malignancies and restriction in normal tissues to expression in mature and precursor B cells, plasma cells, and follicular dendritic cells [11]. It was shown that anti-CD19 CARs were capable of activating T cells in a CD19-specific mechanism that could kill CD19+ primary leukemia cells *in vitro* [174, 175].

1.6.2 iPSCs

Reprogramming of somatic cells has allowed the creation of patient-specific induced pluripotent stem cells (iPSCs). They have the unique properties of self-renewal, large scale expansion, and ability to differentiate into endoderm, mesoderm, ectoderm, or even to hematopoietic stem cells (HSCs) in the presence of stromal cell co-culture or hematopoietic cytokines [176–178]. In as early as the 1960s, it was shown that a pluripotent state could be generated through the reprogramming of fully differentiated cells; essentially, it was demonstrated early on that totipotency could be achieved through alterations in the epigenetic profile [178]. Subsequent somatic nuclear transfer (SCNT), including the "Dolly" experiment, and cell fusion experiments revealed the presence of somatic cell-inducing cytoplasmic diffusible transacting factors in the oocyte/ESC in addition to the proof of reprogrammable terminally differentiated cells.

These results paved the way for one of the landmarks papers by Takahashi and Yamanaka in 2006, which showed the possibility of ectopic expression of a distinct and small set of transcription factors via retrovirus integration into differentiated cells. By identifying and serially reducing this set of genes into the minimal set of factors (Klf4, Sox2, Oct4, Myc) and demonstrating the retention of embryonic stem cell properties in these now 'induced pluripotent stem cells' (iPSCs), they set the stage for subsequent research on refining and implementing various methodologies to edit and induce functional pluripotency in a range of differentiated human cell types. The Yamanaka experiments additionally resolved and avoided the ethical debate around the use of stem cells sans human embryos [178]. Figure 1.3 demonstrates the process for *ex vivo* modification of somatic cells to iPSCs and ultimate correction of disease mutations by genome editing.

Studies using CRISPR/Cas9 editing in the transformation of iPSCs generated from somatic cells have demonstrated homologous recombination-based gene correction that could provide new avenues for treating certain genetic disorders, including β-thalassemia and Duchenne muscular dystrophy, as mentioned before [179].

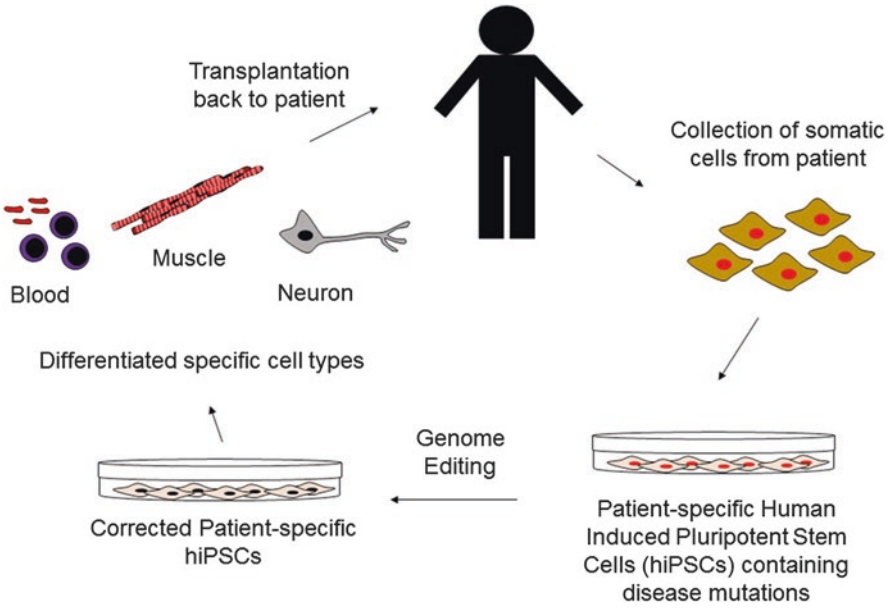

Fig. 1.3 Schematic of isolation of somatic cells from patients, generation of induced pluripotent stem cells, correction of disease causing traits, differentiation into specific cell type and transplantation back into the host

Cas9 methods were used to remove the premature stop codon in the DMD gene leading to Duchenne Muscular Dystrophy and resulted in partial restoration of protein function [180]. Additionally, patient-specific iPSCs generated from Hemophilia A patients were used in conjunction with Cas9-mediated editing to remedy the large-scale chromosomal inversions that underlie the disease process [181].

As for the future of iPSC and CRISPR therapy to treat human disease, many challenges remain. In the clinical setting, treatments generally rely on producing a defined gain of function at desired genes with high frequency; with this method, however, human cells prefer the imprecise pathway of non-homology end joining (NHEJ) repair of the double strand breaks in DNA as opposed to the homology-mediated editing [182]. Therefore, many approaches have been taken to shift the DSB repair pathway from the generation of NHEJ-mediated insertions and deletions to homology-mediated repair; these include cell-cycle dependent control of CRISPR/Cas9 delivery via small molecular NHEJ-inhibitors [183–186]. Additionally, the goal of generating complex hiPSCs with a wide variety of genetic alterations is hindered by the short conversion tracts of human cells and resulting limitation of either NHEJ or HDR mechanism to one side of the DSB [187]. This poses the biggest challenge of broadly applying iPSCs and CRISPR/Cas9 to editing the human genome as well as reveals the unrealized potential of the technology to produce tremendously helpful resources, such as condition human knockout iPSC libraries.

1.7 Conclusion

Genetic medicine has allowed for patient-specific treatment of disease. Progress in modification of patient-specific disease traits in cells, tissues, and whole organ systems has become closer to a reality thanks to multidisciplinary approaches to gene therapy. Modification of cells at the genetic level using CRISPR-Cas systems has revolutionized the ease and efficacy of cell modification, and delivery of genetic material using viral vectors has allowed a level of nuclear access previously unimaginable.

While these advances continue to progress, several key issues need to be solved. One of these issues is the targeting of gene delivery vectors to tissues and organs with spatiotemporal control. Often, genetic disease manifests in only a subset of tissues and organs, meaning that the delivered gene or cell must target that region specifically. Off-target effects of both gene delivery and cellular delivery can result in toxic outcomes and can lead to patient death, as was discussed in the history of viral vectors [62, 111]. Additionally, controlling the activity and timing of therapeutic gene expression or cellular activity may be crucial, as disease progression can be dynamic over time. An added layer of complexity is navigating the host immune system as it serves as a powerful barrier against both viral gene therapy as well as cellular approaches. Going forward, scientists and clinicians will continue to struggle with specificity and control in targeting precision gene therapies. However, the potential for the tools discussed in this chapter will continue to grow. In the coming decades, it is likely that most medicine will be practiced in a precise fashion with tailored cures for each patient's unique genome.

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Chapter 2
Combining Engineered Nucleases with Adeno-associated Viral Vectors for Therapeutic Gene Editing

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Abstract With the recent advent of several generations of targeted DNA nucleases, most recently CRISPR/Cas9, genome editing has become broadly accessible across the biomedical community. Importantly, the capacity of these nucleases to modify specific genomic loci associated with human disease could render new classes of genetic disease, including autosomal dominant or even idiopathic disease, accessible to gene therapy. In parallel, the emergence of adeno-associated virus (AAV) as a clinically important vector raises the possibility of integrating these two technologies towards the development of gene editing therapies. Though clear challenges exist, numerous proof-of-concept studies in preclinical models offer exciting promise for the future of gene therapy.

Keywords AAV • Gene therapy • Gene editing • CRISPR/Cas9 • Zinc-finger nuclease

Abbreviations

AAV	Adeno-associated virus
CCR5	C-C chemokine receptor type 5
CRISPR	Clustered regularly interspaced short palindromic repeats

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crRNA	CRISPR targeting RNA
DMD	Duchenne's muscular dystrophy
Dmd	Dystrophin
Fah	Fumarylacetoacetate hydrolase
FIX	Coagulation factor IX
HBV	Hepatitis B virus
HDR	Homology-directed repair
HSC	Hematopoietic stem cell
NHEJ	Non-homologous end joining
OTC	Ornithine transcarbamylase
PCSK9	Proprotein convertase subtilisin/kinexin type 9
SaCas9	*Staphylococcus aureus* Cas9
sgRNA	Single guide RNA
SpCas9	*Streptococcus pyogenes* Cas9
TALE	Transcription activator-like effector
TALEN	Transcription activator-like effector nuclease
tracrRNA	Trans-activating crRNA
ZF	Zinc finger
ZFN	Zinc-finger nuclease

2.1 Introduction

Gene therapy, the treatment of disease via the delivery of genetic material to cells, has enabled incurable diseases to now be considered as therapeutic targets, including both monogenic diseases with well-defined underlying genetic etiology as well as idiopathic diseases with candidate gene targets. Throughout most of its history, the major barrier to gene therapy has been delivery. A major advance has been the development of safe and effective delivery vectors, and the most prominent for *in vivo* gene therapy have been based on adeno-associated viruses (AAV). Natural AAVs offer reasonable infectivity, a lack of pathogenicity, numerous variants with different tissue tropisms, and negligible genomic integration. As a result, vectors based on AAV have begun to show increasing clinical promise, primarily in studies involving gene augmentation where additional copies of genes are delivered to either replace the functionality of null alleles in recessive diseases or to overexpress a potentially therapeutic factor. In particular, AAV has been successful in trials for monogenic recessive disorders including Leber's congenital amaurosis type 2 (LCA2) [1, 2], hemophilia B [3, 4], spinal muscular atrophy [5], and lipoprotein lipase deficiency [6, 7]. The last of these is the basis for a clinically approved gene therapy product in the European Union, and it is anticipated that a gene therapy for LCA2 may be approved in the US in 2017. In addition, early-stage clinical trials have demonstrated some positive signs in harnessing AAV to treat more complex disorders, such as overexpressing SERC2A in heart failure patients [8] and

expressing the vascular endothelial growth factor (VEGF) inhibitor sFLT-1 in patients with age-related macular degeneration [9].

While gene therapy is thus gathering increasing momentum, particularly for monogenic diseases, a number of disorders are not amenable to gene augmentation therapy. For instance, autosomal dominant genetic diseases require the elimination or modification of the disease-causing allele. In addition, AAV has a limited carrying capacity of <5 kb [10], and mutated genes whose cDNAs exceed this threshold require alternate approaches. Furthermore, while non-integrating vectors like AAV might be safer than integrating vectors, they can be insufficient to treat disease requiring gene delivery in actively mitotic cells due to progressive dilution of the delivered extrachromosomal genetic cargo with each cell division [11].

Gene-editing technology in the form of targeted nucleases, with the capacity to directly and permanently edit and modify the cellular genome, can potentially address such challenges. For example, these nucleases may offer the capability to specifically eliminate dominant disease alleles, correct endogenous genes, or integrate exogenous genes at safe harbors, resulting in permanent changes that are heritable in mitotic cells. These approaches could be applied for direct *in vivo* therapy, and AAV's potential for high delivery efficiency coupled with the enhanced efficacy of AAV genomes as DNA donors for homology-directed repair also offers the capacity for *ex vivo* modification of cells for subsequent engraftment. Coupling the potential of gene-editing technology with the increasingly well-established, safe, and effective gene delivery capabilities of AAV may thus render new classes of genetic diseases accessible to gene therapy.

2.2 Therapeutic Gene Editing

Targeted gene editing has two primary goals—disrupting a sequence or introducing a precisely defined modification to a sequence—and both strategies begin with generating a DNA break at the locus of interest. For disruption, the non-homologous end joining (NHEJ) cellular repair mechanism directly rejoins the two ends and typically introduces small insertions or deletions (indels) at the cut site [12]. When placed near the 5′ end of a coding sequence, such indels generally disrupt the reading frame and thereby effectively knock out the target gene. For precise modification, a DNA template containing both the desired modification and flanking regions of DNA homologous to the target area, known as homology arms, is co-delivered with the nuclease. The homology-directed repair (HDR) pathway can then splice the template in place of the damaged DNA within the region between the homology arms, thereby mediating specific gene modification (Fig. 2.1) [13, 14].

Both strategies require a means to generate targeted DNA strand breaks, and the first such engineerable tool was zinc-finger nucleases (ZFNs). An "alphabet" of individual zinc finger (ZF) DNA binding domains that bind to specific three-nucleotide targets was identified; these ZFs could then be modularly assembled to target new

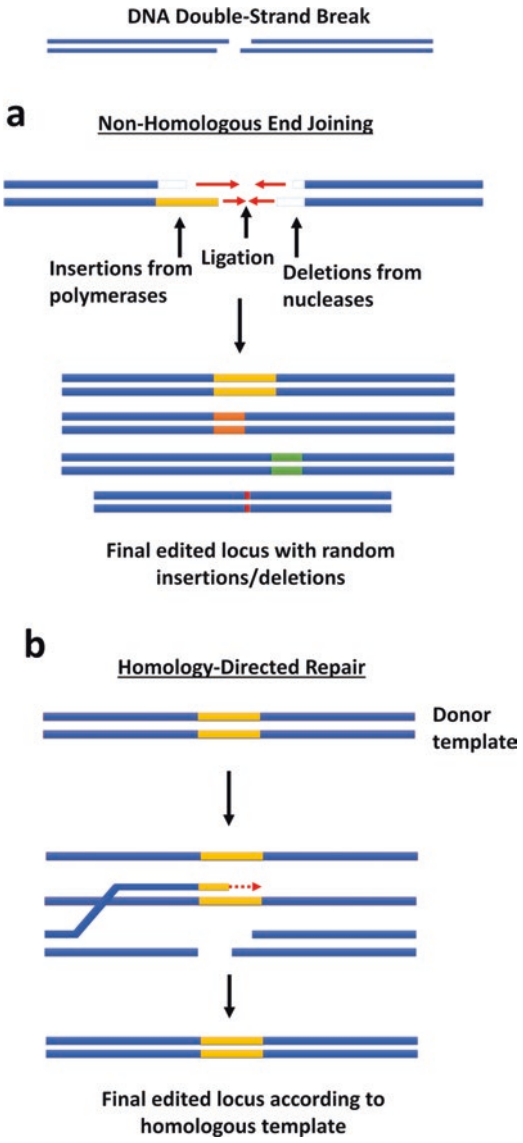

Fig. 2.1 Cellular mechanisms of DNA repair following DNA double-strand break. When a double-strand DNA break occurs, one of two cellular mechanisms repairs the damage. (**a**) In non-homologous end joining, polymerases and nucleases clean up the damaged ends by adding or deleting small numbers of nucleotides until they can be rejoined by ligases. The final ligated product contains small insertions or deletions (indels) at the damage site, often resulting in a frameshift. (**b**) In homology-directed repair, the 3′ overhang strand at a site of DNA damage can displace a strand in a separate DNA duplex with homology to that strand (a donor template). Polymerases extend the damaged end according to the homologous template DNA duplex, and the strand either returns to its original complementary strand, annealing to the other original damaged end, or the donor template strand and the previously damaged strand can undergo complete crossover and recombination. Either option results in a repaired strand

desired genetic loci and fused with *Fok*I nuclease domains to yield custom nucleases [15, 16]. This advance opened the door not only to a broad range of important basic research applications but also to the potential capacity to treat disorders including HIV infection [17], hemophilia [18], sickle cell anemia [19], and others. For instance, the use of ZFNs to knock out the HIV co-receptor CCR5 within T cells, and thus render them resistant to HIV infection, is currently in clinical trials [17]. While established ZFNs are indeed quite effective enzymes, generating new nucleases is difficult since the target specificity of each individual ZF domain can depend on the context of its neighboring domains [20], requiring a high level of expertise, ZFN library selection methods, and thus a time-consuming process to generate specific ZFNs for new targets.

In 2009, the DNA binding domains of the transcription activator-like effector (TALE) class of bacterial transcription factors was found to consist of modular elements [21, 22]. Excitingly, these individual TALE domains were found to bind single nucleotides with strong specificity and, importantly, with minimal context dependence, unlike ZFNs. Thus, TALE DNA binding domains could be linked together with near-ideal modularity to target virtually any desired DNA sequence, and fusion to a *Fok*I nuclease domain yielded TALE nucleases (TALENs) [23]. Simple assembly kits made the generation of new functional TALENs rapid and accessible to researchers. That said, the resulting TALEN constructs were very large and thus challenged the carrying capacity of delivery vectors like AAV, and the repetitive nature of the TALEN coding sequence led to concerns with recombination in the context of these ssDNA viral vectors.

While the simplicity of TALENs seemed unlikely to be surpassed, in 2012, it was demonstrated that the bacterial anti-viral adaptive immune mechanism known as the CRISPR/Cas9 system [24–26] could be re-engineered for targeted gene editing [27], a finding that was subsequently applied for genome editing in human cells [28–30]. Three components of the system from *Streptococcus pyogenes* are necessary and sufficient for enzymatic activity: the CRISPR-associated protein 9 (Cas9) nuclease; the CRISPR targeting RNA (crRNA) that is complementary to a target DNA sequence; and the trans-activating crRNA (tracrRNA) that hybridizes with a crRNA, enables it to bind to Cas9, and helps direct cleavage activity to the encoded locus. Fusing the two RNA components into a single guide RNA strand (sgRNA) further simplified the system such that virtually any desired target strand of DNA could be targeted and cleaved by simply changing the targeting RNA sequence, limited only by the requirement for a small adjacent sequence known as the protospacer-adjacent motif. With this discovery, DNA cleavage and editing no longer required even simple modular protein assembly but merely modification of ~20 nucleotides of the targeting sgRNA. The simplicity and efficacy of the resulting CRISPR/Cas9 system make effective gene editing broadly accessible.

With the potential to effectively modify virtually any locus, CRISPR/Cas9 gene editing offers promise for both *in vitro* and *in vivo* genome editing. Successful application of this work for therapeutic purposes, however, will hinge upon an effective and reliable method for delivering the CRISPR/Cas9 machinery to affected cells.

2.2.1 AAV

Adeno-associated virus (AAV) is the most clinically successful *in vivo* gene therapy vector to date. AAVs are a family of non-enveloped, single-stranded DNA viruses that naturally require the presence of a helper virus, such as an adenovirus, to replicate. The 4.7 kb AAV genome contains two short (~150 nucleotide) viral inverted terminal repeat sequences (ITRs) flanking two genes, *rep* and *cap*, encoding proteins for replication and capsid formation, respectively. Because these genes can function in trans, the virus can be engineered for assembly of virus particles with recombinant genomes containing only the desired genetic cargo flanked by the ITRs. AAV has numerous natural serotypes with somewhat different viral capsid sequences and tissue tropisms, indicating that differences in the viral capsid proteins can lead to differences in infectivity. As a non-integrating virus with a strong safety record, AAV has strong promise as a clinical gene delivery vehicle, and, as mentioned above, there are numerous examples of strong proofs of concept in clinical trials as well as one regulatory approval in the European Union [1–9].

In addition to delivering DNA sequences for direct expression, the single-stranded nature of the AAV genome can serve as an effective template that inherently stimulates the homologous recombination pathway to mediate gene targeting [31]. Specifically, viral delivery of a HDR construct with homology to a chromosomal locus, even without a nuclease component, can result in recombination into the target locus at a rate of up to 1% [32], a rate >1000-fold higher than conventional plasmid donors [33] or other viral vectors [34]. Successful AAV-mediated gene targeting has been achieved in neural stem cells [35], human pluripotent stem cells [36], and hematopoietic stem cells (HSCs) [37], among other cell types. In addition, as discussed below, combining AAV with a nuclease offers even stronger potential.

Despite its success, AAV has significant challenges as a gene therapy delivery system. Natural serotypes of AAV are inefficient at infecting many target cells and tissues, do not have the capacity for targeted delivery to specific cells, and can be neutralized by antibodies prevalent within the human population due to prior natural AAV exposure. Additionally, the packaging capacity of AAV is approximately the size of its wild-type genome (4.7 kb), and cargos that are greater than 10% beyond this size are not possible to package [10]. Finally, while a non-integrating virus offers the potential for lower genotoxicity and thus greater safety than an integrating one, the lack of a specific mechanism for vector integration means that the cargo will be diluted over time in mitotic target cells, and treating diseases with a cargo that must persist for efficacy is thus more difficult.

Different approaches have been taken to address these challenges. For example, directed evolution—or the generation of large AAV variant libraries and iterative selection *in vitro* or *in vivo* for enhanced gene delivery properties—has generated novel AAV variants with greatly improved delivery efficiencies for a range of applications and targets [38]. These include enhanced delivery to lung epithelium in human organotypic culture tissue [39] and a pig model of cystic fibrosis [40],

enhanced retrograde transport for targeting specific neuronal populations *in vivo* [41], greatly improved biodistribution to target tissues such as outer retinal photoreceptors upon simple administration to the vitreous [42], and other applications. In addition, a large cargo can be delivered in AAV by packaging fragments of a gene in two separate vectors, and the full product can then be reconstituted *in vivo* via trans-splicing or homologous recombination of the two separate vectors [43], though with a significant decrease in overall efficiency. At any rate, the potential for highly efficient natural and, in particular, engineered AAV delivery to therapeutically relevant targets makes it a strong choice for gene therapy, including for therapeutic applications of CRISPR/Cas9.

2.2.2 Nucleases and AAV for Therapeutic Gene Editing

One major focus of gene editing has been *ex vivo* engineering of cellular therapies, in which a specific patient's cells are harvested, edited, and re-engrafted. Compared to a direct *in vivo* therapy, more *in vitro* delivery options are available. As a prominent example, CD4+ T cells harvested from HIV-infected patients were edited to disrupt the *CCR5* locus and thereby confer resistance to HIV infection, followed by reintroducion into patients. This approach has been implemented with both ZFNs and TALENs, and the ZFN-based approach—in which the nuclease was delivered with an adenoviral vector—is currently in clinical trials in which the engineered cells were shown to persist following administration [17]. In addition to CCR5 disruption, this *CCR5* locus has been edited within HSCs via AAV donor template delivery and the ZFNs transiently expressing through mRNA electroporation [44].

In addition to *CCR5* disruption for HIV [45, 46], therapeutic treatment of β-globinopathies [47] such as sickle-cell disease and β-thalassemia [48, 49] has been explored. *Ex vivo* cell therapy thus has strong potential to address an unmet medical need, though efforts are currently focused predominantly on the hematopoietic system since its cells can readily be harvested and cultured. *In vivo* delivery will be needed to address most other tissue targets.

For *in vivo* editing, AAV's packaging capacity posed initial challenges for CRISPR/Cas9 delivery, as the combined size of the initially best-characterized *Streptococcus pyogenes* Cas9 (SpCas9), the sgRNA, and promoters for each was simply too large to fit into a single AAV vector. However, two primary approaches for utilizing AAV as a CRISPR/Cas9 delivery vector have since emerged. Since the initial discovery and characterization of SpCas9, thousands of CRISPR/Cas9 proteins have been identified [50], many of which are significantly smaller than SpCas9. The best-characterized alternative Cas9 protein, derived from *Staphylococcus aureus* (SaCas9), is nearly 1 kb shorter than SpCas9 and can thus be accommodated along with its sgRNA in AAV [51]. Other non-Cas9 CRISPR proteins, such as Cpf1 [52], offer new binding and cleavage characteristics in addition to being more compact. With these smaller CRISPR/Cas9 proteins, the entire system can fit comfortably in a single AAV vector, though there are still inflexible limitations on the

maximal size of promoters. As an alternative, some studies have packaged SpCas9 and the sgRNA in separate vectors for co-administration [53]. This approach is particularly useful for HDR-modification applications, where, for example, one vector could be used to deliver the nuclease and sgRNA and a second vector the HDR template.

Both the use of smaller Cas9s and the dual vector approach have been successfully implemented *in vivo* for an increasing number of applications, both to disrupt endogenous gene expression as well as to precisely correct disease alleles. In early 2015, SaCas9 and its sgRNA were combined in a single AAV8 vector to disrupt and thereby knock out expression of a cholesterol regulatory gene, proprotein convertase subtilisin/kinexin type 9 (PCSK9), in the adult mouse liver [51]. The result was reduced circulating cholesterol levels.

Later in 2015, another group demonstrated the ability to correct the ornithine transcarbamylase (OTC) locus, a gene responsible for a potentially life-threatening metabolic disease, in the liver using two vectors [54]. One AAV8 vector contained SaCas9, and the second harbored the sgRNA along with the HDR repair template. Co-delivery successfully corrected a mutation in ~10% of the cells within neonatal mouse liver, leading to significantly greater survival of affected mice.

Two-vector systems have also been successfully used for targeted gene disruption. In 2016, three research groups used either SaCas9 or SpCas9 in a two-vector system to disrupt an exon within the dystrophin gene that harbored a disease-causing mutation within a mouse model of Duchenne's muscular dystrophy (DMD) [53, 55, 56]. Loss of dystrophin expression in the corresponding human monogenic recessive disorder leads to progressive muscle degeneration. To restore expression of this essential protein, they targeted loci within the splice sites flanking the mutation-containing exon 23, and the resulting successful elimination of this non-essential exon from the mRNA led to a functional protein product. AAV was administered via several routes—including direct intramuscular injection, intravenous injection, retro-orbital injection, and neonatal intraperitoneal injection—which resulted in varying levels of functional dystrophin production in muscle tissue. While the fraction of muscle cells corrected was low, as in the OTC liver study, it was sufficient in these models of DMD to restore significant levels of muscle function.

A comparison of each of these gene editing strategies using AAV in combination with engineered nucleases is provided in Table 2.1.

2.2.3 Challenges

While gene editing therapy offers considerable promise, numerous challenges still must be overcome. First, there is a risk of engineered nucleases cutting unintended sites with imperfect but very close homology to the nuclease target site. Such off-target editing is well known to occur within *in vitro* contexts [57], and this risk can be further amplified by viral delivery methods such as AAV that can lead to persistent Cas9/gRNA expression in non-dividing cells for durations far longer than

Table 2.1 Therapeutic applications of gene editing via AAV combined with engineered nucleases

Disease	Target gene	Target cell/tissue	Nuclease used	AAV serotype used	Modification type	Reference	Note
HIV infection	CCR5	CD4+ T cells	ZFN	rAAV6	Disruption	[44]	Donor template only in AAV; nuclease mRNA
Hemophilia B	FIX	Hepatocytes	ZFN	rAAV8	Correction	[67]	
Cardiovascular/cholesterol homeostasis	PCSK9	Liver/hepatocytes	SaCas9	rAAV8	Disruption	[51]	
DMD	Dmd	Muscle	SpCas9	rAAV9	Exon excision	[53]	
		Muscle	SaCas9	rAAV9	Exon excision	[55]	
		Muscle	SaCas9	rAAV8	Exon excision	[56]	
Metabolic disease	OTC	Liver/hepatocytes	SaCas9	rAAV8	Correction	[54]	
Hepatitis B infection	HBV polymerase	HepAD38 cells	ZFN	rAAV2	Disruption	[68]	
Tyrosinemia	Fah	Hepatocytes/liver	SaCas9	rAAV8	Correction	[69]	Donor template only in AAV; nuclease mRNA

needed for the genome editing—an undesirable condition since the likelihood of undesired off-target cutting increases with nuclease residence time [58, 59]. This can been mitigated *ex vivo* though delivery of mRNA encoding the nuclease or even recombinant nuclease proteins [44], but these methods do not translate well to *in vivo* contexts.

Numerous approaches have thus been developed to reduce such off-target effects. One strategy is Cas9 protein engineering. For example, a mutant form of Cas9 capable of only nicking one strand of DNA, rather than cleaving both, was combined with two sgRNAs targeting opposite strands near the desired locus. The resulting paired nicks yielded double-stranded breaks that could be harnessed to generate indels or achieve HDR, but single nicks (such as at an off-target site that matches one sgRNA but not the other) instead lead to high-fidelity repair through the base excision repair pathway. The result is reduced off-target editing [60]. In another approach, rational modifications were introduced into Cas9 to reduce non-specific DNA contacts and thereby decrease binding affinity to non-specific targets without substantially affecting on-target editing rates [61, 62].

A third approach, based on the correlation between residence time and off-target activity, has been controlling the activity of Cas9 after delivery to minimize the total duration of its activity. One approach introduces inteins into the structure of Cas9 that only splice themselves out and generate active Cas9 in the presence of a small molecule ligand. By providing the small molecule for only a short duration, the activity window for editing can be reduced, thereby limiting off-target editing [63]. Another approach is the use of self-inactivating Cas9 vectors, where sgRNA target sites are engineered into the delivered viral genome itself to target the Cas9 expression cassette for destruction at the same time as targeting the desired genomic locus. The result is reduced residence time and off-target editing [64, 65].

While it would clearly be preferable to use a system with reduced off-target cutting, assessing the actual clinical risks of off-target modifications is challenging. *In vitro* assays that detect off-target cutting can be highly sensitive, such that only a subset of at-risk sites are actually cut *in vivo*. Furthermore, off-target modifications can be highly variable in location and sequence, and understanding how sequence changes translate to functional risk of an adverse event is very difficult. Future work may focus increasingly on functional assays of off-target cutting impact, such as cell transformation.

Persistent expression also raises the risk of an immune response to the expression of a bacterial protein in a human cell, which can result in immune elimination of therapeutically corrected cells. For example, expression of AAV-delivered SpCas9 in a mouse has elicited clear immune recognition, though the subsequent cellular damage in this animal model was minimal [66]. Methods of effective transient delivery, such as self-inactivating vectors, may reduce immune responses by limiting the time of exposure.

Efficacy *in vivo* is an additional challenge. While successes in the highly accessible liver bode well for future work, low editing rates in other tissues, while therapeutically sufficient for the strong work in the DMD model, raise concerns for diseases that may require greater levels of correction or for larger animals (or

humans) that tend to be more difficult targets for gene delivery than mouse models. Delivery therefore remains a major challenge, particularly *in vivo*, and improved delivery systems, including novel AAV variants engineered by directed evolution or rational design, are therefore needed for human gene therapy. Improving delivery efficiency to target tissues will increase the efficacy of AAV-mediated genome editing, and improving vector selectivity or targeting to these tissues can enhance the safety profile by reducing potential side effects in tissues unaffected by the disease.

2.3 Conclusion

The era of gene editing has transformed virtually every area of biology, and clinical gene therapy is among the most exciting. The advent of CRISPR/Cas9 has enabled readily engineerable, accessible, and effective gene editing, and this technology is positioned to combine with AAV vectors to assist with *in vitro* editing and to make *in vivo* clinical gene-editing therapy a reality. Addressing additional challenges in the field—including Cas9 target fidelity, Cas9 immunogenicity, and AAVs engineered for optimal gene delivery in the clinic—will enable next generation gene- and genome-editing therapeutics.

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Part II
CRISPR in Model Systems

Chapter 3
From Reductionism to Holism: Toward a More Complete View of Development Through Genome Engineering

Rebecca K. Delker and Richard S. Mann

Abstract Paradigm shifts in science are often coupled to technological advances. New techniques offer new roads of discovery; but, more than this, they shape the way scientists approach questions. Developmental biology exemplifies this idea both in its past and present. The rise of molecular biology and genetics in the late twentieth century shifted the focus from the anatomical to the molecular, nudging the underlying philosophy from holism to reductionism. Developmental biology is currently experiencing yet another transformation triggered by '-omics' technology and propelled forward by CRISPR genome engineering (GE). Together, these technologies are helping to reawaken a holistic approach to development. Herein, we focus on CRISPR GE and its potential to reveal principles of development at the level of the genome, the epigenome, and the cell. Within each stage we illustrate how GE can move past pure reductionism and embrace holism, ultimately delivering a more complete view of development.

Keywords CRISPR • Genome engineering • Development • Genome • Epigenome • Reductionism • Holism • Conrad H. Waddington

3.1 Introduction and Historical Context

From the initial notion that organisms are preformed as miniature versions of themselves to the currently accepted theory of epigenesis—the sequential differentiation into adult tissue from an undifferentiated structure—the question of how multicellular organisms develop from a single cell has puzzled scientists and philosophers for many years [1]. At the heart of this question lies the ultimate quest to bridge the

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gap between genotype and phenotype. How can a single genome code for a diverse array of cellular phenotypes? And, more pertinent to our discussion of development, what is the process, incorporating both spatial context and time, by which this occurs?

The field of Developmental Biology has undergone the influence of a number of theories, but that of Conrad H. Waddington's *Epigenetic Landscape* has proven its staying power [1, 2]. He envisioned development as an inclined, undulating landscape: a ball, representing a cell in an undifferentiated state, rolls down the incline, following one of many valleys—symbols of developmental pathways—to ultimately rest at the bottom as a mature, differentiated cell (Fig. 3.1a).

The significance of Waddington's model goes beyond its specifics; in fact, it may even be the lack of specifics that underlies the importance of the landscape. With an intuitive understanding of the complexity of cell differentiation, Waddington created a "symbolic representation of the developmental potentialities of a genotype in terms of surface" (quoted from [3]). The 3D surface, versus a 2D model, provided space for the potential and vast array of contributing factors and the effects stemming from their interconnectedness.

Central to the model is Waddington's philosophy. Influenced quite profoundly by the thinking of Alfred North Whitehead and his theory of 'organicism,' the epigenetic landscape is a product of "an anti-reductionist systemic view of the organism emphasizing the complex interrelatedness of its developing parts" (quoted from [3]). As an example, Waddington did not explain development as the result of single genes, but rather emphasized the importance of gene networks—this network provided the tethers that secured the hills and valleys of his landscape (Fig. 3.1b).

This holistic mindset quickly fell out of fashion with the rise of molecular biology in the late twentieth century [4]. The shift from morphological to molecular studies set in motion the era of reductionist biology, which favored the idea that complex phenomena, such as development, can be explained entirely by an analysis of their constituent parts [5]. Objectively speaking, this approach has proven successful. It was the application of molecular genetics that lead to the identification of many molecules involved in development, including the discoveries of conserved signaling pathways and identity-bearing transcription factors, such as the Hox genes [6, 7].

But reductionism has its limits, particularly when studying the emergence of properties of multicellular organisms during development [5, 8, 9]. To derive phenotype from genotype requires much more than a parts-list. For example, the same components (e.g. signaling pathways) are used at multiple stages of development yet elicit different responses [10]. Instead, it requires an understanding of the complex interactions between these parts that occur, not only in space and time, but also that traverse the many levels of organization at which development proceeds—namely, the genome, the epigenome, the cell, the tissue, the organ, the organism, and the environment.

The past two decades have ushered in a new era of biology characterized most profoundly by '-omics' technology and an increased ability to view the whole beyond its individual parts. Within cells, for example, we are as close as ever at

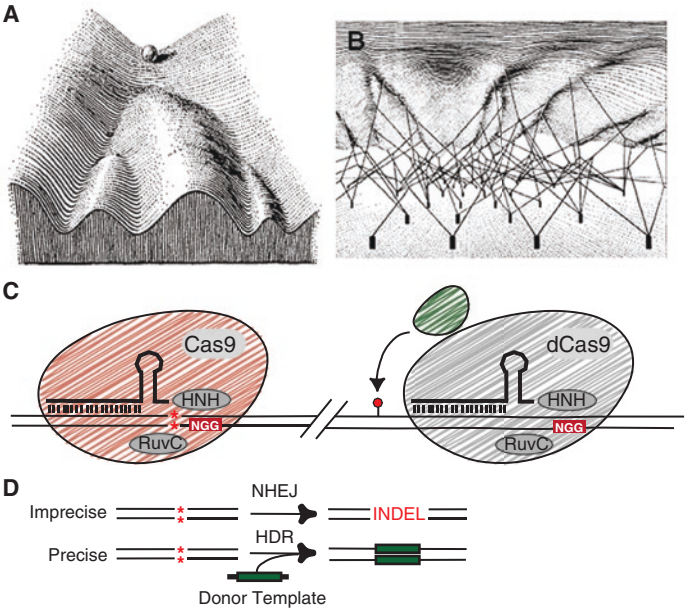

Fig. 3.1 An old idea meets a new technology. (**a**) Waddington's Epigenetic Landscape. A ball, representative of a developing cell, is pulled through one of many developmental pathways to reach the bottom of the hill as a mature, differentiated cell. (**b**) Waddington envisioned that networks of genes and their products shaped the landscape. The black boxes represent genes and the lines, the gene products. (**c**) A schematic of the CRISPR Cas9/guide RNA complex. Cas9 contains two endonuclease domains (HNH and RuvC) that generate a double-strand break positioned three nucleotides upstream of the Cas9-specific PAM, NGG (*Left*). When these nuclease domains are mutated, dead Cas9 (dCas9) no longer generates DNA breaks, but rather serves as a scaffold to recruit additional protein domains (depicted in *green*) that can modify the epigenome. (**d**) Two types of repair can follow Cas9-induced breaks. Repair by non-homologous end joining (NHEJ) results in imprecise repair and the inclusion of insertion and/or deletions (Indels). Repair by homology-directed repair (HDR) using a co-delivered donor template results in precise genomic modifications (in *green*). Figure 3.1**a**, **b** is reprinted from [2] with permission from The Taylor and Francis Group

getting a glimpse of the whole genome, the whole epigenome, the whole transcriptome, and the whole proteome. This technological development—in many cases driven by next generation sequencing (NGS)—has helped create a comprehensive parts-list. In most cases, though, we still lack an understanding of the connections between each of the parts.

The CRISPR/Cas9 (*C*lustered *R*egular *I*nterspaced *P*alindromic *R*epeats/*C*RISPR-*A*ssociated *S*ystem 9) adaptive immune system in bacteria and archaea has provided a simple and efficient means of site-specifically modifying genomes of interest. Applications of the technology, discussed herein, hold the potential to push our understanding of development beyond the parts (reductionism) toward an understanding of how complex phenotypes emerge from the hierarchical and interdependent connections between these parts (holism). Studies highlighted illustrate

the use of CRISPR genome engineering (GE) to more thoroughly map and interrogate gene networks needed to drive cell fate, as well as study gene regulatory regions not as independent units, but within the context of, and influenced by, the native genome (*A Genomics Perspective*). A nuclease-deficient Cas9 (dCas9) has expanded the breadth of CRISPR GE to provide much needed functionality to DNA and histone modifications and expand our understanding of the importance of 3D genome structure, providing a foundation from which to explore the interplay between modifications in *cis* and factors in *trans* in genome regulation (*An Epigenomics Perspective)*. Lastly, CRISPR GE when coupled with cutting-edge *in vitro* differentiation models and when used as a memory-encoding device set the stage to probe how the spatial and temporal dimensions of development converge with genome regulation to decide cell-fate (*A Cellular Perspective*). Together, the research discussed illustrates the capacity of CRISPR GE to broaden our understanding of the interconnected processes underlying development at the level of the genome, the epigenome and the cell.

Reductionist and holistic science are not mutually exclusive; rather, the findings derived from each methodology are complementary [5]. It should not go unnoticed that CRISPR GE, which holds the potential to push our science toward holism, was born from quintessential reductionism (and furthers reductionist science as well). Thus, the most complete understanding of a system as complex as the development of multicellular organisms will best be achieved by merging the two philosophies. Even Waddington understood the importance of this concept. His idea "to explain the complex by the simple, but also to discover more about the simple by studying the complex" is ripe for renewal as we now have the technology to enable it (quoted from [11]).

3.2 CRISPR Genome Editing in Brief

Genome engineering—the controlled introduction of modifications to the genome—is an immensely powerful tool to better understand genome regulation and gene function. For many model organisms—*Drosophila (D.) melanogaster, Caenorhabditis (C.) elegans, Danio rerio* (Zebrafish)—commonly used to study development, the ability to site-specifically modify the genome has only been achieved recently. The utilization of site-specific nucleases, such as transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs), opened the door for GE in a broader array of species and cell-types; but, the difficulty in design and high cost limited the broad use of these tools (reviewed in [12]). The discovery and repurposing of the microbial adaptive immune system, CRISPR, provided an efficient and affordable genome editing tool-kit to circumvent earlier problems [13, 14]. For the purpose of studying development, these advances have expedited the generation of valuable null alleles to study gene necessity, epitope tagged alleles to study protein function, and conditional alleles to asses gene function at different times and in different tissues [15].

The beauty of CRISPR genome editing lies in its simplicity. A single nuclease derived from *S. pyogenes*, Cas9, in complex with a ~ 20 nt hybrid guide RNA (gRNA), recognizes and cuts a genomic sequence based on homology to the gRNA and the presence of an adjacent 'NGG' proto-spacer adjacent motif (PAM, Fig. 3.1c). The ease at which gRNAs can be designed and synthesized allows Cas9—in theory—to target all genomic loci harboring the necessary PAM. Cas9-mediated introduction of a double-strand break (DSB) followed by repair by endogenous DNA repair systems results in either imprecise or precise genome edits (Fig. 3.1d). While the need for a G-rich PAM can be limiting depending on species and/or locus of interest, recently generated mutants of Cas9, as well as the discovery and utilization of nucleases from alternative CRISPR systems, hold the potential to expand the targeting capabilities of CRISPR GE by diversifying PAM recognition [16, 17].

Further, inhibition of the nuclease activity to form dCas9 broadens the utility of the CRISPR system. Without the ability to induce DSB formation, the Cas9/gRNA complex serves as a targetable scaffold on which additional functionalities can be attached (Fig. 3.1c). For the purpose of this review, CRISPR GE will refer to both sequence modification using active Cas9, as well as manipulations using dCas9.

As with all new and exciting technologies, it is tempting to look at CRISPR only with rose-colored glasses and view it as a panacea for both quandaries in basic research and the multitude of diseases that plague humanity; however, even though CRISPR may bring certain experiments and/or therapies "from the realm of the practically impossible to the possible, that is not the same as moving from difficult to easy" (quoted from [18]). There are a number of challenges associated with CRISPR technology as it stands now. From off-target DSB formation, to unpredictable and sometimes inefficient rates of repair, to our current inability to predict the effectiveness of gRNAs based on sequence alone, our understanding of the CRISPR system must necessarily improve in order to bring to light its most promising applications, including those discussed here. Throughout, we touch upon the limitations of CRISPR, but point the readers to more comprehensive reviews covering these issues in more depth [19–24].

3.3 A Genomics Perspective

One significant contribution of Waddington's *Epigenetic Landscape*—and of a more holistic approach in general—is the understanding that cellular phenotypes occur not because of single genes, but rather an entire genotype. The quantitative properties of complete gene networks, the output of which is modulated by its constituent genes, lead to complex and specific phenotypes [25, 26]. CRISPR GE techniques further our ability to identify the components of these networks through high-throughput screens, as well as move beyond single gene perturbations to manipulations of multiple genes at once (*Gene Network Analysis with CRISPR GE*). Further, we have a far better understanding today that genotype is not simply the assemblage of genes, but includes the intervening noncoding DNA. What was once

discarded as junk is now understood to consist of important regulatory regions that control the spatiotemporal expression of genes, as well as the level of expression—matters of utmost importance for obtaining proper gene expression throughout development. While current efforts to dissect and understand regulatory regions often regard them as autonomous units, CRISPR GE expands our ability to probe noncoding DNA at its native locus within the context of the whole genome (*Mapping and Understanding Regulatory DNA within the Genomic Context with CRISPR GE*). Together, these efforts work to improve our understanding of how the genome as a whole guides the development of complex multicellular organisms.

3.3.1 Gene Network Analysis with CRISPR GE

One commonly observed phenomenon is that of the mutational robustness of phenotypes. Because of partial redundancy of gene function and/or the distributed nature of biological systems, knockouts of single genes often result in apparently wild-type phenotypes [27]. Thus, to understand phenotype we must consider the contribution of a network of genes. Despite the use of NGS to profile gene expression, it remains a challenge to (1) identify the component genes involved in a particular phenotypic network and (2) test causality through multiplex perturbation. Recent applications of CRISPR GE have been used to address each of these challenges, specifically through the use of CRISPR-based high-throughput screens to rapidly identify genes involved in phenotypes of interest, as well as through multiplex editing.

The simplicity of designing, synthesizing, and cloning large libraries of gRNAs has been wielded to conduct forward genetic screens in an unbiased and high-throughput manner. Taking advantage of insertions and deletions (indels) following targeted Cas9-mediated DSBs and non-homologous end-joining (NHEJ), several groups have conducted genome-wide loss-of-function (LOF) screens [28–33]. Similar in concept to RNA interference (RNAi), CRISPR LOF screens test the effect of loss of a gene(s) on phenotype. Unlike RNAi, which relies on degradation of the mRNA transcript, CRISPR generates true knockouts through disruption at the genomic level.

The scale of CRISPR LOF screens conducted to date has reached upwards of ~19,000 genes using ~88,000 unique gRNAs [29]. To conduct such large-scale screens, each study has relied on *in silico* synthesis of gRNAs, bulk cloning into the desired delivery vector and transduction (often with lentivirus) into a population of cells *ex vivo* (Fig. 3.2). This ‘pooled’ format relies on the selection of a single phenotype and NGS to determine enrichment or depletion of gRNA sequences in the selected population relative to the initial pool. This approach has been used numerous times to screen genes involved in cell survival and proliferation (in response to a drug or toxin, for example); however, it has also been paired with immunostaining and flow cytometry to isolate LOF mutations that alter expression of a gene of interest [28–32, 34].

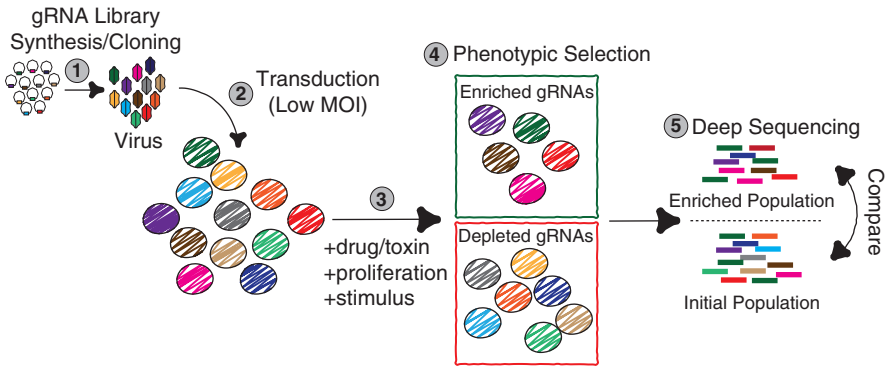

Fig. 3.2 Pooled high-throughput CRISPR GE screens. A schematic details the steps involved in pooled, high-throughput CRISPR GE screens. (*1*) Large-scale production of guide RNAs *in situ* is followed by bulk cloning into a desired vector to generate a gRNA library. (*2*) The library is packaged in virus and used to infect a population of cells at a low multiplicity of infection (MOI) to avoid infection of a single cell with multiple gRNA plasmids. (*3*) Treatment of cells to induce a phenotype of interest, (*4*) followed by selection for the phenotype results in a population of cells enriched for gRNAs that contribute to the phenotype and depleted of those that do not. (*5*) Deep-sequencing of the selected population in comparison to the initial population reveals changes in the relative enrichment and/or depletion of gRNAs, suggesting genes involved in the phenotypic network. Figure adapted from relevant publications

Though these screens provide a means of perturbing large numbers of individual genes to help flesh out a phenotypic network, they do not directly address the combinatorial activity of multiple genes in defining phenotype. To do this, perturbation of multiple genes within the same network is necessary. On a low-throughput scale, multiplexed CRISPR GE has been demonstrated in systems including cell lines, *Drosophila*, Zebrafish, mouse, and monkey, which allows for the simultaneous—and thus, rapid—generation of animals with multiple null and edited genes (up to 5 genes [35]) [35–40]. While many of these approaches have relied on the delivery of gRNAs expressed from individual plasmids or from individual promoters within a single plasmid—a strategy that can limit the number of genes targeted at a single time—a recent study engineered the cleavage and release of multiple gRNAs from a single transcript. This provides much more flexibility in the number of genes that can be targeted simultaneously [15, 41].

Beyond these low-throughput studies, steps have been taken to combine the high-throughput nature of CRISPR screens with multiplex gRNA expression. CombiGEM (*C*ombinatorial *G*enetics en *M*asse), a technique that relies on single pot cloning of a barcoded gRNA library in tandem, allows phenotypic analysis upon perturbation of multiple genes simultaneously. Positive hits from the screen are determined not by sequencing the series of gRNAs (selected for or against in the screen), but the combination of gRNA-associated barcodes. Using this

approach, a library of greater than 23,000 paired gRNAs was employed to discover gene pairs that impart combinatorial influence on cell growth in ovarian cancer cells [42].

Moving beyond CRISPR LOF screens that rely on indels to the use of dCas9 offers additional avenues for multiplexing. While the bulk of the discussion regarding dCas9-based CRISPR GE is included in the section entitled 'An Epigenomics Perspective,' it is worth noting here the utility of dCas9 in screening and multiplexing. Two studies have conducted proof-of-principle pooled high-throughput screens in mammalian cell culture using dCas9 fused to either transcriptional repressors or activators [43, 44]. Again, while these screens targeted single genes at a time, low-throughput advances in multiplexing pave the way for its successful application in a high-throughput manner. Critically, because of the ability to recruit both repressors and activators (Fig. 3.3a), and the ability to use either dCas9 or the gRNA as a scaffold for the recruitment of the effector domain (Fig. 3.3c), multiplexing can include simultaneous gene activation and repression [45, 46].

CRISPR GE requires a number of improvements to make this a routine technology (reviewed in [47–52]); however, an even larger hurdle appears when implementing CRISPR GE screens *in vivo* to reveal gene networks underlying development [15]. While it is likely that *in vivo* screens will be conducted on a smaller-scale with gRNAs that span groups of genes rather than the genome, a handful of studies provide hope for the utility of CRISPR screens in a variety of organisms. Liu et al. have delivered gene-specific gRNAs via bacterial feeding in *C. elegans* [53], which drastically cuts down on time and labor, making it feasible to conduct large-scale studies. Using multiplexed injections followed by phenotypic screening in F0, Shah et al. successfully used 48 gRNAs to screen a set of genes predicted to be involved in synaptogenesis in Zebrafish [54]. Varshney et al., again in Zebrafish, streamlined the screening process by assaying F1 progeny from two targeted founder animals [55]. Finally, the injection of a single plasmid containing both Cas9 and the gRNA into the pronuclei of fertilized mouse eggs can produce mutant organisms at a rate slightly above 50%, with approximately half of the targeting events resulting in bi-allelic disruption [56]. Though these rates are too low to conduct screens on par with those *ex vivo*, it does provide a means of rapidly generating a library of mutant animals that can be used to study a variety of phenotypes of interest. Lastly, as will be discussed below, CRISPR GE in ES cells coupled with *in vitro* development models can also provide valuable information.

3.3.2 Mapping and Understanding Regulatory DNA Within the Genomic Context with CRISPR GE

The regulatory genome, composed of elements termed cis-regulatory modules (CRMs), plays an important role in the translation of genotype to phenotype by tuning the variables of gene expression including space, time, and intensity. The biological importance of the regulatory genome is reinforced by recent genome-wide

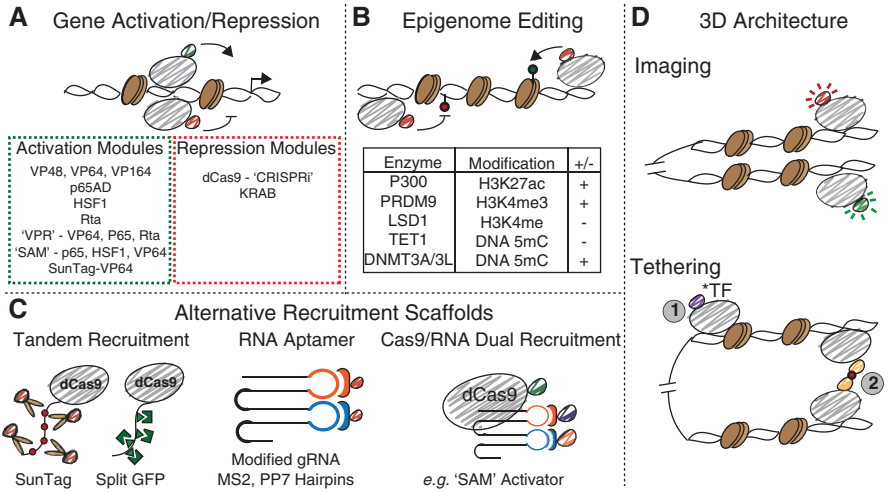

Fig. 3.3 Epigenome modifications with CRISPR GE. (**a**) A schematic depicts the recruitment of dCas9 fused to activation or repression domains to effect changes in gene expression. The activating and repressing modules that have been used are displayed. (**b**) A schematic depicts the recruitment of dCas9 fused to catalytic domains that incorporate (*right*) or remove (*left*) epigenetic modifications. The enzymes (or catalytic domains) that have been used alongside dCas9 are listed, including their targeted modification and whether they work to add (+) or remove (−) the mark. (**c**) Several alternative scaffolds beyond direct protein fusion to Cas9 have been employed. The SunTag makes use of single-chain variable antibody-epitope interactions to recruit several functional moieties to a single molecule of dCas9. Modifications of the gRNA to include aptamers, such as the MS2 and PP7 hairpins, can be used to recruit functional domains to the gRNA, itself, preserving dCas9 as a neutral partner. This allows the targeting of distinct functionalities to different genomic loci simultaneously. Finally, dual recruitment through both dCas9-fusions and gRNA-aptamer scaffolds has been used to enhance the effects of the recruited functionality and recruit distinct moieties to a single genomic locus. (**d**) Dead Cas9-fusions with fluorescent molecules have been used to visualize genomic loci in fixed and live cells. Tethering using dCas9 has not yet been demonstrated, but could conceivably be used to site-specifically recruit transcription factors (TF) of interest and/or force interactions between distal genomic loci with dCas9 molecules harboring hetero-dimerization domains

association studies (GWAS), which reveal that the majority of disease-associated sequence polymorphisms (SNPs) reside within noncoding DNA [57]. Thus, in addition to driving normal development, CRMs, when mutated, have the potential to drive disease.

Despite the recognized importance of the regulatory genome, it has been incredibly challenging to both predict the location and decipher the functionality of CRMs. A number of enhancers, both proximal and distal, and in *cis* and *trans* can control the complex pattern of gene expression of a single gene. In fact, key developmental genes, such as Hox and other selector genes, exhibit some of the most complex regulation [58, 59].

Historically, the identification of CRMs has relied on reporter gene assays in which candidate enhancer DNA is juxtaposed to a minimal promoter driving expression of a reporter gene. NGS has vastly improved both the ability to predict putative

CRMS via genome-wide profiling (of TF binding, histone modifications and nucleosome density), and the ability to rapidly test the functionality of thousands of putative enhancers with *M*assively *P*arallel *R*eporter *A*ssays (MPRAs) [60]. However, MPRAs—like their low-throughput counterparts—require the study of genomic fragments removed from the native locus. While these assays serve to identify elements that are sufficient to activate transcription in a heterologous context, they are unable to identify elements that are (1) necessary but not sufficient for transcription and (2) unable to regulate transcription outside of the native locus for reasons including, but not limited to, potential chromosomal position effects.

[61, 62]. In fact, only a small fraction (~26%) of ENCODE predicted enhancer sequences activate transcription in these assays, calling for new ways to study gene regulation at native loci [63].

Modifications to single CRMs at their native locus can now more easily be performed with CRISPR GE to study the effects on gene expression. CRISPR-mediated deletion of predicted CRMs ~100 Kb from the TSS of the pluripotency factor, *Sox2*, for example, substantiated their importance for Sox2 expression in ES cells [64–66]. Further, interrogation of single CRM elements within the native context can reveal synergistic, antagonistic, or other interdependent relationships between multiple CRMs at the same locus. Deletion of single enhancer elements within the super-enhancer of *Prdm14* in murine ES cells revealed a functional interdependence between constituent elements such that deletion of a single element resulted in a depletion of H3K27ac activating marks at neighboring elements [67]. Finally, CRISPR GE of CRMs can help interrogate the relationship between noncoding SNPs and disease by inserting disease-associated variants in healthy cells or deleting variants from diseased cells followed by gene expression and phenotypic analysis [66, 68, 69].

The efficiency and ease of CRISPR GE enables one to move beyond single targeted mutations to extensive mutagenesis studies and unbiased screens. Cas9-mediated saturation mutagenesis—the tiling of gRNAs to target PAM sites across defined genomic regions—has been used to extensively dissect both coding and noncoding regions of loci of interest [70–72]. While these studies are typically guided by alternate assays that predict the location of CRMs, it is equally possible to use CRISPR GE to scan large tracts of noncoding DNA to discover regulatory regions *de novo*. Following the logic of the high-throughput screens discussed for gene network analysis, CRISPR-mediated indel formation and repression with dCas9-effectors can be used to determine the importance of targeted noncoding regions for gene regulation [51, 72–75]. Many of these screens directly link perturbation of noncoding regions, spanning upwards of 1 Mb of DNA surrounding genes of interest, with phenotypic readouts, such as proliferation [75, 76]. Others focused their screens at the level of gene expression, utilizing knocked-in GFP and IRES-GFP reporters to identify noncoding regions that, upon perturbation, result in a change in expression as measured by fluorescence [73, 74].

Importantly, each of these studies—from low-throughput targeting of single loci, to saturation mutagenesis, to unbiased screens—serves to identify noncoding regions necessary for gene regulation that may not have been discoverable by

traditional enhancer-reporter experiments. Thus, they provide the opportunity to reveal genomic regions that are essential for gene regulation but do not fit the description of a classical CRM. For example, many of the above screens identified genomic regions that were not marked by classical histone marks, could not be predicted by accessibility data such as ATAC-Seq, could not activate transcription in a reporter assay, or only transiently altered gene expression [72–74, 76]. In addition, a number of studies identified the importance of heterologous promoters in the regulation of the target gene and uncovered potential complex connectivity between enhancers and promoters of neighboring genes [73, 75]. Each of these findings pushes us to recognize the importance of genomic regions that serve an important role in gene regulation—perhaps by guiding 3D genomic structure—despite their inability to function independently [77, 78]. With further dissection of native genomic loci, it is likely that additional classical and non-classical regulatory regions will be revealed—as well as the complex interplay between them—ultimately allowing us to reimagine CRMs as integrated components of a whole regulatory system rather than as autonomous units. Of course, it is also this complexity of gene regulation that can obscure our ability to detect the influence of single regulatory elements. Thus, it is imperative that future studies combine CRISPR GE at the native locus with more mechanistic assays to understand regulatory regions both independently and as part of a whole.

The studies discussed above were conducted in cell lines amenable to transduction and rapid screening. Application of these techniques to *in vivo* analysis will present additional challenges, but one can imagine the generation and use of gRNA libraries analogous to RNAi libraries for rapid screening in model organisms with short generation times and efficient genetic modification such as *C. elegans* and *D. melanogaster*.

3.4 An Epigenomics Perspective

Waddington was the first to coin the term epigenetics, defining it as the causal mechanisms by which the genes of the genotype bring about the phenotype [79, 80]. From his perspective, development is inherently epigenetic and each of the interconnected mechanisms that bridge the gap between genotype and phenotype encompass the 'epigenotype.' The output of gene networks, for example, which he used to tether his landscape, falls within this definition. Today, as our molecular understanding of genome regulation has expanded, our definition of epigenetics has narrowed. Now, epigenetics includes the diverse array of covalent modifications to chromatin, including DNA bases and histones. For the purpose of this discussion, we expand upon this definition to include the structure of the genome in 3D—influencing subnuclear position and genomic interactions—which increasing evidence has shown to contribute to the regulation of gene expression [81]. Thus, from a modern perspective, Waddington's landscape is tethered not only by gene networks, but also networks of regulatory DNA (as discussed above), networks of epigenetic

features, and the complex connections between them. Similar to the advantages seen from the genomics perspective, CRISPR GE can be used to flesh out the details of the landscape by offering new techniques to assay genome structure in single, living cells (*Tracking 3D Genomic Structure with CRISPR GE*), as well as pull at the tethers of the landscape through targeted perturbations of the epigenome to better understand their role alongside trans-acting factors in regulating gene expression and cell-fate (*Manipulating DNA and Histone Modifications with CRISPR GE*).

3.4.1 Manipulating DNA and Histone Modifications with CRISPR GE

NGS and '-omics' technology have enabled the discovery and profiling of numerous modifications to the epigenome in a diverse array of cell-types. Each of these epigenetic marks has been demonstrated to display some level of cell-type specificity and dynamic behavior during cell differentiation. DNA methylation and histone modifications vary widely between ES cells and differentiated cells [82, 83]. In fact, a recent report found that chromatin accessibility data (ATAC-Seq) performed better than RNA-Seq in defining unique cell identities and rebuilding lineages during hematopoiesis [84, 85]. The importance of epigenetic marks for cell identity is supported by the fact that altered epigenomes are commonly found in cancer cells [82]. However, while there are clear correlations between distinct epigenetic marks and gene activity, very little evidence exists to point to causality. Without such information, it is challenging to understand how distinct epigenetic marks function independently, within the epigenetic network, and in coordination with gene and gene regulatory networks to determine cell-fate. Targeted dCas9-mediated modifications to gene activity and to the epigenome provide a road forward to address these complex processes. As with other applications of CRISPR technology, these ideas are not entirely novel. Targeted modifications have been achieved with other DNA binding proteins (TetR, LacI, ZFNs, TALENs); however, the ease of CRISPR vastly expands these capabilities [86, 87].

The realization that dCas9 can be used as a targetable scaffold to recruit functional domains to loci of interest catalyzed a series of reports using the tool to activate and/or repress gene expression (Fig. 3.3a). Whereas weak repression was shown to occur due to steric hindrance of dCas9 binding alone, much more efficient repression occurs via the recruitment of a Kruppel Associated Box (KRAB) repressor domain [43, 45, 88–91]. Similarly, successful gene activation has been observed via the recruitment of a variety of activation domains alone and in combination [43–46, 88, 92–98] (Fig. 3.3a). In most cases, tiling of gRNAs to recruit multiple copies of the Cas9-activator fusion is necessary to achieve significant upregulation; however, recent developments to recruit multiple activation domains to a single dCas9/gRNA complex reduce the number of binding events necessary. Toward this goal, fusion of multiple activation domains

in tandem and/or the recruitment of activation domains to modular gRNA scaffolds have been used (Fig. 3.3a, c) [43–46, 96, 97, 99].

While these approaches do not directly modify the epigenome, the recruitment of activation and repression domains has been reported to result in remodeling of the chromatin landscape. The recruitment of KRAB to a distal enhancer of the globin locus, for example, induced H3K9me3, as well as decreased chromatin accessibility at both the enhancer and its targeted promoter [91]. Similarly, gene activation via recruitment of the activator VP64 to genes encoding neuronal transcription factors resulted in enrichment of the activating histone marks, H3K27ac and H3K4me3 [100]. These findings underscore the correlation between histone modifications and gene regulation, but still do not directly address the function of these marks.

Several reports have detailed the use of dCas9 to alter the chromatin state of a targeted region without altering the underlying genomic sequence [101–108]. Though the list of inducible epigenetic marks comes nowhere near the complete list of all observed modifications, researchers have successfully used dCas9 to site-specifically induce histone methylation (to H3K4me3 by PRDM9 [104]) and demethylation (of H3K4me2 by LSD1 [101]), histone acetylation (to H3K27ac by P300 [102]), and DNA methylation (with DNMT3A [103, 107, 108]) and DNA demethylation (with TET1 [105–107]) (Fig. 3.3b). Each of these studies demonstrates that, at the tested loci, modification of the epigenetic code is sufficient to induce changes in gene expression, providing evidence of a causal relationship between the epigenome and transcription. Interestingly, modifications induced at distal enhancers, including histone demethylation and acetylation, were sufficient to alter gene expression at their target promoter [101, 102].

Most notably, these studies emphasize the connectivity of individual epigenetic modifications with one another and with other nuclear factors. First, some loci are less responsive to epigenetic editing than others, suggesting the influence of the local chromatin context in dictating the effects of single perturbations. Second, epigenetic editing can indirectly effect the enrichment of other epigenetic marks, suggesting cross talk between modifications. As an example, demethylation of H3K4me2 by targeted LSD1 resulted in a decrease in local enrichment of H3K27ac [101]. Finally, a number of reports suggest that the maintenance of epigenetic state and gene activity through cell division depends on a network of modifications. Targeted H3K4me3 of promoters to activate gene expression resulted in sustained activation in a manner dependent on the presence of H3K79me and the absence of DNA methylation [104]. Similarly, co-targeting of KRAB, DNMT3A and DNMT3L resulted in enhanced stability of gene silencing [108]. As more of these studies are conducted, we will be able to fill out the connectivity within epigenetic networks, as well as study the result of epigenetic editing on other layers of gene regulation, including transcription factor binding and chromatin looping. As a start, methylation of the binding motif for the insulator and looping factor, CTCF (CCCTC-Binding Factor), in mouse ES cells resulted in reduced binding, altered looping, and aberrant gene activation [107].

The studies presented thus far have been conducted in cells *ex vivo*, but a handful of reports have demonstrated the feasibility of these techniques *in vivo*. TALE- and dCas9-based activators and repressors have been used during the development of *D. melanogaster* [109, 110]. Interestingly, whereas TALE-repressors acted in a dominant fashion, TALE-activators could not significantly activate transcription outside of the boundaries of normal gene expression [109]. Similarly, another study found that a dCas9-activator could induce gene activation, but only in a subset of cells in which dCas9 was expressed [110]. Again, these studies hint at the importance of cellular state—including the epigenome and set of trans-acting factors—in modulating the effect of additional epigenetic perturbations. Additional ZF-targeted epigenetic modifications, including histone and DNA methylation, have been conducted *in vivo* by (1) surgery and viral infection of murine brain regions and (2) injection of viral-transduced cell lines into immuno-compromised mice [111, 112].

Of particular importance for conducting functional epigenetics in the context of development is the ability to manipulate the epigenome in a temporally and spatially specific manner. Cell- and/or tissue-specific expression of dCas9 can be achieved by driving expression with regulatory regions (i.e. drivers) active in a subset of cells. This can be further restricted by using multiple drivers to express independent components of a split Cas9 system [113–119].

Temporal control is typically much harder to achieve, but the fusion of a small-molecule responsive destabilization domain to Cas9, and the development of inducible split Cas9 systems enables Cas9 activity to be tuned temporally using exogenous signals [115, 116, 118, 120–122]. Split Cas9 effector systems, in particular, provide an elegant means to induce Cas9 activity despite ubiquitous expression. Systems controlled by the addition of a drug, as well as optogenetically, have been generated, with the latter allowing for the reversibility of Cas9 activation.

3.4.2 Tracking 3D Genomic Structure with CRISPR GE

In addition to the more classical epigenetic modifications, several pieces of evidence collectively suggest the importance of the spatial organization of the genome within the nucleus and interactions between genomic loci for the spatiotemporal regulation of gene expression (reviewed in [81]). While chromosome conformation capture (3C) studies have provided evidence that topologically associated domain (TAD) structure is relatively cell invariant, differences in high-level genome organization and enhancer promoter looping have been noted between cell-types and throughout cell differentiation [123–132]; other studies, such as one in *D. melanogaster,* found enhancer-promoter looping to be invariant throughout embryogenesis [133]. Thus, we still have no comprehensive understanding of how genome structure interfaces with other cellular factors to regulate gene expression during development. The majority of progress at the interface of CRISPR and genome architecture involves labeling and tracking subnuclear genomic location with fluorescent molecules. While these experiments do not technically fall within the

category of GE, we present them here for two reasons: (1) they help to inform on the correlative relationship between genome structure and gene expression—a necessary foundation to move toward engineered perturbations and (2) the tools developed for these experiments can also be employed to modify the 3D genome in a targeted fashion.

Both 3C studies and fluorescence *in situ* hybridization (FISH)—the two most common methods of assaying genome structure—can only deliver a static snapshot of genome interactions at the point at which the cells were harvested and fixed for analysis. To understand the dynamics of genome structure in the context of a developing system it is necessary to incorporate genomic labeling with live imaging. The insertion of a repetitive tract of binding sites for known DNA binders (e.g. LacI [134, 135], TetR [136]) into the genome has been used for this purpose; however, this requires the additional step of GE and the insertion of long repetitive regions that could disturb normal gene function. Dead Cas9, while hindered by its own set of hurdles, provides a means to label and track loci within their native position and without prior engineering. A handful of studies in the past 3 years have conducted proof-of-principle experiments to label and/or track loci in cell culture (Fig. 3.3d) [96, 137–142]. Each of these studies, thus far, relies on either targeting repetitive regions or tiling gRNAs (>26 [143]), such that multiple dCas9-fluorescent molecules are recruited to enhance the signal at the focus relative to the diffuse signal in the nucleoplasm. Streamlined methods (e.g. CRISPR EATING [142]) that rely on enzymatic processing of entire (small) genomes or genomic regions have been developed to simplify the necessary tiling of gRNAs. Further, the development of tools, such as the SunTag and split fluorescent proteins, allow the recruitment of many fluorescent molecules in tandem to a single molecule of dCas9 to enhance the signal (Fig. 3.3c) [96, 144].

Additional advances to CRISPR imaging expand the number of loci that can be visualized at once, enabling genomic interactions to be viewed in real-time. Co-expression of Cas9 variants derived from distinct species, each with unique gRNA scaffolds and PAM specificities, can be used to tag as many loci as there are variants in the system. Importantly, each of the variants tested (nmCas9, saCas9, stCas9) perform with equal efficiency to spCas9 [138, 140]. Further, modifications of the gRNA scaffold enable simultaneous recruitment of diverse functional moieties or fluorescent proteins to distinct loci. Expansion of the gRNA structure to include multiple copies of MS2 and/or PP7 hairpins allows for the recruitment of different fluorescent molecules to independent loci or the co-recruitment of multiple molecules to a single loci to expand the color profile through spectral overlap [137]. Finally, a creative use of MS2 repeats allows for the co-imaging of transcriptional activity and the nuclear position of a gene. The insertion of a 1.3 kb MS2 repeat into the *Nanog* gene in mESCs served to illuminate the nascent transcript in addition to the genomic locus [145].

Our ability to use dCas9 as an imaging tool is still limited. However, as the technology improves, pairing genomic imaging with current advances in fluorescence super resolution microscopy provides some exciting possibilities. Single molecule imaging of fluorescently-tagged TFs has enabled visualization and tracking of individual TFs

as they bind and diffuse in a live nucleus [146]. Pairing this type of imaging with the labeling of genomic loci can, for example, reveal how TF binding regulates subnuclear position and/or specific genomic interactions. More generally, it will augment our understanding of how the shape of the genome and the factors that act on it work together to properly regulate cell-identity.

To go beyond the parts-list and get at the connections that underlay the emergence of phenotypes, it is helpful to perturb components of the system and measure the associated change in output. For the 3D genome, this means going beyond imaging. Already, CRISPR GE has been used to highlight a causal relationship between 3D structure and gene expression. For example, inversion of binding sites for CTCF using CRISPR GE resulted in altered enhancer-promoter looping with effects on gene expression [147]. While this requires alteration of the underlying genomic sequence to perturb 3D structure, the dCas9-based imaging experiments discussed above suggest that dCas9 CRISPR GE can overcome this. In theory, rather than recruiting a fluorescent moiety to the dCas9/gRNA complex, the targetable complex can be used as a means to tether proteins to regions of interest or even tether two genomic regions together (Fig. 3.3d). Already, fusions of the *β-globin* looping factor, LDB1, with a targeted ZFP have been used to force enhancer-promoter looping and drive low levels of gene expression in the absence of necessary trans-factors [148–150]. This can be expected to get easier with dCas9 as the design and synthesis of gRNAs is much more accessible.

3.5 A Cellular Perspective

The development of phenotype depends not only on the internal state of the cell, but also on its connection with the external environment. Even prior to the introduction of molecular techniques, scientists understood the importance of cellular context in directing the differentiation of individual cells to alternate fates [151]. In addition, development occurs in a manner that progressively limits potential fates as differentiation proceeds. Thus, the lineage of a cell is equally important in guiding developmental decisions. Despite this, there remains much to learn about how positional and temporal information is integrated with the regulation of gene expression to specify cell fate. Very recent work using CRISPR GE as a lineage tracing tool attempts to reveal cell relationships and differentiation pathways within whole, complex multicellular organisms—building a necessary foundation to understand the temporal progression of development (*Lineage Tracing with CRISPR GE*); and the union of CRISPR GE with *ex vivo* models of tissue morphogenesis and organogenesis provides a tractable system in which to interrogate the effects of genomic and/or epigenetic perturbations at the cellular and organ level (*CRISPR GE and Ex Vivo Organogenesis*). More than in the other two perspectives, the studies discussed here are in their very early stages; however, we believe that the exciting potential they hold, particularly in providing a holistic approach to study development, warranted their inclusion.

3.5.1 Lineage Tracing with CRISPR GE

A key piece of information required to understand the development of multicellular organisms is a map that outlines the history of each cell and its relationship with all other cells throughout time. This will aid not only in our understanding of how perturbations at the genomic, epigenomic, or extracellular level are reflected in differentiation pathways, but is also crucial for our attempts at directing differentiation *in vitro*.

The only complete lineage map thus far is that of the roundworm, *C. elegans*—the completion of which was aided by its visual transparency and relatively small size [152]. For less tractable organisms, clever techniques to mark cells and their progeny have been developed [153]. The most common technique currently used takes advantage of cell-specific expression of a recombinase (e.g. Cre/Flp) to activate the expression of a conditional reporter gene (often a fluorescent protein). In effect, all progeny derived from the cell with the active recombinase are permanently marked with the expression of the reporter. While this technique has been successful at delineating sub-lineages within complex organisms, its utility in generating complete lineage maps is limited by (1) its inability to discern relationships amongst the many descendants of a single progenitor, and (2) the number of reporter genes available to unequivocally label many distinct lineages [153].

A recent application of CRISPR GE coupled with NGS aims to use mutations generated through Cas9-induced cleavage and NHEJ-mediated repair to reconstruct cell lineage maps, potentially throughout whole organisms [154–156]. In theory, if each cell contains a unique DNA sequence—a barcode—generated through multiple rounds of Cas9 activity throughout development, the relationship of each barcode to all others can be decoded to determine the lineage history of each cell within a single organism (Fig. 3.4a). An increase in the number of editable sites (size of barcode, number of copies) and the diversity of edited products within each site allows this technique, in theory, to be scalable to whole organisms—or at least organs, aiding in our efforts to map neurons in the brain, for example.

A handful of proof of principle studies have been published recently (as well as deposited on the bioRxiv and arXiv preprint servers [157, 158]), which collectively highlight the promising potential and identify the challenges of Cas9-mediated lineage tracing [154–156, 159]. Though similar in motivation, each study utilizes slightly different approaches. Experimenting with a short synthetic tract of 10 Cas9 target sites, Mckenna et al. establish the vast diversity of repair products achieved by Cas9. Greater than 1500 uniquely mutated barcodes were achieved after only 7 days of culturing HEK293T cells, and a median of 225 (range: 86–1323) revealed in individual Zebrafish embryos 30 h post-fertilization and injection of the Cas9/gRNA complex at the single-cell stage. Though not able to completely lineage trace the Zebrafish using this method, they revealed that the majority of adult cells arise from few embryonic progenitors due to the predominance of a small number of specific barcodes in cells derived from a single organ [154]. Despite its successes, this study also serves to illustrate the main problem associated with a bar-

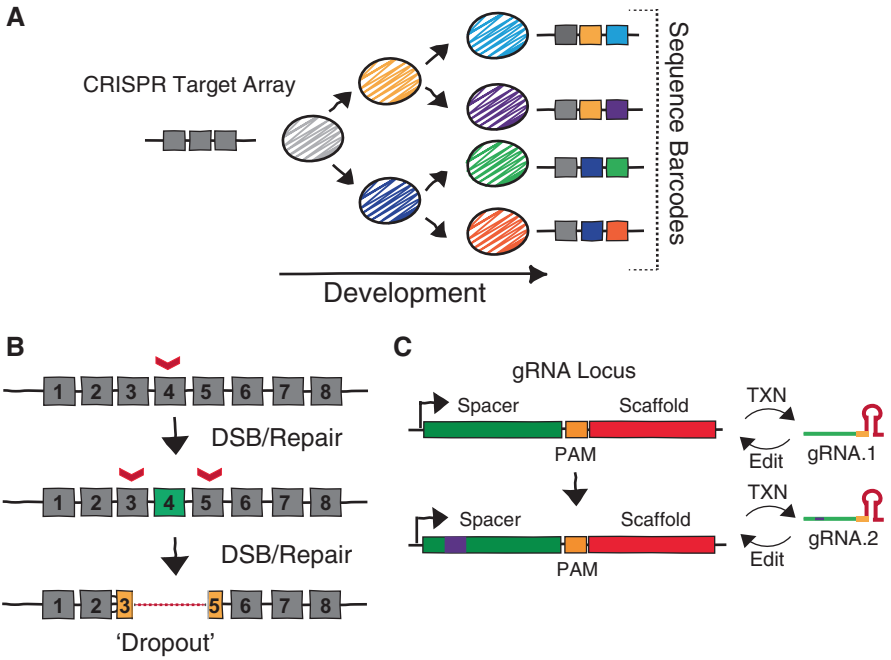

Fig. 3.4 Lineage tracing with CRISPR GE. (**a**) A schematic depicts an idealized example of lineage tracing with Cas9. An array of CRISPR targets is inserted into the genome and subject to the activity of the introduced Cas9/gRNA complex. Mutations induced by Cas9 within the array are replicated and maintained throughout cell division. Thus, the CRISPR array of a mature cell serves as a memory of all Cas9 events that occurred throughout development and acts as a unique barcode signifying its developmental history, or lineage. The relationships between these barcodes (determined by NGS) can then be used to reconstruct a lineage map. (**b**) An example of an inter-target deletion, or 'dropout.' In the first round of CRISPR-mediated DSB and repair, only the fourth target is modified (change in color to *green*). However, during the second round, Cas9 induces DSBs in both the third and fifth target, leading to a deletion of the previously modified fourth target. This dropout event results in a loss of information. Red arrowheads depict DSB induction. (**c**) An example of a homing or self-targeting gRNA. The sequence of the gRNA is engineered to contain a PAM site between the spacer and scaffold portions of the gRNA, thus allowing the gRNA to target the locus from which it was derived. Multiple rounds of self-targeting result in the accumulation of mutations within the spacer sequence. A single round is shown with the induced mutation depicted as a purple bar. Transcription is denoted as 'TXN,' and Cas9/gRNA-mediated editing as 'Edit'

code containing a series of Cas9 target sites: the loss of information stemming from inter-target deletions, also termed *dropouts*. Ideally, each of the target sites are edited independent of one another; however, deletion of unused target sites or sites previously edited can occur, leading to loss of information (Fig. 3.4b).

An alternative published strategy targeted a single site within the genome—exemplified by the design and use of self-targeting gRNAs (stgRNA, *aka* homing gRNAs), which allows for a single, evolvable locus that can be retargeted throughout development (Fig. 3.4c) [155]. Modification of the gRNA sequence to include a GGG PAM site enables a single site to serve both as a source of gRNA and as its

target. In theory, as long as the PAM is not disrupted, this approach allows for multiple rounds of editing, which can be decoded computationally to reveal lineage relationships. The authors establish the self-targeting ability of their modified gRNAs and the generation of a diverse set of mutations upon induction of Cas9 in HEK293T cells. While promising, this approach is currently limited by several factors. First, the majority of mutations that occur in response to Cas9-induced DSB formation are deletions. This results in the progressive shortening of the gRNA and its eventual inactivity. Increasing the length of the initial gRNA sequence prolongs its activity, however also leads to a concomitant loss in efficiency. Second, because the repair product in response to Cas9 DSBs is not easily predictable, it is difficult to track the progression from one cycle of mutation to the next, hindering our ability to definitively map lineages. This computational challenge of delineating single editing events also exists for the other methods, particularly when dropouts are a possibility.

The most recent advance in CRISPR lineage tracing actually relies on the deletion of sequence information to work. Utilizing RNA-FISH rather than NGS as a readout, Frieda et al. inserted several copies of a Cas9/gRNA target, each paired with a unique barcode sequence, into the genome of a mES cell line [159]. Cas9 activity—during development, for example—results in the deletion of the target, but maintenance of the barcode. RNA-FISH using probes against the target region as well as the barcode region reveals Cas9 activity through the presence or absence of the co-localization of the barcode signal with the target signal.

While this iteration removes the complexity of NGS and the problem of dropouts, it still suffers from additional challenges intrinsic to Cas9, which are shared by all CRISPR lineage tracing techniques. Sequence bias of Cas9 and of endogenous repair processes can lead to non-uniform editing, as well as the independent generation of duplicate editing events, giving the false impression of relatedness amongst distinct lineages of cells [154]. The dosage of Cas9 can also critically alter the outcome of editing, with higher doses correlating with increased inter-target deletions [154]. Thus, it is imperative to consider the delivery method of Cas9/gRNAs to optimize the concentration of complex, as well as methods to prolong Cas9 activity throughout development and couple it with cell-cycle progression.

Despite its current shortcomings, lineage tracing with Cas9 would not only allow a comprehensive understanding of cell-relatedness during normal development, but also in models of developmental disorders and during the progression of cancer [154]. In the longer term, coupling of Cas9 lineage tracing technology with improved single-cell profiling, including *in situ*—omic techniques that retain anatomical information, will help to bridge the gap between molecular factors that dictate development and the temporal progression of cellular differentiation.

Fundamental to lineage tracing *in vivo* is the ability to permanently encode memory of the past in a cell. For the purpose of mapping cell relationships, the past is simply the series of precursor cells from which the cell of interest derived. However, one can imagine using Cas9 to encode additional information, such as exposure to cell signaling molecules, as long as the signaling event can be linked to Cas9/gRNA

expression or activity. This type of tool could potentially be used to create a permanent record of cell signaling inputs occurring throughout development.

A proof-of-principle study published recently established the possibility of a Cas9-based recording device [156]. Using an stgRNA approach coupled with an NFκB-responsive element to link Cas9 expression with NFκB activity, Cas9-induced mutation of the stgRNA cassette was detected in response to inflammation, demonstrating that a transient signal can be permanently recorded in the DNA. On a population level, induction of inflammation by varying amounts of stimulus resulted in mutation of the stgRNA cassette such that increased strength and/or duration of signal resulted in increased mutation; however, because of the difficulty in precisely controlling and/or predicting the mutation event in response to Cas9 cleavage, it is not yet feasible to directly translate mutational load to signal intensity and/or duration on a single-cell level. This would require first creating a calibration metric by generating a transition probability matrix for each gRNA—a process that could potentially vary depending on cell-type and cell-cycle state and the favored repair mechanisms associated with each. In addition, as was seen in Kalhor et al., the use of stgRNAs necessitates the use of long gRNAs to compensate for the propensity of Cas9 DSBs to result in deletions [155].

3.5.2 CRISPR GE and Ex Vivo Organogenesis

The prior perspectives have emphasized the importance of the output of whole networks in regulating cell-identity during development. However, they largely maintained their focus on mechanisms occurring within a single cell, whereas the development of whole tissues and organs involves the co-development of distinct cell types not as autonomous units but rather as parts of a whole with complex interrelationships. A complete view of development, thus, relies on an understanding of how the external environment, including the intercellular network, guides development, with particular emphasis on how it is coupled with cell-internal genome and epigenome regulatory networks to maintain cell- and tissue-identity.

The use of directed differentiation experiments *in vitro*, which use growth and/or signaling factors in the culture medium to guide the development of particular lineages from pluripotent stem cells (PSCs, either embryonic (ESCs), or reprogrammed (iPSCs, [160])), are useful tools to ask developmental questions at the level of a single cell, but are poor representations of the intercellular communication involved in tissue development. Recent developments in 3D–culture systems—using 3D matrices as a surrogate extracellular matrix (ECM)—push beyond traditional 2D cultures to better mimic the diversity of cell types and interactions within a developing tissue environment (reviewed in [161–166]). Termed ‘organoids,’ these 3D mini-organs resemble their *in vivo* counterpart in composition, structure and (at least some) function. They can be derived from PSCs (as well as neonatal tissue stem cells and adult stem cells (AdSCs)), which after initial stimulation toward the desired germ layer and subsequent lineage, largely form through

a process of self-organization—stemming from cell-cell interactions, as well as spatially restricted differentiation [163]. Thus, organoids result from guiding and fostering emergent cell behavior. As this technology develops, it will provide new avenues forward to model human disease derived from patient-specific cells and test the efficacy and toxicity of drugs. However, organoids also serve as an intermediate between 2D cultures and *in vivo* experimentation to better understand development: they represent a more physiological model, but remain experimentally tractable. This is particularly important for studying human development as the use of animal models cannot always faithfully recapitulate human physiology, and remains ethically challenging [167].

The marriage of organoid technology and CRISPR GE presents the possibility of interrogating the intercellular network (e.g. by targeting intercellular signaling components), but also of better understanding intracellular networks in the context of this complex environment. The applications of CRISPR GE discussed throughout this discussion can each be applied to organoid systems to elucidate principles of development. Genomic and/or epigenomic perturbations can modify components of the intercellular network or the signaling cascade that links the external and internal state of a cell; selective perturbations in subsets of cells within organoids can reveal the effect of identity in one cell on the phenotype of another; and the use of CRISPR GE to tag proteins and genomic loci with fluorescent molecules coupled with advanced imaging techniques will allow the visualization of genome regulation in the context of the intercellular network [168].

Only a handful of examples of CRISPR GE in organoids exist. Matano et al. and Drost et al. both used CRISPR GE to mutate tumor suppressor genes and oncogenes to develop tumorigenic intestinal organoids not dependent on stem cell niche factors; and Schwank et al. repaired a mutation in the cystic fibrosis transmembrane conductor receptor (CFTR), commonly mutated in cystic fibrosis, to restore functionality to the organoid [169–171]. Despite these few examples, a number of studies have successfully used CRISPR GE to generate LOF and conditional LOF mutants, tagged alleles, and reporter alleles in human PSCS (hPSCs)—a feat that remained unsuccessful prior to the introduction of site-specific nucleases [172–179]. In addition, dCas9 fused to activator and repressor domains has been used successfully in hPSCs [97, 180]. These advances can be directly translated into organoids derived from PSCs. Further, just as in 2D directed differentiation experiments, these genomic and epigenomic perturbations can be used to assess functionality at different stages of organoid development [173, 174].

One of the ultimate goals of this line of work is tissue engineering—the *in vitro* generation of tissues and organs that completely recapitulate their *in vivo* counterpart. While traditional tissue engineering focuses on providing cells with instructive signals for differentiation, the organoid approach strikes a balance between exogenous delivery of signals and the self-organizing capacity of cells to more accurately recapitulate tissue development [181]. How specifically to generate this dynamic environment requires a better understanding of the intercellular network formed in space and time during development that the use of CRISPR GE can help unravel. What is clear, though, is the utility of tissue engineering for advancing

human health. The ability to generate healthy tissues and organs from a patient's own cells will transform the field of medical transplantation. And, when we think about the causes of human health more holistically and consider environmental factors, advanced tissue engineering can facilitate the production of cultured meat *in vitro*, curbing the negative effects of animal agriculture on climate change and human health [182–184].

3.5.3 Final Thoughts

More than the applications of CRISPR GE to further our understanding of development discussed herein, is the impact this technology, along with other recent advances, can have on how we approach biological questions. Modern biology has developed the tools necessary to flesh out the ideas of Waddington and other holistic thinkers, placing us in a superb position to understand complex systems. While this holds significance for basic research, it will also prove valuable to our understanding and treatment of human disease.

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Chapter 4
A Transgenic Core Facility's Experience in Genome Editing Revolution

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Abstract The use of animal models, particularly rodents, has been immensely important to nearly all aspects of biomedical research, from basic science exploration to translational discoveries into clinical applications. The transgenic core facility that provides animal model production, preservation, and recovery services has been fundamental to the success of research efforts using animals. Recent advances in genome editing technologies, especially the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) enzyme system, have transformed the tedious animal model production into a simple and effective procedure. We, as a transgenic core facility established in 1993, adopted the CRISPR/Cas9 technology in early 2014 and have experienced the dramatic shift in the practice of animal model production, from the conventional embryonic stem cell approach to the direct genomic editing in rodent embryos. In this chapter, we describe the lessons that we learned from more than 200 genome editing projects performed in this core facility within the past 3 years. We also provide the practical guidelines for efficient generation of animal models using this technology and the insights into where new technologies lead us.

Keywords CRISPR/Cas9 • Microinjection • Transgenic facility • Animal model • Genome editing • sgRNA

Abbreviations

Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
ES cells	Embryonic stem cells
HDR	Homology-directed repair

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NHEJ	Non-homologous end joining
sgRNA	Single-guide RNA

Manipulating the genome of laboratory animals, particularly rodents, has been of instrumental importance for innumerable biomedical advancements. *In vivo* gene function, cell differentiation, development, disease progression, and drug discovery are just a few of the scientific mysteries that genetically modified animals have helped to elucidate. Nearly 40 years since the development of the first transgenic mouse [1], transgenic and gene targeting methods have been refined, and many new techniques have been introduced. A transgenic core facility has been the major place to perform these techniques to provide the animal model production service for researchers. The latest genome-editing technology, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, offers a previously unattainable speed and efficiency in targeted gene mutagenesis that has revolutionized the practice of rodent model generation and is being implemented in transgenic facilities around the world.

Genetically engineered laboratory animals can be broadly divided into three categories: (1) transgenic animals that carry a foreign DNA fragment, known as a transgene, introduced into the genome in a randomly integrated or targeted (e.g. recombinase-mediated cassette exchange) fashion; (2) targeted mutant animals where the mutation (e.g. gene disruption, replacement, or insertion) is introduced to a specific locus via an embryonic stem (ES) cells approach, followed by chimeric animal production, or a programmable nuclease-mediated gene editing technique; (3) animals carry random point mutations induced by chemicals (e.g. ethylnitrosourea). Transgenic and targeted mutant animals, generated by transgenic core facilities via specialized zygote microinjection techniques, are the major animal models used in research. The most common zygote microinjection techniques include pronuclear injection, ES cell injection, and cytoplasmic injection. While these injection methods have not changed much since their inception, they have been retrofitted for new uses, particularly nuclease-mediated genome engineering.

4.1 Pronuclear Microinjection

The pronucleus is the nucleus of a spermatozoon or an ovum containing the haploid paternal or maternal DNA. After fertilization, at which a sperm enters an egg, two pronuclei are present in the resulting one-cell embryo and eventually fuse to form a diploid nucleus. Foreign DNA or the genome editing materials are backloaded into a filamented glass capillary needle with a fine tip (~0.5 μm in diameter)

and injected into either of the embryo's pronuclei under a microscope. Pronuclear microinjection remains the predominant method of delivering exogenous materials into fertilized zygotes.

4.2 ES Cell Injection

The mouse ES cells derived from the inner cell mass of the blastocyst-stage embryos are a pluripotent cell type with the potential to develop into all tissues of the body. To manipulate the genomic DNA sequence, ES cells can be transfected with a targeting vector carrying the desired mutations (e.g. knock-out, knock-in, or conditional allele) as well as selectable markers flanked by homologous sequences. After screening for mutations or selectable markers, correctly targeted ES cell clones are selected for microinjection into embryos at either 8-cell or blastocyst stage [2, 3]. Modified ES cells contribute to part of the host body and develop into chimeric animals which are able to transmit the targeted allele to the offspring via the germ line at a certain frequency. For injection into eight-cell embryos, two to eight ES cells are injected into the perivitelline space between the zona pellucida and the blastomeres using a beveled and sharp-tipped glass needle (15–20 μm in inner diameter) [4]. If injecting ES cells into blastocysts, 10–15 ES cells are injected into the blastocoel cavity. Injected embryos are subsequently transferred to pseudopregnant females for continued development [2]. We prefer to inject ES cells into eight-cell embryos rather than blastocyst embryos because it gives a higher rate of high-percentage chimeric mice. From our experience, about 10% of the chimeric mice we generated were fully ES cell-derived animals.

4.3 Cytoplasmic Microinjection

Foreign DNA, or other genome editing materials, can be delivered into the cytoplasm of fertilized zygotes using a similar technique to pronuclear microinjection, except that the glass pipette does not penetrate the pronucleus. Another method is to utilize a piezo-driven microinjection technique, by which the pipette holder is equipped with a piezoelectric actuator that creates a quick mechanical pulse to vibrate the pipette tip and pierce the zona pellucida and oocyte membrane (oolemma) without a damaging effect. The exogenous editing materials loaded into the glass pipette are able to pulsate into the cytoplasm when the glass pipette punctures the oolema. The oolema then heals itself after the glass pipette is retrieved [5]. We typically use a blunt-end glass needle of 8 μm in inner diameter and obtain more than a 90% survival rate following the injection.

4.4 A New Era of Animal Model Production by the CRISPR/Cas9 Technology

Soon after the discovery of the profound genome-editing potential of the CRISPR/Cas9 system in human cell line studies [6–8], Rudolf Jaenisch's group tested the system in mouse zygotes and was able to show that targeted mutant mice can be generated with a previously unattainable speed and efficiency [9, 10]. One microinjection procedure delivering CRISPR/Cas9 into mouse zygotes can directly edit the mouse genome. The injected zygotes are subsequently transferred to recipients and give rise to mice with intended mutations at a high frequency [5]. The types of genome modification mediated by CRISPR/Cas9 include, but are not limited to, genetic alterations in single or multiple loci, large DNA inversions and deletions, point mutations, targeted knock-ins, reporters, and conditional alleles. The CRISPR/Cas9 method omits the use of embryonic stem cells and dramatically reduces the time needed for generation of a new mouse model. The simplicity and efficiency of the CRISPR system has triggered a revolution in the way the research animals are made.

Our facility, the Transgenic Animal and Genome Editing Core in Cincinnati Children's Hospital Medical Center, was established in 1994 and since that time has provided all of the classical transgenic services, such as pronuclear injection, blastocyst injection, and sperm and embryo cryopreservation and recovery. In early 2014, we incorporated the CRISPR/Cas9 genome engineering capacity into the facility, as inspired by the work of the Jaenisch group [9, 10]. Since then, we have provided our customers with a comprehensive genome-editing service, beginning with a design to achieve their desired mutation, construction of a CRISPR/Cas9 editing system, and, ultimately, production of genotype-confirmed founder animals. In 3 years, we, as a mid-sized transgenic facility, have generated 164 rodent models by CRISPR, including 63 knockouts/large deletions/large inversions, 72 small knock-ins using donor oligos, 12 large knock-ins using donor plasmids, and 17 conditional alleles, averaging one project per week. Compared to the conventional embryonic stem cell targeting approach, the remarkable effectiveness of the CRISPR/Cas9 technology on mutant rodent production is clearly evident. In addition to these rodent models, we have completed a dozen cell editing projects in human Induced Pluripotent Stem (iPS) cells, mouse ES cells, and cancer cell lines.

The success of the CRISPR/Cas9 genome-editing experiments depends on the choice of guide RNA. Guide RNA can be either a two-RNA (crRNA and tracrRNA) composition or single-guide RNA (sgRNA; a chimeric RNA that combines both crRNA and tracrRNA). Similar to other programmable nucleases (e.g. ZFNs and TALENs), the performance of the CRISPR/Cas9 system is affected by two major parameters: on-target activity and specificity. Although all the initial publications demonstrated the high efficiency of the CRISPR/Cas9-mediated genome editing in rodent zygotes [9–14], we quickly realized that not all sgRNAs work. About 10% of sgRNAs (4 in the first 40 sgRNAs we injected) failed to produce any editing at the target loci. Given that any unsuccessful targeting is costly, we established a reliable

cellular assay to evaluate the sgRNA activity, allowing us to avoid the use of weak sgRNAs (details below). Then, several groups performed large library-based screens in a variety of cell lines and organisms and graciously created the web tools available to the public [15–20]. We further combined our sgRNA selection strategy with these published scoring systems to increase the targeting efficiency. Another important consideration when selecting sgRNAs is the potential for off-target effects [6, 7, 21]. Undesired genome editing can be avoided by using an engineered Cas9 which has a higher fidelity, as well as by referencing published scoring tools to computationally select specific target sequence. Below, we describe the experience acquired from 200 genome editing projects and the key factors that determine successful targeting.

4.5 Guide RNA Activity is the Key

The principle of genome editing relies on successful production of a sequence-specific DNA break by a programmable nuclease. The break then triggers DNA repair responses inside the cell via either non-homologous end joining (NHEJ) to create sequence disruption or homology-directed repair (HDR) to create intended DNA replacement. From our experience, the efficiency of guide RNA-mediated DNA break is the major factor that determines the success of the genome editing events. The activity requirement is particularly obvious for the large KI projects. Given the laborious and time-consuming nature of genome engineering in mice, it is crucial to confirm the activity of guide RNA before use. It is best to validate the activity of guide RNAs directly in mouse zygotes via injection or electroporation and *in vitro* culture to blastocyst stages for DNA-editing analysis. However, when this approach is not readily accessible, the validation can also be done in cultured cells though chromatin accessibility, and epigenetic states in certain genomic regions are expected to be different from those in mouse zygotes. Nevertheless, the expenses of cultured cell-based validation are lower, and a larger number of guide RNAs can be tested per batch in cultured cells.

In the beginning of our CRISPR service, we picked mouse kidney epithelial mK4 cells to validate sgRNA activity by the T7E1 cleavage assay because mK4 cells are highly transfectable [22]. To establish the minimum sgRNA activity required for efficient gene targeting in mouse embryos, we cloned 29 sgRNAs into a pX458 vector (addgene #48138), which expresses both sgRNA and Cas9 protein. After transfection into mK4 cells, the sgRNA's relative activity was obtained by comparing with a *Tet2* sgRNA (target sequence: GAAAGTGCCAACAGATATCC) and the result ranged from 26 to 195% (Fig. 4.1a). We then injected these sgRNAs individually into fertilized mouse zygotes, followed by embryo transfer to produce live offspring. After genetic editing analysis of these offspring, we determined that for a specific sgRNA to be able to induce genetic modifications in mouse zygotes, this guide must exhibit at least 85% of *Tet2* sgRNA activity in a cell-based T7E1 assay. Based on this observation, we further surveyed 204 sgRNAs targeting 74

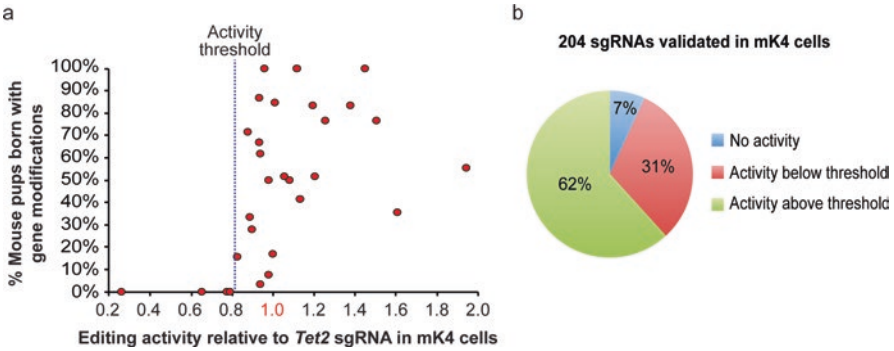

Fig. 4.1 Identification of the minimum sgRNA activity required for genome editing in mouse embryos. (**a**) sgRNAs targeting 29 different loci, mostly in exons, in the mouse genome were cloned into the pX458 vector (addgene #48138) that contains U6 promoter-driven sgRNA and ubiquitously expressed SpCas9 and GFP. Individual sgRNA vectors were transfected into mK4 cells in 24-well plates using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). Cells were examined for GFP expression under a fluorescence microscope to ensure equivalent transfection efficiency between wells. Two days after transfection, cells were harvested for DNA extraction, and the T7E1 mismatch cleavage assay was performed. The editing efficiency was measured according to band intensity on the gel, relative to that of *Tet2* sgRNA. Values represent an average of two replicates for each sgRNA. Then, the same set of sgRNAs were *in vitro* transcribed and purified. 50 ng/μL sgRNA and 100 ng/μL *Cas9* mRNA (and 100 ng/μL single-strand DNA oligo for some samples) were injected into fertilized mouse zygotes by a piezo-driven cytoplasmic injection. Injected zygotes were transferred to pseudopregnant females immediately. Pups were born and genotyped by PCR and Sanger sequencing. The editing efficiency in animals are expected to be underestimated because small indels could be overlooked. In addition, deleting the entire function of the essential genes causes embryonic lethality, favoring the pups with the wild-type alleles to survive. The *dashed line* indicates the recommended activity threshold. (**b**) A survey of the editing activity of 204 sgRNAs targeting 74 different loci in the mouse genome was performed in the same cellular assay. The activity threshold was set at 85% of *Tet2* sgRNA activity

different genetic loci, mostly in exons, using the same cellular assay. We found that 78 of these sgRNAs (38%) have activity below the minimum threshold, including 14 (7%) with zero activity (Fig. 4.1b). This suggests that even though the majority of sgRNAs show some editing activity in cultured cells, more than a third of the sgRNAs do not pass the activity threshold. sgRNAs below this threshold are unlikely to show efficient editing in mouse zygotes. Due to the prevalence of low-efficiency sgRNAs, it is critical to utilize available resources to assess a sgRNA's editing activity before costly zygote injection.

4.6 Design of the CRISPR/Cas9-Mediated Targeting

Given that a sgRNA's activity is the key to the successful genome targeting in mouse embryos, a substantial amount of research efforts have been devoted to optimize the system and increase efficiency. Below we describe the strategies that have been

implemented into our sgRNA selection procedure to ensure effective genome editing in rodent embryos.

1. *Selection of candidate guide RNAs according to on- and off-target scoring web tools*. The current best practice for genome editing in animals is to carefully select guide RNAs to minimize potential off-target effects and test 2–3 per target site to ensure that at least one of them has sufficient on-target activity for the experiment. The choice of the guide RNA sequences can be facilitated by on-line scoring algorithms for the prediction of the off-target effects [23–34] and the on-target activity [15–18, 20, 35, 36]. Conveniently, Haeussler, et al., built the web tool, CRISPOR (http://crispor.tefor.net), that integrates several existing algorithms for a comprehensive assessment of the candidate guide RNAs [37]. Authors also recommend referring the scores from Moreno-Mateos, et al. web tool [18], known as CRISPRscan, if guide RNAs are used for mouse zygotes editing, because the algorithm was derived from the experiments in zebrafish using *in vitro* transcribed sgRNAs (similar to what we used in mouse zygotes), as opposed to those collected data from the U6 promoter-driven *in vivo* expression in other algorithms. To test the prediction accuracy, we compared the guide RNAs' scores from Moreno-Mateos, et al. system and their editing efficiency in mice (Fig. 4.2). We found that Moreno-Mateos, et al. scoring system predicts better than Doench, et al. algorithms [16, 17] that were based on *in vivo* sgRNAs transcription driven by a U6 promoter. The latter ones show a higher rate of false negatives and are harder to define a cut-off. We recommend selecting the guide RNA that has the highest scores across all algorithms and uses a score of 30 from Moreno-Mateos's algorithm as a cut-off. For targeting the non-coding region, we also take chromatin accessibility into consideration. We choose the guide RNAs that target the open chromatin regions, based on the DNaseI hypersensitivity map.
2. *Careful design of sgRNA for the specific type of genetic modification desired*.
    - For basic gene knockout, sgRNA validation is not necessary. Because there is no location restraint, we typically look into all the available exons that are early in the coding sequence and shared by all transcript isoforms to be deleted. We then pick two sgRNAs per gene with high predictive on- and off-target scores from the CRISPOR or other algorithms. We also avoid GC-rich regions that are known to be resistant to guide RNA and Cas9 targeting [17, 38, 39]. We routinely target up to 4 genes with up to 8 sgRNAs per zygote injection.
    - For large DNA fragment deletion, inversion, or duplication, we recommend using two pairs of close-by sgRNAs (four sgRNAs total); each pair cuts the start and end point of the sequence. Although two single sgRNAs are sufficient for this type of DNA manipulation in many cases [40–42], including ours, using a pair of sgRNAs on each side further increases efficiency, as well as the range of the DNA length to be mutated [43].
    - For smaller gene knock-in using a single-strand donor oligo (e.g. point mutations, epitope knock-in, etc.), we generally select 2–3 sgRNAs near the insertion site (<20 bp to the cut site is preferred) and validate their activity in the

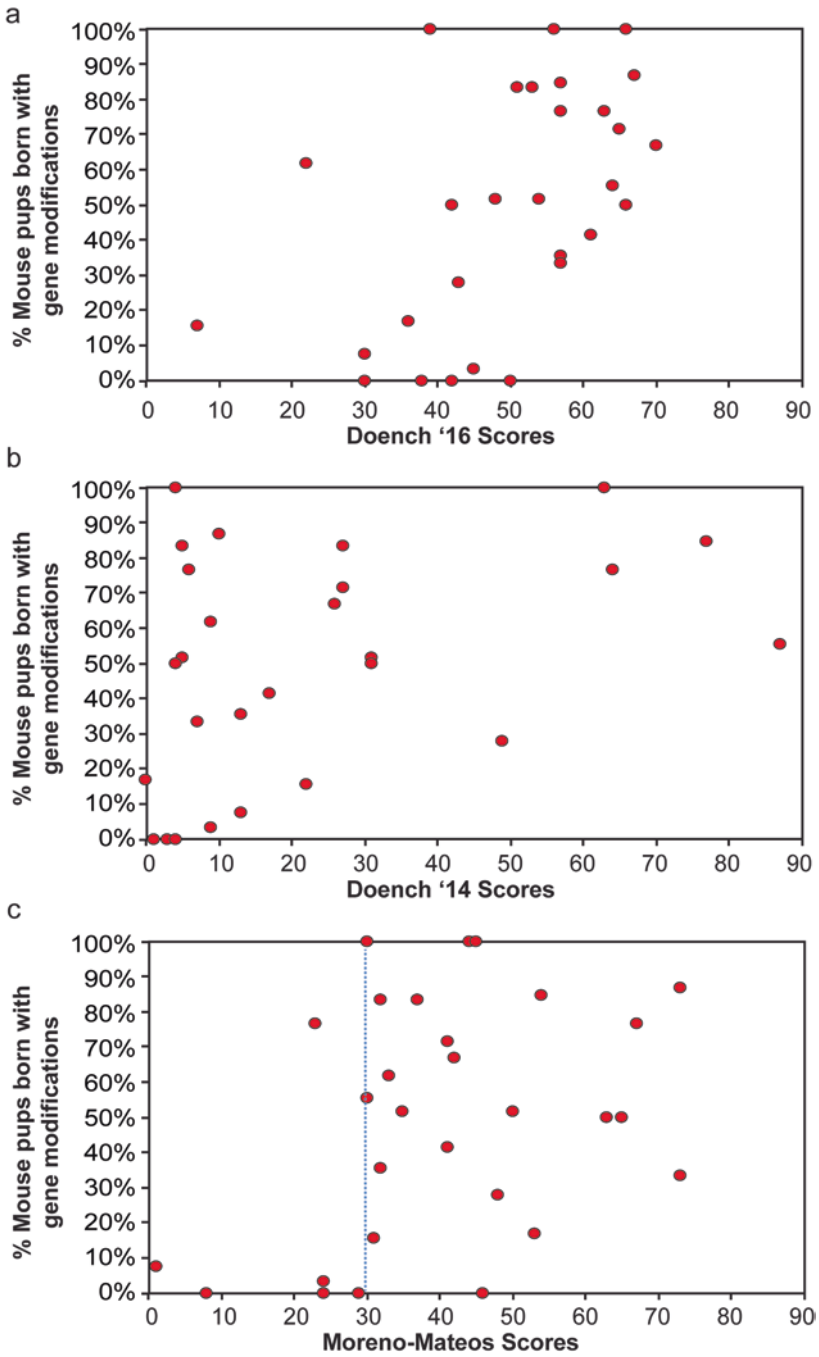

Fig. 4.2 Comparison of on-target scores of 29 sgRNAs from the algorithms generated by (**a**) Doench '16 [16], (**b**) Doench '14 [17], and (**c**) Moreno-Mateos [18] with their actual editing efficiency in mice. A score of 30 from Moreno-Mateos's algorithm as a cut-off (*dashed line*) is recommended

cells or directly in mouse embryos to ensure sufficient activity of the sgRNA. The principles for the donor design are: (1) to include the intended mutation, as well as silent mutations based on similar codon usages, to block sgRNA re-targeting; (2) to introduce new restriction enzyme sites via silent mutations for easy genotyping, particularly useful for identification of point or small mutations. Among more than 70 projects we have performed, we failed only one due to the low complexity of the homologous arms. The single-strand donor oligo can be designed in several different ways (e.g. PAM vs. non-PAM strand, symmetric vs. asymmetric homologous arms, etc.), and we prefer to follow an asymmetric design on a non-PAM strand [44, 45].

- For larger gene knock-ins using a donor plasmid, a sufficient sgRNA cutting activity is very critical for successful targeting. Therefore, validation of the sgRNA's activity prior to the construction of the donor plasmid is necessary. The donor plasmid is then designed based on the sgRNA selection, because the insertion site should ideally be placed near the sgRNA cut site. The length of the homologous arms is also an important factor. We recommend having a total length of at least 4 kb (e.g. 2 + 2 kb or 1.5 + 2.5 kb) of homologous arms. An even longer length is required when the homologous arms contain a significant portion of repeats, particularly the transposable elements and tandem repeats. The plasmid should be injected in the circular form because it significantly reduces the toxicity and the rate of random integration compared to the linearized form.
- For generation of the conditional allele, we use a strategy similar to that of the large knock-in project. We design a pair of non-overlapping adjacent sgRNAs to target each desired loxP insertion site, so that a total of four sgRNAs are used for an efficient deletion between two loxP insertion sites in the genome. The donor plasmid is constructed to contain the floxed exon and flanking homologous arms, but the sequence encompassing the sgRNA recognition sites is disrupted by the loxP sequence to avoid the sgRNA re-targeting. The conditional allele can also be made by inserting the loxP sequence to the DNA simultaneously or sequentially using two sgRNAs and corresponding single-strand donor oligos. The frequency of the former is quite low because it requires two knock-in events to happen in the same embryos and *in cis*. The outcome is unpredictable and usually takes a lot more rounds of injections and resources to achieve it. We recommend doing the latter by inserting the first loxP and then breeding the one-loxP mice for targeting the second loxP, which allows the mice to be made in a more predictable way.

3. *Use of optimized sgRNA scaffold*. sgRNA contains a ~20 nucleotide user-defined target sequence, followed by the scaffold that consists of a duplex and three stem-loop structures. The sgRNA scaffold is essential for Cas9 binding and the full catalytic activity of the complex [7, 8, 46, 47]. Because the optimized scaffold was reported to further enhance the sgRNA activity [48, 49], we modified our sgRNA scaffold accordingly by flipping an A-U base pair and extending the

Cas9-binding duplex. The sgRNAs in the optimized vector, named pX458M, increase the editing efficiency in some cases and does not elicit a damaging effect when compared side-by-side with the those in the unmodified pX458 vector (Fig. 4.3). The sgRNA with the optimized scaffold has been used routinely in our service.

4. *Cas9 protein vs. Cas9 mRNA*. Cas9 can be delivered in either DNA, mRNA, or protein form. When a DNA expression plasmid is used, it is expected to have a delayed expression because it requires transcription, and one-cell-stage mouse embryos are transcriptionally silent until the entry of the S phase [50, 51], leading to a high rate of mosaicism. In addition, the DNA vector can be randomly integrated into the genome of a subset of embryos, and the prolonged expression in embryos before its degradation potentially increases the off-target effects. On the other hand, Cas9 mRNA and protein are not associated with these concerns, therefore these two forms of delivery are preferred for zygote injection. When using Cas9 protein, it should be incubated with sgRNAs at 37 °C for a short time before injection to allow for the formation of ribonucleoproteins (RNPs). We typically use Cas9 protein because it displays a higher targeting efficiency than *Cas9* mRNA (Fig. 4.4), in line with other reports [38, 41, 52, 53].
5. *Selection of injection methods*. The editing reagents are commonly delivered into the fertilized zygotes using a standard pronuclear microinjection technique with some modifications. For instance, the needle can be slightly delayed on pulling out of the zygote, so that the editing materials can deposit in the cytoplasm, in addition to the pronucleus. The technique can also be used for the cytoplasmic injection without penetrating the pronucleus. Although the overall targeting efficiency between pronuclear and cytoplasmic injections is comparable, the latter method gives a higher rate of live born pups [54, 55]. In our practice, we use a piezo-driven cytoplasmic microinjection technique for the projects that do not involve a donor plasmid, such as making knockout mice and knock-in mice with single-strand donor oligos. The advantage of this technique is that it provides superior zygote survival and birth rates following injection (Table 4.1). As a result of these high survival rates, we only need 40–60 zygotes for each project. For a project requiring a donor plasmid, such as generation of mice with Cre or fluorescent protein knock-in or conditional allele, pronuclear injection is preferred. Although the number of pups yielded from this injection method is low, we normally obtain a higher KI efficiency compared to the cytoplasmic injection. We reason that the pronuclear injection increases the concentration of DNA donors in the pronucleus where the homologous recombination takes place. Electroporation poses an attractive alternative delivery method that is simple and high-throughput, and it does not require highly technical training to operate as opposed to the microinjection [56–59]. However, it is currently limited to generation of animal models with gene deletions or knock-in using single-strand donor oligos.

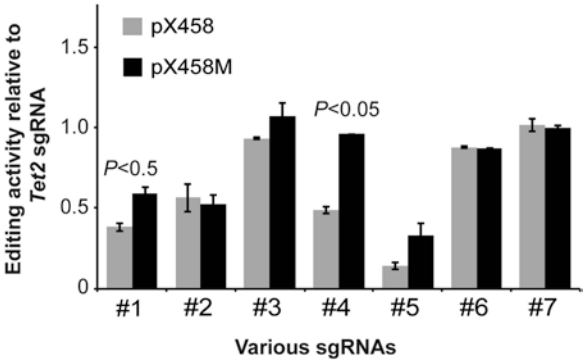

Fig. 4.3 Use of optimized sgRNA scaffold increases editing activity in some cases without a damaging effect. The sgRNA target sequences were cloned into both original pX458 and optimized pX458M vectors, and transfections were done side-by-side in mK4 cells. Editing activity was assessed by the T7E1 assay relative to that of *Tet2* sgRNA. Values here are means ± standard deviation from two independent experiments. *P* two-tailed Student's *t*-test

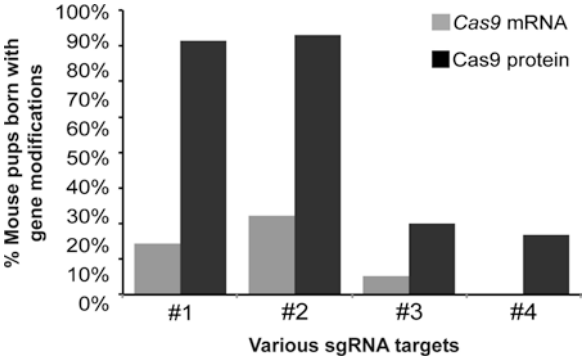

Fig. 4.4 Use of Cas9 protein enhances editing activity, compared to *Cas9* mRNA. sgRNA mixed with either *Cas9* mRNA or Cas9 protein was injected into fertilized mouse zygotes, and injected zygotes were transferred into pseudopregnant females for continued development. Editing activity in live born pups was assessed by genotyping PCR and Sanger sequencing

Table 4.1 Percentage of embryo survival and birth rate following pizeo-driven cytoplasmic injection

Strain	Embryo survival after injection	Birth rate of transferred embryos
C57BL/6	90.1 ± 5.5%	32.1 ± 13.2%
B6D2F2	86.5 ± 7.6%	30.8 ± 10.9%
FVB/N	89.0 ± 8.0%	34.9 ± 9.2%

Data were collected from the service we performed between June 2014 and September 2015

4.7 Conclusion and Perspectives

Animal models remain the most powerful and widely used genetic tool for studies of *in vivo* gene functions and human diseases. The conventional ES cell approaches are laborious and time-consuming, and the types of the genetic modifications that can be engineered are limited. The CRISPR/Cas9 system, combined with conventional zygote injection methods, has opened a new era of possibilities for faster and cheaper animal model production. In our practice, it takes less than a week to prepare the editing reagents for making a simple knockout or small knock-in mouse model. After zygote collection from donor females, microinjection, and transfer to recipients, which is a 1-day process, the animals with desired mutations are born in few weeks. We have experienced firsthand the dramatic change in the practice and witnessed the power of the genome-engineering technology to shorten the length required for animal model production and advance the progress of science.

Additionally, the flexibility of the CRISPR/Cas9 system allows for the creation of genetic tools that were previously unobtainable. We are no longer limited to a simple genetic manipulation (knockout or knock-in of a short distance of DNA) for a single gene at a time. We routinely knock out as many as four genes with a single microinjection. We have also deleted or inverted a DNA fragment as large as 133 kb and 1.7 Mb, respectively, in mouse zygotes directly by a dual sgRNA strategy. Nonetheless, the size of the DNA segments to be deleted or inverted can be much larger than our record [43, 60]. In addition to strategically adding or removing base pairs, the ability of the CRISPR/Cas9 system to target specific sequences can be harnessed to alter the genome in novel ways. When sgRNA is applied with a catalytically inactive Cas9 (dCas9), the complex can target the specific DNA sequence without cleaving it [61]. Therefore, one can fuse a functional protein domain or an effector to dCas9, and then the dCas9/sgRNA complex can bring the effector to the specific DNA location to exert its function. For instance, dCas9 can be fused to a cytidine deaminase that converts cytidine to uridine, and this fusion protein can then be delivered into mouse zygotes along with specific sgRNAs. Kim et al. [62] showed that single-nucleotide substitutions (targeted point mutations) are able to be induced at a specific locus in mice at a high frequency without using a donor oligo. Liu et al. [63] showed that when dCas9 is fused to Tet1, which induces DNA demethylation, dCas9-Tet1 can change the methylation status of a promoter region in postnatal mice following a lentiviral-mediated delivery. Therefore, the CRISPR/Cas9 system has greatly expanded the genetic toolbox for biomedical research. Future animal models will go beyond DNA modification (knockout or knock-in) and give researchers the tools to study areas that were not possible to manipulate in the past, such as epigenetic modifications, RNA editing, chromosome architecture, and genome organization. However, the CRISPR/Cas9 system is not perfect yet. Refinements in targeting specificity are still needed. Although off-target mutations are infrequently detected in CRISPR-targeted rodents [9, 10, 64, 65], it remains a concern in the field. Progress has been made to increase the specificity of Cas9 without losing its on-target activity based on protein structure engineering [66, 67]. Other major

challenges that have not been fully addressed include unpredictable editing outcomes (NHEJ vs. HDR) and a high rate of mosaicism among the founder animals. To tackle these challenges, it is crucial to develop a better understanding of the timing and mechanisms of CRISPR/Cas9 targeting and its role in the DNA repair process in one-cell embryos to maximize the chance of achieving the desired genome editing products.

The generation of animal models is no longer the tedious and time-consuming process that it was in the recent past. A mid-sized core facility like ours can generate enough mutant alleles for one CRISPR project each week, in addition to other routine work on vector construction, transgenic animal production, embryo/sperm cryopreservation, *in vitro* fertilization, and embryo transfer. For each CRISPR project, the number of the mutant alleles we generate is usually more than what researchers actually need. This illustrates the power of CRISPR/Cas9 genome-engineering technology to rapidly advance genetic studies. Given its remarkable flexibility, adaptability, and accessibility, we believe that the application of CRISPR can be further developed for many more uses, far beyond the capacity of our current knowledge and imagination.

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Chapter 5
Genome Editing to Study Ca^{2+} Homeostasis in Zebrafish Cone Photoreceptors

Susan E. Brockerhoff

Abstract Photoreceptors are specialized sensory neurons with unique biological features. Phototransduction is well understood due in part to the exclusive expression and function of the molecular components of this cascade. Many other processes are less well understood, but also extremely important for understanding photoreceptor function and for treating disease. One example is the role of Ca^{2+} in the cell body and overall compartmentalization and regulation of Ca^{2+} within the cell. The recent development of CRISPR/Cas9 genome editing techniques has made it possible to rapidly and cheaply alter specific genes. This will help to define the biological function of elusive processes that have been more challenging to study. CRISPR/Cas9 has been optimized in many systems including zebrafish, which already has some distinct advantages for studying photoreceptor biology and function. These new genome editing technologies and the continued use of the zebrafish model system will help advance our understanding of important understudied aspects of photoreceptor biology.

Keywords Zebrafish • Photoreceptors • Mitochondria • Genome editing • Calcium

5.1 Introduction

This review briefly summarizes the importance of studying Ca^{2+} homeostasis in photoreceptors and how newly developed genome editing strategies in zebrafish can be used to help answer outstanding questions in photoreceptor biology.

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5.2 Ca^{2+} Homeostasis in Photoreceptors

Vertebrates contain two types of photoreceptors: rods responsible for vision in dim light, and cones, which respond to bright light and mediate color vision. Photoreceptors are highly polarized and consist of three main subcellular domains: the outer segment (the site of phototransduction), the cell body (the site of major organelles and the nucleus), and the synapse (the site of neurotransmitter release). This segregation of function requires that the different cellular compartments carefully regulate signaling molecules involved in multiple cellular processes.

Ca^{2+} plays vital roles in cellular processes in all compartments [1]. For example, Ca^{2+} regulates photoresponse recovery and adaptation in the outer segment [2], metabolism [3–7] and protein trafficking [8, 9] in the cell body, and synaptic transmission at the synapse [10–13]. Furthermore, perturbations in cellular Ca^{2+} are associated with photoreceptor cell death. Mutations in photoreceptor phosphodiesterase and guanylate cyclase activating protein both result in sustained high Ca^{2+} in the cell and cause retinal degeneration [14–16]. Sustained light exposure, rhodopsin kinase knockouts, and arrestin knockouts cause sustained low intracellular Ca^{2+} and also cause retinal degeneration [17–19].

Each compartment of the photoreceptor uses different mechanisms for regulating Ca^{2+} levels [1]. Kinetics of Ca^{2+} clearance from the outer segment and the cell body/synapse are markedly different, with the outer segment extruding Ca^{2+} at a much faster rate than the rest of the cell [20]. Ca^{2+} in the outer segment must be cleared quickly to mediate rapid visual responses to changes in illumination. Ca^{2+} in the cell body coordinates cellular processes such as gene expression and metabolic flux, which occur on a slower time scale. Ca^{2+} flow through the cell is mediated by the endoplasmic reticulum (ER), which extends from the synapse to the cell body [21, 22]. Mitochondria tend to cluster in photoreceptor cell bodies where they both regulate intracellular Ca^{2+} levels and functionally integrate cellular Ca^{2+} dynamics [23, 24]. In zebrafish cones, large clusters of 80–100 individual mitochondria aggregate at the apical end of the inner segment [24, 25]. These mitochondria vary drastically in morphology and size most likely reflecting different cellular roles. Most of the details regarding the role of the ER and mitochondria in photoreceptor Ca^{2+} homeostasis are unknown.

The recent identification of the mitochondrial calcium uniporter (MCU) provides the opportunity to genetically dissect the consequence of altered Ca^{2+} uptake into mitochondria on photoreceptor viability and function (Fig. 5.1). The membrane-spanning MCU, together with regulatory proteins, is thought to control influx of Ca^{2+} into mitochondria [28]. Ca^{2+} has vital roles in regulating mitochondrial function. Uptake of Ca^{2+} into mitochondria regulates bioenergetics by lowering Km's of dehydrogenases that produce NADH (pyruvate dehydrogenase, isocitrate dehydrogenase, and α-KG dehydrogenase) [29–31]. Ca^{2+} also increases NADH consumption by increasing F1-F0 ATP synthase activity [5, 32]. However, excess mitochondrial Ca^{2+} can be very detrimental to the cell and lead to activation of cell death pathways [33]. Ca^{2+} can increase free radical production through elevated

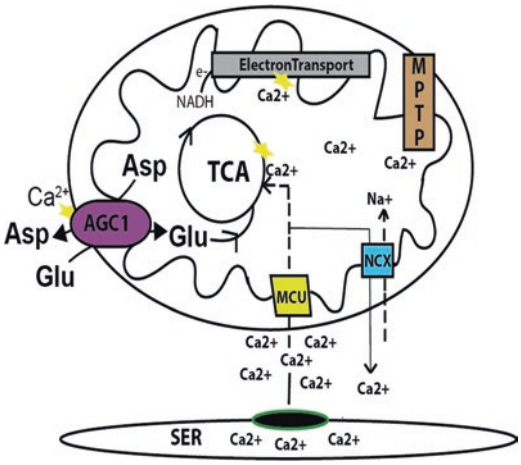

Fig. 5.1 The many roles of Ca^{2+} in mitochondria. Ca^{2+} stimulates aerobic metabolism (*yellow stars*) by activating proteins involved in substrate uptake, production of reducing equivalents and electron transport. Ca^{2+} can enter mitochondria through the MCU at areas of high [Ca^{2+}] such as ER junctions (SER) and is extruded by Ca^{2+}/Na^{+} (NCX) or Ca^{2+}/H^{+} exchangers (HCX). Ca^{2+} binds and activates AGC1, a major component of the malate aspartate shuttle [26, 27]. This enhances complete oxidation of glucose and increases O_2 consumption [3]. Reactive oxygen production and MPTP opening occur when mitochondrial [Ca^{2+}] is too high (not shown)

oxidative phosphorylation and consequent oxidative damage. Excess mitochondrial Ca^{2+} also can overload the buffering capacity, stimulate opening of the mitochondrial permeability transition pore (MPTP) and release mitochondrial components [34]. As described below, new advances in genome editing make it straightforward to generate tissue-specific deletions of important genes such as the MCU.

Other recent findings have further highlighted both the importance of regulation of cellular Ca^{2+} levels in photoreceptors and the many unanswered questions in this field. For example, knockout mice each specifically lacking the Na^{+}/K^{+}-Ca^{2+} exchanger in either rod or cone outer segments were able to slowly recover from flashes of light [35, 36]. This surprising result suggests an alternate and currently unknown route of Ca^{2+} efflux from the outer segment. At the synapse, depolarization maintains high Ca^{2+} levels in the ER in rods due to diffusion from the soma [22]. This mechanism allows for CICR-triggered synaptic release at non-ribbon sites, which is thought to be significant in rods. Moreover, our recent work demonstrates a role for Ca^{2+} uptake by mitochondria as critical for maintaining distinct Ca^{2+} pools in the cell body and outer segment. Pharmacological inhibition of the MCU leads to a redistribution of Ca^{2+} throughout the cell. This same study also demonstrates large increases in mitochondrial Ca^{2+} due to overload in outer segment Ca^{2+} suggesting a critical role for mitochondrial Ca^{2+} buffering in prolonging the health and survival of photoreceptors during degeneration due to disease-causing mutations [24].

5.3 Genome Editing in Zebrafish

For many years, researchers have focused on developing methods to rapidly and efficiently edit the zebrafish genome. These efforts came to fruition about 10 years ago with the development of programmable site-specific nucleases; first ZFNs and then TALENs and most recently with the programmable bacterial endonuclease Cas9 [37]. These methods, particularly CRISPR/Cas9, which is the easiest and most efficient strategy, are transforming biological research enabling new discoveries. They have also led to the disturbing discovery that morpholinos (the previously accessible and straightforward gene knockdown tool in zebrafish) caused phenotypes that were often not recapitulated in genetic knockouts [38]. This finding may be partially explained by genetic compensation that can occur in response to genetic mutations [39]. Nevertheless, new guidelines in the zebrafish field now recommend morpholino phenotype corroboration that includes genome editing [40, 41].

In zebrafish, CRISPR/Cas9 genome editing has already been used in many different ways; to generate targeted knockouts and knockins, in transgenic strains expressing Cas9 in specific tissues, and for forward genetic screens to identify new players in different biological processes. Excellent and comprehensive recent reviews summarize the research underlying the uses and advances of genome editing in zebrafish [37, 42, 43]. The most straightforward and successful strategy so far is the use of CRISPR/Cas9 genome editing to generate gene knockouts. This approach can be cheaply and efficiently done in all labs competent in basic molecular biology techniques. As an example, I highlight our approach generating knockout models of the MCU.

Making global knockouts in zebrafish using CRISPR/Cas9 is simple and inexpensive.

Briefly, our strategy is as follows. More experimental details can be found in the excellent protocols widely used by the zebrafish community [44, 45].

1. Multiple databases exist for the selection of the 20nt complementary base pairing sequence that directs Cas9 activity to a specific gene [37]. This 20nt sequence is part of the single guide RNA (sgRNA) that also contains a 42nt Cas9-binding hairpin and a 40nt terminator sequence. We use at least two different programs to identify target sequences and pick sequences that are predicted in more than one program to not have off target sites. We also try to choose sequences within an exon near the 5′ end of the gene.
2. To generate the sgRNA, we subclone our target sequence into the pT7-gRNA from the Chen and Wente lab [46]. This plasmid is designed for the rapid and efficient cloning of target sequence into the sgRNA backbone. Similarly we have synthesized Cas9 mRNA using the version developed by this same group that has nuclear localization sequences at both the amino and carboxyl termini and has further been optimized for expression in zebrafish [46].
3. After *in vitro* transcription of both the Cas9 mRNA and the sgRNA and subsequent purification, we inject these into fertilized zebrafish eggs at the 1–2 cell stage. We do several experiments in which we first optimize the concentration of sgRNA to minimize lethality, but maximize mutagenesis.

4. We assess the mutagenic efficacy of different sgRNAs using high resolution melting (HRM) analysis and analyzing larvae between 1 and 2 days post fertilization.

We recently used this strategy to generate insertion and deletion (indel) mutations in the zebrafish MCU gene. Mutations generated and HRM data are presented in Fig. 5.2.

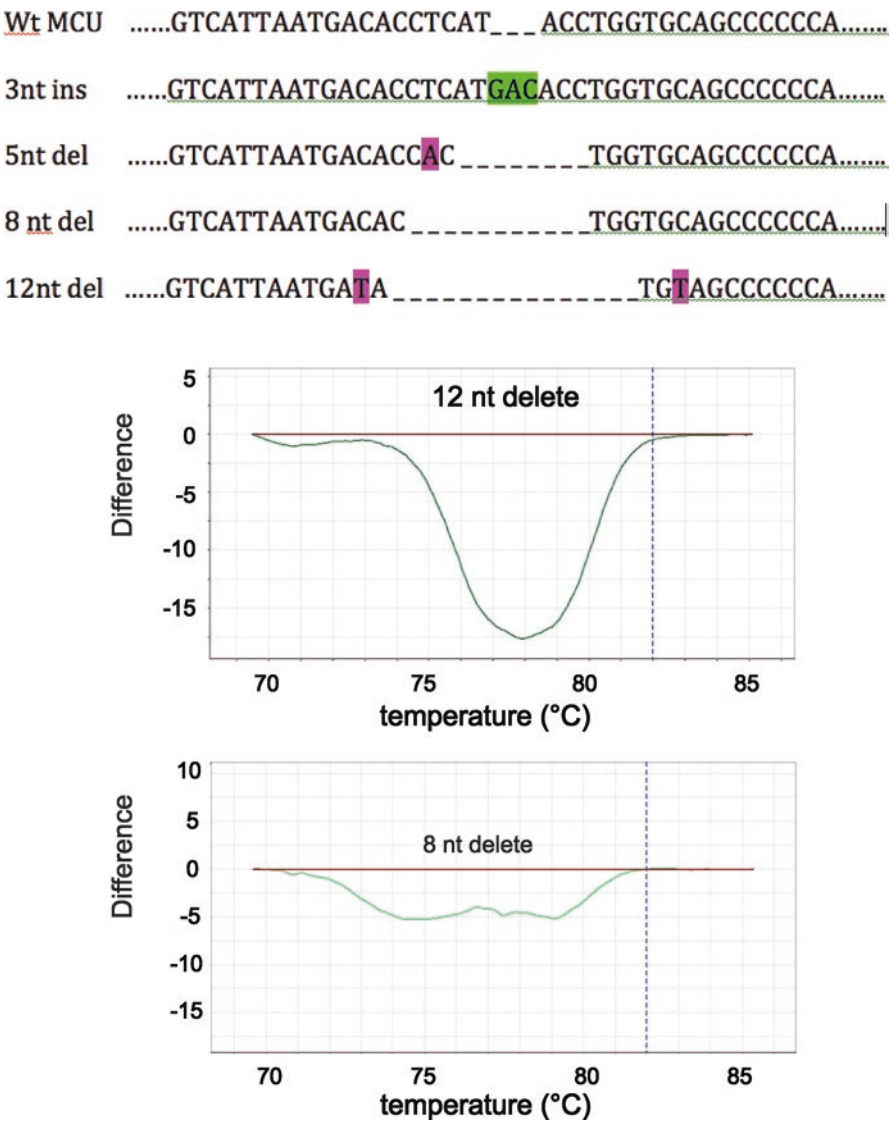

Fig. 5.2 CRISPR/Cas9 mutations in the zebrafish MCU gene. *Top panel* shows four different indels generated by following the procedure outlined in step 1–4 to generate a global knockout of the MCU gene. *Bottom panels* show HRM difference analysis of F1 larvae containing 8nt or 12nt deletion. *nt* nucleotide

While global knockouts are extremely straightforward to make, a potentially more useful approach is to generate knockouts in specific tissues. This strategy can be particularly useful for genes that are globally expressed or have unique functions in different tissues. There are two strategies for generating tissue specific Cas9-mediated genomic changes. Both rely on generating transgenic fish, which stably express Cas9. In one approach, a single Tol2 transposon expresses both Cas9 and the targeting sgRNA [47]. This single DNA construct makes it possible to inactivate a target gene in a specific tissue in one generation, which is additionally facilitated by the inclusion of a fluorescent marker for screening of carriers of the transgene. Cas9 is expressed under a tissue-specific promoter and the sgRNA is expressed ubiquitously using a U6 splicesomal RNA promoter. The authors of this system have made generating the transposon even more straightforward by modifying it so that it can be used with the Gateway cloning system [48]. Thus combining different promoters and sgRNAs becomes simply a matter of mixing appropriate plasmids together for a mix and match approach.

The second approach requires generating two different Tol2 transposons and two transgenic lines, which are then mated with one another [49]. One transposon expresses a tissue-specific or otherwise regulatable Cas9 gene and the other one expresses one or more sgRNA molecules under the control of distinct U6 promoters. The identification of transgenic carriers is again facilitated by the presence of fluorescent markers in both transposons. Once carriers are identified, crossbreeding of the different transgenic lines introduces mutations in specific Cas9-expressing tissues. This system is slower than using a single transposon, since two different transgenic lines need to be identified, but it is advantageous to express multiple sgRNAs at once to ensure that a loss of function allele is identified in the target gene of interest. These tissue-specific transgenic systems are still relatively new, but they hold promise for being used in many different studies. Continued improvements to these systems will come from additional conditional Cas9 activation strategies, such as small molecules or light, which are rapidly being developed and optimized [50, 51].

Several recent studies have used the new genome editing strategies in zebrafish to study retina and RPE [52–58]. Many of these studies are already exploiting the speed and ease by which CRISPR/Cas9 can be used to generate mutant alleles. Zebrafish are well known for distinct experimental advantages. Genetic screens have been used to identify many genes essential for photoreceptor function [59, 60] and development [61, 62]. Recent work has further exploited the transparency of larvae and the ease of generating transgenic strains to conduct sophisticated imaging experiments using fluorescent markers. These studies, which are too numerous to list all here, have provided insight into many aspects of photoreceptor biology. They include analyses of photoreceptor behavior during development [63–65], regeneration [66, 67] and disease [68, 69]. They also provide insight into intracellular events in photoreceptors such as autophagy [21, 70], protein trafficking [71, 72] and dynamics of essential second messenger molecules such as Ca^{2+} [24, 73]. The recent rapid development of sophisticated genome editing techniques ensures that zebrafish will continue to provide novel

and important insights to the study of photoreceptor function and physiology. This will provide a key asset to dissecting critical unanswered questions in the field of photoreceptor biology.

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Chapter 6
CRISPR: From Prokaryotic Immune Systems to Plant Genome Editing Tools

Anindya Bandyopadhyay, Shamik Mazumdar, Xiaojia Yin, and William Paul Quick

Abstract The clustered regularly interspaced short palindromic repeats (CRISPR) system is a prokaryotic adaptive immune system that has the ability to identify specific locations on the bacteriophage (phage) genome to create breaks in it, and internalize the phage genome fragments in its own genome as CRISPR arrays for memory-dependent resistance. Although CRISPR has been used in the dairy industry for a long time, it recently gained importance in the field of genome editing because of its ability to precisely target locations in a genome. This system has further been modified to locate and target any region of a genome of choice due to modifications in the components of the system. By changing the nucleotide sequence of the 20-nucleotide target sequence in the guide RNA, targeting any location is possible. It has found an application in the modification of plant genomes with its ability to generate mutations and insertions, thus helping to create new varieties of plants. With the ability to introduce specific sequences into the plant genome after cleavage by the CRISPR system and subsequent DNA repair through homology-directed repair (HDR), CRISPR ensures that genome editing can be successfully applied in plants, thus generating stronger and more improved traits. Also, the use of the CRISPR editing system can generate plants that are transgene-free and have mutations that are stably inherited, thus helping to circumvent current GMO regulations.

Keywords CRISPR • Cas9 • Cpf1 • Plants • Genome editing • Editing tools

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6.1 Introduction

The past decade has seen massive strides in the direction of development and identification of technologies that would help in precise targeting of the genome to create mutations at specific locations or precise insertion of desired sequences in a particular location. Genome editing, as it is commonly known, is now a routine and easy practice that is performed in laboratories around the world and on a variety of organisms. This involves the use of synthetic nucleases that can create DNA double-stranded breaks (DSBs) and mutations that subsequently arise when the break is repaired by the endogenous DNA repair mechanism of the organism. Initially, technologies such as homing-endonucleases or meganucleases [1, 2], zinc finger nucleases (ZFN) [3, 4], and transcription activator-like effector nucleases (TALENs) [5, 6] were adopted for targeted edits or changes in a genome. But, in the past few years, a new technology has come to the fore. Isolated and derived from the prokaryotic immune system, the clustered regularly interspaced short palindromic repeats system (or CRISPR system for short) has had a massive effect on increasing the feasibility of precision genome editing [7–11].

One of the most attractive features of the CRISPR system is its flexible nature, allowing greater leeway for targeting locations of interest within the genome and hence causing it to be adopted widely [12–16]. The CRISPR system is a single sequence or stranded DNA recognition tool and can cause breaks in a specific location within the genome. Another added advantage is that the CRISPR/CRISPR-associated protein (CRISPR/Cas) system can help create modified plants that can avoid regulatory classifications generally associated with transgenic plants in certain countries [17]. Moreover, the simplicity of the components required for such an experiment is also an added advantage, since CRISPR/Cas depends on only two components to show its activity: single guide RNA (sgRNA) and CRISPR-associated protein or effector.

Taking into account the current agricultural scenario and the always present need to have crops with stronger and improved traits such as increased yield or enhanced pathogen resistance, genome editing has been performed on plants with great success. The CRISPR system has been quite widely used with plants in the last couple of years, with research encompassing plants from a wide range of plant families and genera. CRISPR has been applied to model plants such as *Arabidopsis thaliana* [18–20] and *Nicotiana benthamiana* [10] and other important crop plants such as *Solanum tuberosum* [21], *Triticum aestivum* [11, 22], and finally *Oryza sativa* [7, 11, 19, 20]. Consistent research on food crops will greatly benefit the world as more improved varieties can be identified and obtained by introductions of targeted mutations and new traits. In this chapter, we will take a brief look at the origin of the CRISPR system and its journey from existence as a bacterial adaptive immune system to a genome-editing tool. Light will be shed on the application of the CRISPR system in crop plants and plants in general along with recent modifications that increase the efficiency of the system.

6.2 What Is CRISPR?

Bacteria and archaea are the largest group of life forms on Earth. They are grouped under prokaryotes, generally indicating single-celled organisms that do not possess highly complicated cells or genome structures. This lack of complexity makes them prime targets of viruses and phages. But, since every organism is primed with a sense of survival, it does not come as a surprise that prokaryotes possess some sort of defense mechanism to protect them against such attacks. But, viruses pose a very real threat to bacteria and archaea in the way that they can easily bypass most defensive measures that are undertaken by the latter. Therefore, for survival, bacteria and archaea developed a complex adaptive immune system that allows them to counter this threat. Since for every bacterial cell there are a lot virus-based predators, an adaptive immune system is helpful, and it is the CRISPR system that acts like an adaptive immune system for prokaryotes [23].

Clustered regularly interspaced short palindromic repeats (CRISPR for short) are a set of DNA sequence repeats, present inside the genome of prokaryotes, which can function individually to help confer adaptive immunity to bacteria. The combined CRISPR-Cas system is able to target both DNA and RNA viruses depending on the type that attacks the bacteria [24]. The CRISPR locus was observed for the first time in the genome of *Escherichia coli* accidentally [25], and at that time was not understood in great detail. Later experiments by different groups confirmed the presence of direct repeats in a number of prokaryotes. The recognition elements that are used to neutralize viral elements are termed "spacers" and are incorporated between direct repeats. After infection by a new virus, new spacers can be identified and incorporated in the genome of the host bacteria or archaea, serving as potential recognition elements in the future [26]. Also, for the immune system to work perfectly, it needs a set of CRISPR-associated or *cas* genes that are located next to the CRISPR locus [23, 27].

The CRISPR-Cas system comprises Cas proteins that enact different activities, such as those of helicases or nucleases [28]. The CRISPR-Cas system is classified on the basis of the signature protein it expresses. The system contains two classes that are subdivided into six types, with three in each class. Each type has further subtypes. Two proteins, Cas1 and Cas2, are ubiquitous to most of the CRISPR systems, the function attached to them being the adaptation of new spacers in the CRISPR array [27]. Another conserved region across all CRISPR systems is a short sequence that is located upstream (according to the direction of the transcription) to the CRISPR array known as the leader sequence [29]. Class 1 is divided into types I, III, and V as in all three types multiple Cas proteins are required to cleave the target DNA. The class 2 system is divided into types II, IV, and VI because only a single large protein is employed by these to cleave the target DNA [30]. These six types are further subdivided into 19 subtypes according to the signature protein expressed [31]. It is important to understand how the immunity against an infection proceeds in a prokaryote to better understand the CRISPR system. The entire process has three stages: adaptation, expression, and interference (Fig. 6.1). Each stage

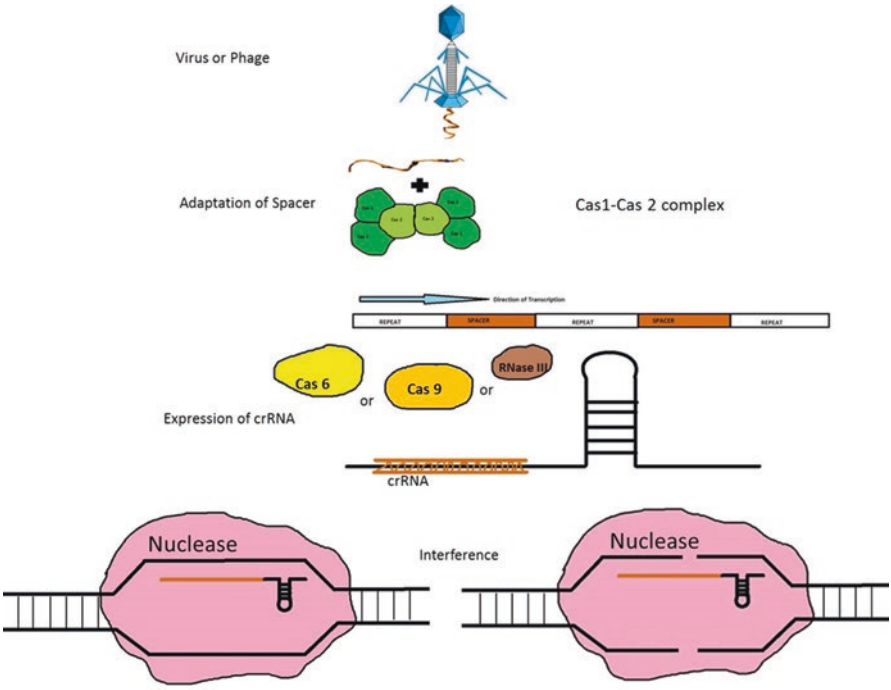

Fig. 6.1 The steps involved in the adaptive immunity in bacteria and archaea that is achieved with the help of the CRISPR-Cas system. It involves three phases or stages: adaptation, expression, and interference

is characterized by a particular set of activities or sequences that lead up to the interference of the foreign viral particle. In the adaptation phase, the infection is recognized and potential spacer sequences are identified and are inserted within the genome. In the expression phase, the CRISPR locus is transcribed into a precursor RNA (pre-crRNA). The adjacent *cas* genes are transcribed to form the Cas proteins, which then cleave the pre-crRNA to a mature crRNA. In the final interference stage, the mature crRNA acts in concert with a few of the expressed Cas proteins. It recognizes the target nucleic acid region and destabilizes it. Thus, in its entirety, this process grants immunity to prokaryotes.

CRISPR adaptation is the first stage or phase of the entire process and is important as it provides memory of a prior infection and thus helps in the expression and interference phases that come in later. Adaptation involves spacer acquisition, which is not completely understood yet. It is apparent that two of the conserved Cas proteins, Cas1 and Cas2, play an important role in identifying and acquiring spacers into the CRISPR array for subsequent steps of expression and interference against the phage-virus attacking the prokaryotic genome. This entire process can be whittled down to two levels, selection of the protospacer and subsequent generation of the spacer, and that is succeeded by integration of the spacer into the CRISPR array

of the prokaryote. As mentioned earlier, Cas1 and Cas2 play an important role in spacer acquisition. They form a complex in which Cas2 forms one dimer, which joins with two Cas1 dimers, in which the Cas1 depends on the Cas2 to bind the CRISPR DNA [32]. Spacer selection, though, is not arbitrary; certain sequences are present in the target that drive the spacer selection. The presence of a short 3–5-nucleotide long motif called protospacer adjacent motif (PAM) next to a potential target is extremely important for target selection. PAM is very important to the prokaryote CRISPR as it helps in distinguishing between self and non-self [33]. In type II-A, the signature protein, Cas9 nuclease, is tasked with the identification of PAM in prospective targets [34].

6.3 History of CRISPR

The CRISPR locus or array was first isolated unintentionally by Ishino et al. [25] from *Escherichia coli* while they were cloning the *iap* gene. The function or the effect of such sequences were not known and understood at that time. For many years, the existence of such direct repeats was just considered to be a quirk of the bacterial genome. Multiple research groups then observed similar interrupted direct repeats in the genome of multiple prokaryotes, giving rise to understanding that CRISPR sequences occur in a lot of prokaryotic genomes. The real research into understanding the use of CRISPR began in Danisco, a dairy company where Philippe Horvath and Rodolphe Barrangou, while sequencing the *Streptococcus thermophilus* genome, came across CRISPR repeats.

Initially, the reason behind the existence of such repeats was not understood, but as they sequenced multiple strains of the bacterium, it was theorized to be linked to an immune system that would act as a defense mechanism against phage infection. Since phage-based infection is a massive problem for the dairy industry as it needs starter cultures to make cheese and yogurt, research into an immune system that could stop phage infections was high on the agenda. As they confirmed that CRISPR actually confers phage resistance, Barrangou et al. [23] later began to use the inherent bacterial CRISPR system to immunize the strains against different phages. The surviving bacteria were then used to create starter cultures that were immune to phage attacks. This was very important as Danisco is part of DuPont and the parent company owns a huge share of the global dairy market. Since they used improved or modified bacterial strains to create cheese and yogurt, there is a good chance that we have ingested CRISPR-based food. But, it is not to be considered a GMO since no recombinant technology is involved.

But, scientists soon figured out that, although the CRISPR system works naturally in bacteria and archaea, the components of the system can be used to create specific mutations in the genome of most organisms due to the precise targeting of the CRISPR system. This led to massive interest in research on the CRISPR system and its subsequent application in genome editing. The organism of choice was *Streptococcus pyogenes*. Jinek et al. [8] characterized and engineered the type II

CRISPR effector Cas9 to create a two-component system that featured tracrRNA and crRNA fused into one single chimeric gRNA. This gRNA would then work in concert with Cas9 and could be programmed by changing the nucleotides to target and cleave a DNA sequence of choice [8].

A group led by Feng Zhang from M.I.T. using the CRISPR/Cas9 system showed functional genome editing of human cell cultures for the first time [35]. Later development of multiplexing platforms to target multiple regions in a genome [36] and the development and identification of other CRISPR effectors such as Cpf1 [37] have had a burgeoning effect that has led to widespread research and application of the CRISPR system for efficient genome editing. Not only human and rat cells but also other model organisms such as zebrafish and even plants have been successfully edited using the CRISPR system.

6.4 Cas9 and Cpf1: The Lead Players in CRISPR-Based Genome Editing

The CRISPR system has been harnessed for genome editing efficiently in the past couple of years. Although the applications and methods to use the system have varied from organism to organism, the nuclease that is used to create a double-stranded DNA break in the target region remains more or less constant in all experiments. The ribonucleoproteins Cas9 [8] and Cpf1 [37] have been ground-breaking discoveries that are driving forward the genome-editing tool CRISPR into greater applications daily. Both these CRISPR effectors can show action with just the presence of a mature crRNA without requiring any of the processing. Thus, targeting varied regions of the genome is made possible by designing a synthetic guide RNA or gRNA, which can be a combination of both the crRNA:tracrRNA (Cas9) or just crRNA (Cpf1), and it harbors a 20nt sequence that is complementary to the target sequence and is located next to an appropriate PAM. Software that can generate the 20nt sequence (e.g., DESKGEN) can be used to create it. This entire system with the CRISPR effector and the synthetic gRNA can then be cloned into a vector and used to transfect targeted cells.

We will first talk about CRISPR-associated protein 9 or Cas9 for short. Cas9 is the signature protein of the type II system of the CRISPR system. Most commonly isolated from *Streptococcus pyogenes*, Cas9 is a protein that is involved in both crRNA maturation and crRNA-guided DNA degradation [8]. As is the hallmark of the type II system, Cas9 requires a crRNA that is base paired with a small RNA known as tracrRNA. To ensure proper degradation of the intended target, it is not only necessary that the crRNA:tracrRNA bind to the target DNA on the basis of complementarity but the degradation also depends on the location of the PAM sequence next to a target region that the Cas9 can identify correctly. After this base pairing at the correct location, Cas9 creates a blunt double-stranded break. It is

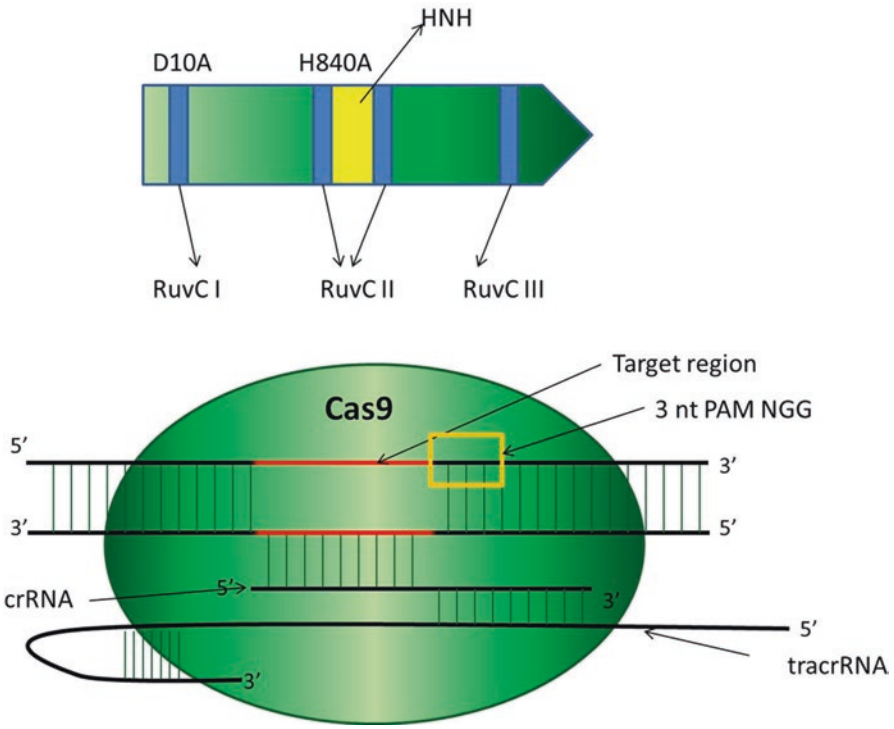

Fig. 6.2 Organization and working of Cas9

interesting to note that the DNA cleavage is at a site that is three base pairs upstream from that of the PAM.

It is also important to understand the structure of Cas9 and how it acts. Cas9 protein has two domains that are homologous to endonucleases RuvC and HNH that enable it to cleave both the strands at the same time. Jinek et al. [8] found out by experimenting which domain cleaves which strand, with the result that RuvC-like domain cleaves the non-complementary strand whereas the HNH-like domain cleaves the complementary strand. It has been found that a seed sequence present in the crRNA, a continuous stretch of 13 base pairs at the 3′ end of the crRNA, is important for cleavage. But, along with that, the PAM sequence location next to a potential target plays an equally important role. PAM helps in identifying the target and is also important for target binding. In the case of Cas9, it requires a PAM sequence that is G:C rich and characterized by the sequence of NGG. Figure 6.2 gives a diagrammatic representation of Cas9 and how it acts. Cas9 has become a popular choice for genome editing and several different variants of Cas9 have been engineered. Also, Cas9 from other species has been isolated and is in wide use. A few examples of Cas9 variants are Cas9 nickase or *Cas9n* [18], in which one of the endonuclease domains of Cas9 (RuvC) is inactivated so as to generate single-strand cuts, *NmCas9* or Cas9 isolated from *N. meningitides* that has an alternative PAM

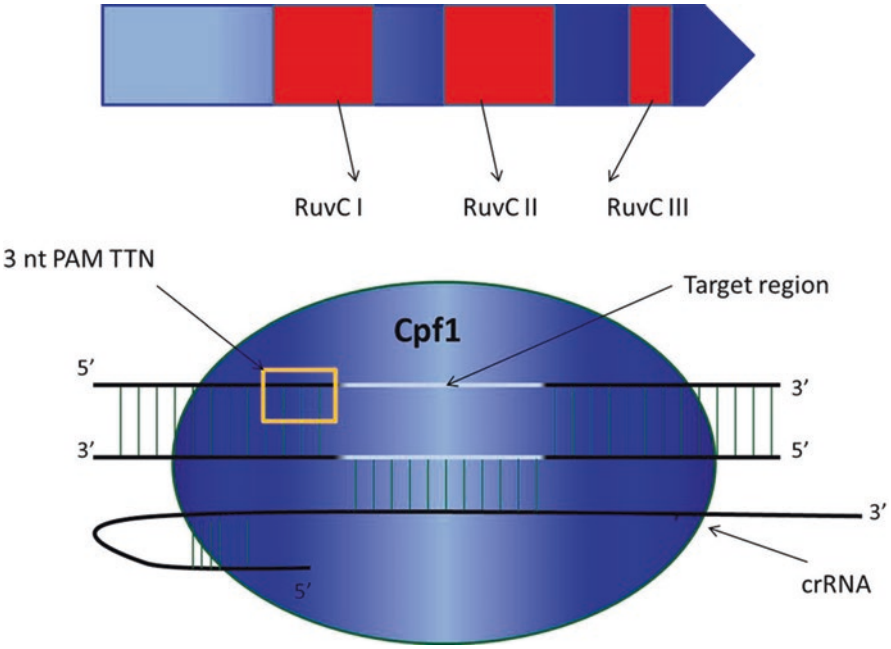

Fig. 6.3 Organization and working of Cpf1

site and reduced off-target ability [38], or even dCas9, which is a catalytically dead Cas9 that can be used for transcription-based studies [39].

Another Class 2 type V CRISPR effector from *Prevotella* and *Francisella* 1, or Cpf1, is slowly gaining importance as an enzyme that can create double-stranded DNA breaks. Although it belongs to the same class of effectors as Cas9, it is markedly different from it. Cpf1 does not require an extra tracrRNA for the functioning of the mature crRNA; second, the PAM region associated with that of Cpf1 is A/T-rich, with the sequence being TTN; and, last, although Cas9 introduces a blunt break, Cpf1 introduces a staggered double-stranded DNA break, resulting in a 4–5nt 5′ overhang [37]. Another reason for the difference between Cas9 and Cpf1 is that although Cas9 possesses both RuvC- and HNH-like domains, Cpf1 carries two RuvC-like domains (Fig. 6.3). The seed region for Cpf1 exists at the 5′ end of the protospacer, which is an advantage because cleavage by Cpf1 at the target will be away from the seed region. Thus, the indels introduced will be away from the target site and the target site will be available for other rounds of Cpf1-based targeting and cleavage.

After a double-stranded break is created by both of these systems, the host organism then repairs the damaged DNA through non-homologous end joining (NHEJ), which is error-prone and can give rise to mutations in the target region. It can also follow homology-directed repair (HDR) that may help in inserting new loci into the target region and can be achieved by introducing a homology arm. The aforemen-

tioned CRISPR effectors and their other variants are helping in broadening the horizon and the application of CRISPR in genome editing. With their ability to target different regions of the genome, both G/C- and A/T-rich regions of a genome it will result in a greater ability of precise genome editing at various locations.

6.5 Modification of the CRISPR System in Plants

Since the Class 2 CRISPR system has been widely in use for genome editing, several modifications have been made to the system itself so as to make it functional within different organisms. Its journey from an adaptive immune system of bacteria and archaea to a genome-editing tool has involved many changes to its components. This starts from Jinek et al. [8] when they used the type II system Cas9 and constructed a chimeric RNA that had both regions of crRNA and tracrRNA to create one gRNA that harbored the target and goes to current research scenarios in which the vectors for the CRISPR system are specially designed to make delivery of the system easier through a lot of small, subtle, and holistic changes. The CRISPR effector itself is modified to allow optimum activity in an organism. The *Cas9* gene is inserted into a vector for it to express the Cas9 protein after it has been delivered. Codon usage patterns differ between species, which suggests that, even though an amino acid generated may be the same, the codon used by an organism may be different in different species [40]. Thus, expressing a foreign gene in another organism may not yield the perfect result as each organism has its own codon bias. Codon optimization is a routine process in which the codons in a gene are changed or modified to suit a particular organism (subject to the most prevalent occurrence of a codon triplet coding for an amino acid in the species) without changing the protein structure and composition of the protein formed. For example, *SpCas9* has been optimized to form *hSpCas9*, in which the codons were modified in the *Streptococcus Cas9* to better suit human codon usage and bias [36]. Figure 6.4 shows a diagrammatic representation of the arrangement of the CRISPR effector and the gRNA cassette.

For plants, the CRISPR effector gene can be codon optimized for the organism of choice like it has been done for *Arabidopsis* [18] or for rice [11, 41]. Cas9 has also been optimized in *Chlamydomonas rheinhardtii* for enhanced expression in plants because of the codon usage patterns and bias mentioned above [19]. A nuclear localization signal is attached to the gene to express a protein that would be easy to import into the nucleus by nuclear transport and is sometimes flanked by detectable signals such as 3XFLAG to allow easier purification and detection of the protein. The effector gene cassette is then driven by a promoter that can be either a CaMV 35S promoter or an ubiquitin (e.g., maize ubiquitin)-based promoter and terminated by either a Nos terminator or any other terminator of choice.

The gRNA is the portion that contains the 20nt target sequence that is normally designed in such a manner that it consists of the 20nt sequence that will bind to the target sequence and next to an appropriate PAM sequence. The gRNA also harbors

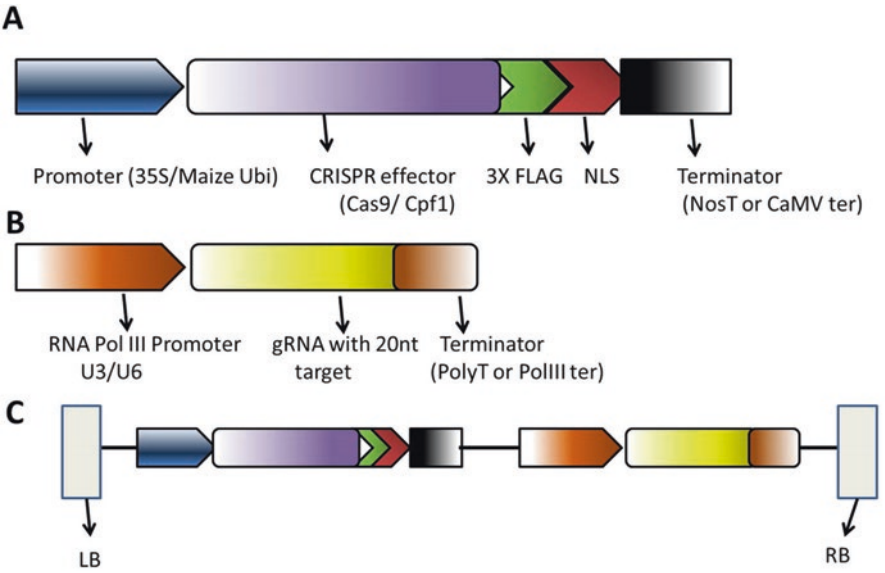

Fig. 6.4 Diagrammatic representation of (**a**) CRISPR effector gene cassette; (**b**) gRNA gene cassette; and (**c**) a general plant binary vector featuring CRISPR effector gene and gRNA

a scaffold (tracrRNA:crRNA or crRNA) that precedes or follows the 20nt sequence depending upon the CRISPR effector that is being used in concert with it. The target region in the gRNA can be replaced by digestion with a restriction enzyme and replacing it with the sequence of choice. This oligonucleotide fragment is driven by an RNA polymerase III promoter, U3 or U6 promoters. Species- and organism-specific promoters can also be used to drive the gRNA cassette such as AtU6 promoter for *Arabidopsis* and OsU3 or OsU6 for rice [42, 43]. This fragment is then terminated by any generic terminator or by a specific Pol III terminator such as U6. As Cas9 requires both crRNA and tracrRNA, the scaffold for this can be either crRNA and tracrRNA driven by separate promoters or one contiguous gRNA harboring both crRNA and tracrRNA.

The entire fragments or cassettes containing either the CRISPR effector gene or the gRNA cassette can then be cloned into plant binary vectors to transform the plant. The fragments can either be cloned separately into different vectors and be co-transformed or sequentially transformed or they can be cloned into one vector using appropriate cloning strategies. A popular strategy to obtain an all-in-one gRNA-CRISPR effector vector is based on the Gateway™ cloning method. In this, the gRNA is cloned into an entry vector. The CRISPR effector is cloned into a location in the destination vector. Then, following the protocols for general Gateway cloning, the gRNA is transferred into the destination vector, thus creating an all-in-one construct.

After the transformation and the subsequent generation of transgenic lines mutagenesis can be checked using several methods. The first is to use a restriction

enzyme digestion assay; most target sites selected include the presence of a restriction enzyme site. After being subjected to a DSB by CRISPR and subsequent repair by the NHEJ mechanism, the site is lost. A PCR performed by amplifying a fragment with primers located in the flanking regions of the target site will generate an amplicon that will be cleaved at the location of the restriction enzyme site in the wild type, giving two bands in a gel, but no result will be obtained in the mutated plants. Another method to detect the mutation is to use the Surveyor® Assay (Integrated DNA Technologies, Catalog #706025/20/21). Surveyor is an enzyme that creates a break in DNA after it encounters a base pair mismatch. The region of the target to be mutated is amplified in boththe wild type and the transgenic line. They are then combined to form a heteroduplex between the two. This is then subjected to the Surveyor enzyme, which will cut the heteroduplex after encountering a base pair mismatch due to the mutation in the transgenic line at the target location. This can then be sent for sequencing to identify the exact nucleotide sequence of the mutation and the zygosity.

6.6 Application of CRISPR in Plants

6.6.1 Model Plants

Since 2013, the application of CRISPR-based genome editing and site-directed mutagenesis technology in plants has been widespread. The ease with which we can develop improved traits and the ability to develop improved varieties have been driving the research forward. Although there have been multiple studies in a wide range of plants, initial studies focused on model plants such as *Arabidopsis thaliana* and *Nicotiana benthamiana.* Most genes that were initially targeted were easily verifiable due to their phenotypic effects like those of PDS or *Phytoene Desaturase,* a gene whose disruption leads to an albino and dwarf phenotype [44].

Genome-editing studies in model plants have paved the way for our understanding of the efficacy of the CRISPR system for application more widely in plants. Generating mutations that were bi-allelic in the target region and obtaining mutations that were stable in the later generations, etc., all displayed the potential for obtaining plants that were modified at desired locations. The fact that a wide variety of genes were targeted shows the potential of the system and technology in use. In *Arabidopsis*, disruption of genes such as *CHLOROPHYLL OXYGENSASE 1* (*CAO1*) and *LAZY1* has shown the expected phenotypes. The fact that in this case each gene had two targets that were simultaneously targeted gives credence to the idea that multiplexing of the CRISPR system for targeting in plants is also possible [45]. The mutation frequency observed in such cases was high and the mutations were stable across multiple generations. In the case of *Nicotiana*, two genes such as *PDS* and *GFP* were tested for genome editing and showed the mutation as expected using the CRISPR system [10, 19]. A list of a few targeted genes for model crops appears in Table 6.1.

Table 6.1 List of plants modified and genes targeted with CRISPR

Name of the plant	Genes targeted and their function	Delivery method	References
Model plants			
Arabidopsis thaliana	Disrupted *GFP* (reporter gene, fluorescence)	*Agrobacterium*-mediated transformation	Jiang et al. [19]
	AtPDS3 (carotenoid biosynthesis pathway), *AtFLS2* (pseudogene in flavonoid synthesis pathway)	*Agrobacterium*-mediated transformation and protoplast transfection	Li et al. [9]
	AtRACK1b (receptor for activated C kinase)		
	AtRACK1c (receptor for activated C kinase)		
	ADH1 (alcohol dehydrogenase, reduction of acetaldehyde to ethanol), *TT4* (chalcone synthase, transferase activity)	*Agrobacterium*-mediated transformation	Fauser et al. [18]
	RTEL1 (regulator of telomere elongation helicase 1)		
	BRI1 (brassinosteroid signal transduction), *JAZ1* (jasmonate signaling)	*Agrobacterium*-mediated transformation	Feng et al. [7]
	YFP (reporter gene, fluorescence)		
	PHYB (phytochrome B, photoreceptor, controls expression of multiple nuclear genes) and *BRI1* (brassinosteroid signal transduction)	Protoplast transfection by RNP:RGEN	Woo et al. [20]
Nicotiana benthamiana	*NbPDS3* (carotenoid biosynthesis pathway)	*Agrobacterium*-based infiltration	Li et al. [9]
	NbPDS (carotenoid biosynthesis pathway)	*Agrobacterium*-based infiltration	Nekrasov et al. [10]
	NbPDS (carotenoid biosynthesis pathway)	*Agrobacterium*-based infiltration	Belhaj et al. [12]
	Disrupted *GFP* (reporter gene, fluorescence)	*Agrobacterium*-based infiltration	Jiang et al. [19]
Crop plants			
Glycine max	*Glyma07g14530* (putative glucosyl-transferase)	*Agrobacterium*-mediated transformation	Jacobs et al. [46]
	GFP (reporter gene, fluorescence)		
	DD20 and *DD43* (genomic sites)	*Agrobacterium* biolistics	Li et al. [47]

(continued)

Table 6.1 (continued)

Name of the plant	Genes targeted and their function	Delivery method	References
Oryza sativa	*ROC5* (modulating leaf rolling), *SPP* (metalloendopeptidase, peptide cleavage), and *YSA* (chloroplast development in early seedling leaves)	*Agrobacterium*-mediated transformation	Feng et al. [7]
	OsSWEET14 and *OsSWEET11* (sugar transporters and TAL effector-based susceptibility to blight)	PEG-induced protoplast transfection was used	Jiang et al. [19]
	BEL (resistance to herbicide bentazon)	*Agrobacterium*-mediated transformation	Xu et al. [48]
	CDKB2, CDKA1, CDKA2, and *CDKB1* (cell cycle regulation)	*Agrobacterium*-mediated transformation	Endo et al. [42]
	OsAOX1a, OsAOX1b, OsAOX1c, and *OsBEL* (resistance to herbicide bentazon)	*Agrobacterium*-mediated transformation	Xu et al. [49]
	ALS (first enzyme for amino acid synthesis for leucine, isoleucine, and valine, specific mutation confers resistance to bispyribac sodium)	*Agrobacterium*-mediated transformation	Endo et al. [50]
	OsPDS (carotenoid biosynthesis pathway) and *OsBEL* (resistance to herbicide bentazon)	*Agrobacterium*-mediated transformation	Xu et al. [51]
	OsDL (floral homeotic gene) and *OsALS* (first enzyme for amino acid synthesis for leucine, isoleucine, and valine, specific mutation confers resistance to bispyribac sodium)	*Agrobacterium*-mediated transformation	Endo et al. [52]
Solanum lycopersicum	*SlAGO7* (RNA silencing pathway, Argonaute gene family)	*Agrobacterium*-mediated transformation	Brooks et al. [53]
Solanum tuberosum	*StIAA2* (related to the functions of Aux/IAA family)	*Agrobacterium*-mediated transformation	Wang et al. [21]
Sorghum bicolor	*DsRED2* (reporter gene, bioluminescence)	*Agrobacterium*-mediated transformation	Jiang et al. [19]

(continued)

Table 6.1 (continued)

Name of the plant	Genes targeted and their function	Delivery method	References
Triticum aestivum	*TaMLO* (stress repsonse)	Transfection of protoplast	Shan et al. [11]
	Tainox (catabolism of inositol) and *TaPDS* (carotenoid biosynthesis pathway)	*Agrobacterium*-mediated transformation	Upadhyay et al. [54]
	TaMLO-1 (stress response)	Biolistics	Wang et al. [22]
Vitis vinifera L.	*IdnDH* (tartaric acid biosynthesis)	*Agrobacterium*-mediated transformation	Ren et al. [55]
Zea mays	*ZmIPK* (inositol hexakisphosphate biosynthesis)	Transfection of protoplast	Liang et al. [56]
	LIG1 (maize leaf development), *Ms26, Ms45* (related to male fertility), *ALS1,* and *ALS2* (first enzyme for amino acid synthesis for leucine, isoleucine, and valine)	Biolistics	Svitashev et al. [57]
	ARGOS8 (negative regulator of ethylene response)	Biolistics	Shi et al. [58]
	Zmzb7 (methyl-D-erythritol-4-phosphate (MEP) pathway)	*Agrobacterium*-mediated transformation and protoplast transfection	Feng et al. [59]

6.6.2 Application in Crop Plants

After successfully applying the CRISPR system for genome editing and validating its potential in model plants, the technology was soon applied to crop plants. The technique promised the generation of altered traits and genes that could be achieved easily because of the precise nature of the CRISPR system. The CRISPR system has been applied to many crop plants across a wide spectrum with many different target genes. Plants such as *Sorghum bicolor* [19], *Glycine max* or soybean [46, 47], *Solanum lycopersicum* [53], *Solanum tuberosum* [21], and even plants such as grapes or *Vitis vinifera* L. [55] have been targeted and modified.

Research and application of the CRISPR system have not been limited to vegetable crops as mentioned above but have also been holistically applied to important food crops such as wheat and rice. Wheat and rice are important both economically and agriculturally as they are responsible for feeding most of the world's population. Accelerated research in *Triticum aestivum* [11, 22, 54] and *Oryza sativa* [11, 19, 48, 60] is being conducted to generate better varieties of these crop plants to satisfy the growing need for them. A list of the crop plants that have been subjected to the CRISPR system has also been included in Table 6.1.

6.6.3 Off-Target Effects

For any technology that targets editing of a genome, an important and large consideration should be the possibility of unwanted off-target modifications. Although the CRISPR system is highly precise in its activity of targeting a particular location, it is not entirely perfect and sometimes off-targeting has been noticed. Thus, it is imperative that, when designing the gRNA for a particular target, consideration should be given to the possible occurrence of off-targeting. Software and applications that can preempt possible off-targets of a selected gRNA sequence are already available on the internet. One of them, known as the Cas-OFFinder [61], can be used to generate potential off-target locations by changing the parameters of stringency. After the off-target sites have been located on the genome, they can then be verified by conducting a PCR with flanking primers to the region and then sequencing the amplicons to check whether any unwanted modifications have taken place in them.

For example, in the CRISPR study conducted in Bandyopadhyay Lab [68], the Cas-OFFinder was used to generate off-target sites by changing the parameters of stringency:. base pair mismatches allowed and the RNA and DNA bulge allowed. Ten targets that could be the location of off-target effects with highest probability were selected and were aligned against the IR64 genome. As mentioned above, primers were designed for the flanking regions of the target site and the amplicons were generated in both the transgenic and the wild type. Sequencing chromatograms for both the transgenic and the wild type were the same at all ten loci, indicating that no off-target effect was observed.

6.7 Future Prospects of Genome Editing in Plants

The CRISPR-based genome editing system is an exceptional system because it can create varieties of plants that are transgene-free and that are homozygous in fewer generations, thus helping in circumventing the current regulatory scenario for genetically modified organisms. With the ability to target a DNA location of choice in any genome, this will lead to faster improvement of crop varieties by increasing the ease with which new traits can be added via introducing knockdowns or insertions of a trait of your choice that in turn will accelerate plant breeding. Continued research into development of the technology is giving rise to innovations that can further help us understand the different characteristics of the CRISPR system and further enhance its applications.

One of the major applications can be the removal of unwanted genes to help confer resistance against pathogens [22]. This will further help in deriving varieties that can be more resistant to attacks by pathogens. Functional analysis of genes can be conducted by performing knockout studies using CRISPR. Unknown genes

located in known gene clusters can now be characterized easily as CRISPR has been shown to be able to cleave large chromosomal sections by virtue of targeting multiple regions [62]. Normally in an organism, the exons are targeted. But, since the plants mainly follow the NHEJ repair mechanism, the resulting mutations may create non-functioning genes. To circumvent this and to introduce new traits or genes or gene segments, intervening introns can be targeted to cleave the DNA at the introns adjacent to the exon. This can later be replaced by a modified sequence. Intron targeting, as it has been named, uses the host NHEJ repair machinery to replace a part of the gene, resulting in a new trait [63]. All of this is due to multiplexing. Targeting multiple regions with the same CRISPR effector is now possible through the platform of multiplexing; that is, to create vectors harboring multiple gRNAs to target multiple loci at the same time and thus reduce the workload [36].

Plants generally use the NHEJ repair mechanism to repair their DNA. But, using a homology arm that is provided right after the DNA has been cleaved by the CRISPR system can lead to HDR. In this, the new homology arm acts as the template for the repair machinery. By manipulating the nucleotide sequence of the homology arm and using the precise targeting of the CRISPR system, allele swapping can be achieved [50, 52]. A different approach to this would be to introduce single-stranded oligonucleotides (ssODNs) with the CRISPR effector to induce HDR and thus endow new traits [64]. Although vector-based plant transformation is the most widely used method of delivery of the CRISPR system, it often gives rise to foreign genetic elements in the plant genome. To avoid this, researchers are now employing a technique in which the CRISPR ribonucleoprotein (purified CRISPR effector protein) is combined with the sgRNA in RNA form to create a ribonucleoprotein and RNA-guided endonuclease (RNP:RGEN) complex and is thus introduced into the plant system. This combination can then be introduced in a protoplast by transfection [20]. The combination immediately acts as soon as it is introduced [65] and after action is most likely destroyed by host endonucleases to reduce off-targeting possibilities.

Derivations of the existing CRISPR effectors to create effectors that can perform specialized functions and the discovery of CRISPR effectors from different species are broadening the scope of application of the CRISPR system. Along with previously mentioned derivations, specialized proteins such as eSpCas9 [66], which helps in reducing off-targeting, and destabilized Cas9 or Cas9-DD [67], which helps in reducing the efficiency of NHEJ-mediated gene insertion, have given rise to an increased understanding and applicability of the technology. Cas9 Nickase or Cas9n [18] is specifically designed by the inactivation of RuvC-like endonuclease domain to create an effector that can cleave only a single strand. Modifications and innovations of this nature will only help us perform gene discovery and functional characterization much more easily. These modifications and further research will help researchers perform site-specific integration of the desired traits and perform gene expression regulation studies and, more importantly, create transgene-free edited plants in the future.

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Part III
The Future of CRISPR

Chapter 7
Target Discovery for Precision Medicine Using High-Throughput Genome Engineering

Xinyi Guo, Poonam Chitale, and Neville E. Sanjana

Abstract Over the past few years, programmable RNA-guided nucleases such as the CRISPR/Cas9 system have ushered in a new era of precision genome editing in diverse model systems and in human cells. Functional screens using large libraries of RNA guides can interrogate a large hypothesis space to pinpoint particular genes and genetic elements involved in fundamental biological processes and disease-relevant phenotypes. Here, we review recent high-throughput CRISPR screens (e.g. loss-of-function, gain-of-function, and targeting noncoding elements) and highlight their potential for uncovering novel therapeutic targets, such as those involved in cancer resistance to small molecular drugs and immunotherapies, tumor evolution, infectious disease, inborn genetic disorders, and other therapeutic challenges.

Keywords Genome engineering • Pooled CRISPR screens • Functional genomics • Cancer • Drug resistance • Infectious disease • Metabolism • Target identification

7.1 Introduction

The recent development of RNA-guided CRISPR nucleases for genome editing has created new opportunities for understanding the genetic basis of disease. With the development of pooled screens utilizing RNA-programmable nucleases, thousands of genes can be interrogated simultaneously to test many genetic hypotheses in parallel. Beyond their initial application for loss-of-function screening, pooled CRISPR screens have also been adapted for gene overexpression, repression, and enhancer region modulation. Here, we first present an overview of pooled screen workflows and how different CRISPR effectors can be harnessed to activate, repress, or knockout genes in different disease models (Fig. 7.1a). We also survey

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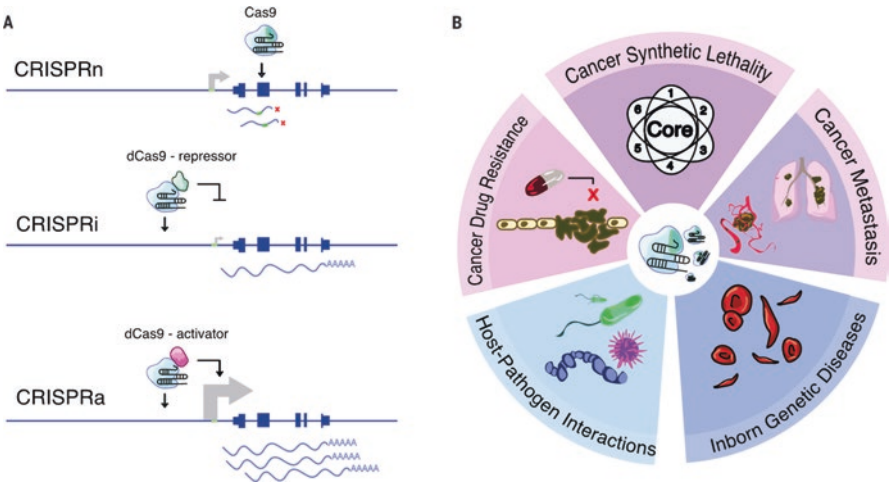

Fig. 7.1 Pooled CRISPR screen platforms and applications. (**a**) Different CRISPR effectors for gene manipulation. *CRISPRn*: CRISPR nuclease target coding exons, where double-strand break repair introduces indel mutations that can result in gene knockout. *CRISPRi*: CRISPR interference fuses a KRAB repressive element to a catalytically inactive form of Cas9 that is capable of binding its genomic target but does not cut. This results in gene repression when targeted near the promoter. *CRISPRa*: CRISPR activation fuses one or more transcriptional activation elements (e.g. VP64, p65, HSF1, Rta, etc. [10]) to a catalytically inactive form of Cas9. This results in gene activation when targeted near the promoter. (**b**) Key disease areas in which pooled CRISPR have been used to understand genetic mechanisms and find new therapeutic targets

applications of CRISPR screens in cancer, infectious diseases and inborn genetic disorders (Fig. 7.1b and Table 7.1). We highlight how these screens have been used for target discovery and potential therapeutic developments from identified target genes/genetic elements.

7.2 Technologies for CRISPR Screens

7.2.1 From Gene Editing to Pooled Screens

Programmable nucleases, such as the clustered regularly interspaced short palindromic repeat-associated nuclease Cas9 (CRISPR/Cas9) have ushered in a new era of precise genome manipulation. For targeted modification in mammalian cells, it is necessary to express both the Cas9 nuclease and a single-guide RNA (sgRNA) [1–3]. The sgRNA contains a 20 nt sequence complementary to the target

Table 7.1 CRISPR pooled screens by disease area and therapeutic implications

Disease category	Disease	Screening phenotype	Therapeutic implications
Cancer	Melanoma	Resistance to chemotherapy drug (a BRAF protein kinase inhibitor vemurafenib [6, 24, 32]); lethal/anti-proliferative phenotype [43, 85]	Screened human coding genes for loss-of-function (LoF) and gain-of-function (GoF) mutations that confers vemurafenib resistance in BRAF mutant melanoma cancer cell line, validated known resistance genes, and identified novel targets (LoF genes such as *NF2* and *CUL3* [6] and GoF genes such as *ITGB3* and *P2RY8* [22]). Further study expanded the screening region to search for adjacent noncoding regulatory elements that mediate the expression of vemurafenib resistance genes (e.g. *CUL3*) and identified a list of previous uncharacterized regulatory elements and mechanisms [32]. Screened human coding genes and identified melanoma-specific cancer dependencies [43, 85]
	Myeloid leukemia	Resistance to chemotherapy drugs such as etoposide [7], cytosine arabinoside (Ara-C) [48] and ATR kinase inhibitor [49]; protein stability reporter assay [86]; lethal/anti-proliferative phenotype [87–89]	Screened human coding genes for LoF mutations that confer drug resistance in myeloid leukemia cell lines, validated known resistance genes (e.g. *DCK* gene for Ara-C resistance in acute myeloid leukemia, (AML)) [48], and identified new resistance genes (e.g. *CDK6* for etoposide resistance in chronic myeloid leukemia, (CML) [7], *SLC29A* in Ara-C resistance [48] and *CDC25A* in ATR inhibitor resistance in AML [49]), provided therapeutic insights into combinatorial drug actions based on patient's genotypes (e.g. prednisolone for *DCK* negative cases [48] and WEE1 inhibitors for ATR inhibitor resistant cases [49]). Another study screened human coding genes for regulators of resistance factors and identified new genes regulating CDC25A protein stability in ATR inhibitor resistance [86]. AML-specific genetic vulnerabilities have been identified with a genome-wide screen of human protein-coding genes [87–89] and saturation mutagenesis of 192 chromatin regulatory domains in the mouse genome [90]. These studies have expanded the list of druggable genetic dependencies in AML, identified new regulatory microRNAs required for proliferation [89], and highlighted new candidates (such as *KAT2A* [87], *ENL* [88]) for pharmacological inhibition.

(continued)

Table 7.1 (continued)

Disease category	Disease	Screening phenotype	Therapeutic implications
	Non-Hodgkin's lymphoma	Apilimod cytotoxicity [91]; induction of lymphoma (*in vivo* transplant of mutated cell pool and assess lymphoma onset) [92]	To study pharmacogenomics of a newly identified chemotherapy drug, apilimod, a genome-scale CRISPRn screen was applied to search for human protein-coding genes that mediate apilimod sensitivity [91]. The study identified novel targets associated with lysosomal homeostasis (genes such as *OSTM1*, *CLCN7*, *SNX10*, and *TFEB*), highlighting the potential to treat B-cell non-Hodgkin's lymphoma with apilimod [91]. To functionally validate sequencing data of rare mutations in human Burkitt's lymphoma, a CRIPSRn screen interrogated the murine orthologues of a list of rare mutations in a genetically engineered mouse model that recapitulates features of human Burkitt's lymphoma and identified two candidate tumor suppressors (*PHIP* and *SP3*). LoF of *PHIP* or *SP3* accelerates lymphomagenesis, suggesting that restoration of these tumor suppressors could treat Burkitt's lymphoma [92].
	Hepatocellular carcinoma	Lethal/anti-proliferative phenotype [30]; induction of liver tumor [46, 93]	Deletion-based screen of human long noncoding RNA (lncRNAs) in HuH-7 to identify 51enriched and depleted lncRNAs in a proliferation assay [30]. PiggyBac-based transposon screen *in vivo* to identify tumor suppressor genes associated with liver tumorigenesis (such as *Cdkn2b*) [46]. Screened mouse genome for tumor suppressor genes via *in vivo* transplantation of library-transduced cell pool and identified candidates such as *NF1*, *PLXNB1*, *FLRT2*, and *B9D1* contributing to tumorigenesis. This work suggests meta-transcriptional regulator HMGA2 (part of the *NF1* pathway) as a potential inhibitory target to treat liver cancer [93].
	Lung adenocarcinoma	Induction of primary tumor and lung-metastatic phenotype [45]; lethal/anti-proliferation phenotype [94]	Genome-wide *in vivo* pooled competition assay for pro-growth and pro-metastasis LoF mutations. After *ex vivo* transduction, the cell pooled was transplanted and both primary tumor and distal organs were monitored for tumor cell growth over several weeks [45]. Screened human coding genes for LoF cancer dependencies in EGFR-mutant lung adenocarcinoma cell line; validated driver mutations, and discovered putative dependencies such as the *TBK1* gene [94].
	Neuroblastoma	Lethal/anti-proliferation phenotype	Screened human coding genes for LoF cancer dependencies in NRAS-mutant neuroblastoma cell line; validated driver mutations and their downstream kinases in the screen, and discovered putative dependencies including *TRIB2* [94].

	Sarcoma	Lethal/anti-proliferation phenotype [44]; myogenic differentiation [95]	Established a patient-derived sarcoma cell line, and screened a selective set of druggable human genes combining CRISPRn, RNAi and small-molecule screening approaches. The study identified *CDK4* and *XPO1* as potential therapeutic targets [44]. Screened class I and class II histone deacetylases (HDACs) genes in established rhabdomyosarcoma cell line; identified HDAC3 deacetylase as a major suppressor of myogenic differentiation, suggesting *HDAC3* as a potential therapeutic target in differentiation therapy (where malignant cells are encouraged to differentiate into non-proliferative cells) [95].
	Breast cancer	Oncogene-induced senescence (anti-proliferation phenotype) [96]	Screened 90% of p53-bound enhancers and 60% of ERα-bound enhancers for functional elements. The enriched functional elements for the senescence (for p53) or growth (ERα) phenotypes were enhancers near cell-cycle genes (*CDKN1A* and *CCND1*) [96].
	Ovarian cancer	Lethal/anti-proliferation phenotype	Screened 50 epigenetic regulators with a paired-gene targeting sgRNA library to study cooperative regulation and possible combinatorial cancer therapeutics to treat ovarian cancer; identified sets of epigenetic regulators that confer synthetic lethality and proposed a combination of a *KDM4C* inhibitor and *BRD4* inhibitor to reduce ovarian cancer cell proliferation [34].
	Pancreatic cancer	Lethal/anti-proliferation phenotype	Screened human coding genes in RNF43-mutant pancreatic ductal adenocarcinoma cell line, for cancer-dependent specificity in proliferation; identified and functionally validated the FZD5 receptor as target for potential targeted therapy using antibody [97].
	Colon cancer	Lethal/anti-proliferation phenotype	Screened human coding genes for context-specific cancer dependencies, discovered distinct vulnerabilities between common *KRAS*-mutant colorectal cancer cell lines: DLD1, appears to be dependent on EGFR signaling (despite the cells being KRAS-mutant), whereas HCT116 relies on ETC complex I function and can be inhibited selectively by metformin [43].
Infectious disease	*Clostridium septicum* infection	Resistance to either clostridium septicum alpha (α)-toxin or 6-thioguanine	Screened mouse genome for resistance to α-toxin or 6-thioguanine; identified 27 previously characterized and 4 novel genes involved in resistance [98].

(continued)

Table 7.1 (continued)

Disease category	Disease	Screening phenotype	Therapeutic implications
	Synthetic bacterial toxins	Sensitivity to diphtheria and chimeric anthrax toxins [57]; sensitivity to a cholera-diphtheria toxin [16]	Screened 291 genes for host factors essential for anthrax and diphtheria intoxication; identified four genes (*PLXNA1*, *FZD10*, *PECR* and *CD81*) as candidates in protective agent-mediated anthrax toxicity [57]. Screened human coding genes for sensitizing and resistant host factors that regulate cellular response to cholera-diphtheria intoxication [16].
	Gram-negative bacterial infections	Inflammatory cytokine tumor necrosis factor (*Tnf*) positive after lipopolysaccharide (LPS) stimulation	Screened mice genome for induction of *Tnf* after LPS stimulation, identified novel genes that regulate TLR4 signaling pathway in response to LPS [99].
	Staphylococcus aureus infection	Sensitivity to α-hemolysin toxin (αHL)	Screened human coding genes and identified 10 host targets required for αHL susceptibility; validated proteins (such as SYS1, ARFRP1, and TSPAN14) that regulate host receptors [100].
	Vibrio parahaemolyticus infection	Sensitivity of type III secretion system (T3SSs)-dependent cytotoxicity	Screened human coding genes for distinct host factors facilitating T3SSs-dependent cytotoxicity, discovered processes underlying host-pathogen interactions [59].
	West Nile virus (WNV) infection	Resistance to West Nile virus (WNV) infection [63, 64]	Screened human coding genes for host factors required for West Nile virus infectivity; identified genes including 7 strongly protective genes (*EMC2, EMC3, SEL1L, DERL2, UBE2G2, UBE2J1*, and *HRD1*) in the ER-associated protein degradation (ERAD) pathway as potential therapeutic targets to prevent WNV induced cell death [63]. Another CRISPRn screen identified and specifically detailed the role of SPCS1 in modification and secretion of flaviviral particles, and suggested that inhibition of SPCS1 might reduce viral replication [64].

	Dengue virus (DENV) and hepatitis C virus (HCV) infection	Resistance to dengue virus (DENV) and hepatitis C virus (HCV) infection	Screened human coding genes for host factors required for DENV and HCV infectivity; identified oligosaccharyltransferase complex as essential elements for DENV replication and discovered role of intracellular flavin adenine dinucleotide during HCV replication which suggests new host targets for antiviral drugs [65].
	Human immunodeficiency virus (HIV)	Lethal phenotype	Screened human coding genes and identified five HIV host cell factors: co-receptors CD4 and CCR5, TPST2, SLC35B2, and ALCAM, which suggest new cellular pathways for antiviral therapy [66].
	Chronic viral infection	Altered PD-1 expression profile	Screened ~23.8 kb enhancer and eight additional regulatory regions adjacent to *Pdcd1* locus in murine T cells; discovered that exhausted $CD8^+$ T cells have a unique enhancer and transcription factor binding landscape which suggests an option to edit exhaustion-specific enhancers for engineered T-cell therapy [52].
	Apicomplexan parasites	Lethal phenotype	Screened *T. gondii* parasite genome for factors that facilitate infection; identified ~200 new fitness genes and investigated critical factors (such as CLAMP protein) involved in host cell infection [70].
Inborn genetic disorders	β-hemoglobin disorders	Fetal hemoglobin (HbF) enrichment [31, 33]	Screened BCL11A composite enhancer DNase I hypersensitive sites in human hematopoietic stem and progenitor cells; identified a conserved GATA1 motif as the essential element of BCL11A enhancer for human erythroid BCL11A expression and HbF repression, which suggests a potential therapeutic genome editing site for β-hemoglobin disorders [31]. Screened *HBS1L-MYB* intergenic region (whose variants modulate HbF level) for regulatory elements that control *MYB* expression; identified putative regulatory elements that control *MYB* expression [33].
	Mitochondrial diseases	Lethal phenotype [74, 75, 77]	Screened human coding genes for protective factors during mitochondrial respiratory chain (RC) inhibition; identified von Hippel-Lindau (VHL) factor and proposed a hypoxia treatment for mitochondrial disease [74]. Screened human genome for novel genes essential for oxidative phosphorylation; identified 191 hits and discovered *NGRN*, *WBSCR16*, *RPUSD3*, *RPUSD4*, *TRUB2*, and *FASTKD2* that form a mitochondrial 16S rRNA regulatory module [75]. Screened human genome for LoF mutations that can rescue Mitochondrial complex I-impaired cytoplasmic hybrid cells in conditions that require cellular oxidative phosphorylation; identified *BRD4* whose loss enhances oxidative phosphorylation activity, suggesting it as a promising target to overcome mitochondrial defects [77].

genomic region, and a part of a palindromic repeat that forms the secondary structure for Cas9 docking [4]. Directed by sgRNA, Cas9 nuclease identifies a target genomic region and introduces a double-stranded break (DSB). Chromosomal DSBs are typically repaired through cellular repair mechanisms such as homologous recombination (HDR) or non-homologous end joining (NHEJ). In the NHEJ repair pathway, the Ku DNA-binding heterodimer first binds to the DNA terminus to initiate end processing and recruits enzymes such as Artemis-DNA-PK_{CS} to trim the incompatible ends, polymerases to fill the gaps, and ligases (XRCC4-DNA-ligase-IV complex) to seal the nick [5]. Cellular repair mechanisms such as NHEJ often create deletions or insertions (indel mutations) at the DSB site. If Cas9 targets a coding exon, indel mutations can result in a frameshift mutation and a premature stop codon, thereby knocking out the target gene expression. If Cas9 targets an intron, enhancer, or other noncoding region, mutagenesis can disrupt functional elements such as transcription factor binding motifs or chromatin anchoring sites, which can alter regulation of gene expression.

CRISPR forward genetic screens take advantage of the same genome editing machinery to pair many different genetic changes with a phenotypic assay [6, 7]. Specifically, the screen quantifies which genetic manipulations are enriched or depleted in a disease-relevant phenotype. The workflow for CRISPR screens can be summarized in five steps: (1) choose genomic regions or genes of interest and design a sgRNA library to target these elements, (2) generate cell populations with various genetic perturbations introduced through this sgRNA library, (3) select a biological phenotype of interest, (4) trace back from the selected phenotype to its associated gene/genomic targets, and (5) confirm the function of the identified targets through additional validation studies [8].

CRISPR screens can be performed in either an arrayed or pooled format. In an arrayed CRISPR screen, each well receives one sgRNA delivered into all cells. In comparison, a pooled CRISPR screen can perturb thousands of genes simultaneously—with each cell in the pool receiving one genetic perturbation. This is most often achieved via lentiviral delivery of the CRISPR library to a large cell pool. Each construct in the pooled lentiviral library contains a unique sgRNA. To ensure that each cell only receives a single CRISPR construct, the viral titer is adjusted such that the multiplicity of infection is less than 1 (i.e. fewer viral particles than cells). Successful genomic integration of the virion results in expression of the sgRNA in a Cas9-expressing cell line. Alternatively, both sgRNA and Cas9 nuclease can be packed into the same virion to infect wild type cell lines. To remove non-transduced cells, the construct also includes a selectable marker such as drug resistance or fluorescence. After lentiviral integration, the unique 20 nt sgRNA guide sequence serves as a barcode for the construct. This barcode is used to measure enrichment or depletion of the specific sgRNA after phenotypic selection. Significant enrichment or depletion of a sgRNA barcode suggests functional association between the sgRNA target locus and the phenotype of interest. To reduce false-positive hits, genes/genome target regions should be validated with newly-designed

sgRNAs that are not in the original library. Validation of individual sgRNAs should also include analysis of indels (e.g. Surveyor/T7E1, sequencing, etc.) and/or gene expression changes (e.g. qPCR, quantitative protein blotting, etc.). After initial hit validation, further in-depth studies may involve genetically-engineered mouse models, perturbations of related genes in the same pathway, and validation across a panel of cell lines to examine the effects of genetic background.

7.2.2 Types of CRISPR Screens

CRISPR screens to date have mainly focused on applying CRISPR nuclease (CRISPRn) Cas9 to identify loss-of-function mutations in protein-coding genes associated with disease traits. In addition to their use as a targeted nuclease, CRISPR systems have also been deployed as a general DNA-targeting platform to bring new effector domains to specific regions of the genome [9–11]. Beyond Cas9, there are also exciting possibilities for applying other DNA and RNA targeting CRISPR systems to take advantage of the metagenomic diversity of CRISPR systems [12]. These different CRISPR systems and effector domains can greatly diversify the genetic manipulations available for screening gene loci and noncoding regions.

There is a variety of effector fusions that have been developed to activate or repress gene expression. Gene repression via effector domains is distinct from nuclease-based gene loss-of-function. Cas9 nuclease targeting typically results in loss-of-function due to formation of indel mutations in coding exons and nonsense-mediated decay of mRNA transcripts. In contrast, CRISPR interference (CRISPRi) screens use a deactivated Cas9 (dCas9) fused to a Krüppel-associated box domain (KRAB) repressor [13]. Deactivated Cas9 (via alanine mutagenesis of a catalytic residue in the nuclease domain) retains the ability to form Cas9-sgRNA complexes that bind target sites [14]. The KRAB repressor is one of the most commonly used effectors for gene repression. Once at the target site, KRAB recruits nuclear proteins to form a heterochromatin complex that can facilitate histone methylation and deacetylation [15]. CRISPRi screens using dCas9-KRAB have been applied to study protective factors in cellular toxin-resistance [16] and identify regulatory elements in the vicinity of oncogenes such as *GATA1* and *MYC* [17]. For upregulating gene expression, there are three major types of dCas9-based gene-activating approaches (CRISPRa): tethering dCas9 directly with one or multiple activators (dCas9-VP64 [18, 19], dCas9-VPR [20], dCas9-P300 [21], and dCas9-VP160 [22]); engineering a polypeptide scaffold to dCas9 for tagging multiple activator copies (Suntag [23]); modifying sgRNA scaffold hairpin region to recruit activators (SAM [24] and others [25]). A recent comparison of dCas9 activators found that activators with multiple, distinct activation domains (dCas9-VPR, SAM and Suntag) were capable of higher and more robust gene activation compared to effectors with

a single type of domain (e.g. dCas9-VP64, which contains four tandem repeats of the VP16 domain) [10].

In addition to gene activation and repression, other effectors have been incorporated into CRISPR systems to manipulate DNA methylation, histone acetylation and base editing. DNA methylation is catalyzed by DNA methyltransferases (Dnmt) and typically results in gene silencing [26] whereas DNA demethylation is facilitated by ten-eleven translocation (TET) dioxygenases and can result in gene activation [27]. Catalysts of DNA methylation and demethylation can be fused with dCas9, such as dCas9-Dnmt3a and dCas9-Tet1 respectively, and have been used to precisely edit CpG methylation [27]. Recent studies have shown that DNA methylation correlates with certain neuropsychiatric disorders such as schizophrenia, Rett syndrome, and immunodeficiency-centromeric instability (ICF) syndrome [26]. CRISPR screen effectors dCas9-Dnmt3a or dCas9-Tet1 could be used to identify regions of the genome that harbor control elements sensitive to changes in methylation. In addition to DNA methylation, post-translational modifications to histone tails can also modulate gene expression. Fusing the catalytic unit of acetyltransferase to dCas9 can robustly activate gene expression by catalyzing acetylation of histone H3 lysine 27 at enhancer/promoter sites [21]. Additionally, the base pair editing tool dCas9-cytidine deaminase fusion protein has been used for making C to T (or G to A) point mutations [28]. Another point mutation generator system: "CRISPR-X" used dCas9 and a modified sgRNA with two MS2 hairpins to recruit a cytidine deaminase [29]. These systems can act as re-purposed CRISPR screens to provide alternatives to the kinds of mutations that result from CRISPRn-driven NHEJ.

Recently, pooled screens that pair CRISPR nucleases with multiple guides have been used to analyze multi-gene interactions and larger deletions. To study noncoding elements such as long noncoding RNAs (lncRNAs) or super-enhancers, pairs of sgRNAs can create deletions that span the beginning and the end of larger genomic regions. A deletion screen targeting multiple long noncoding RNAs successfully demonstrated targeted genomic deletions to pinpoint regulatory lncRNAs associated with liver cancer [30]. For higher-resolution tiling in the noncoding region, single sgRNA saturation mutagenesis has been particularly helpful in identifying functional elements such as transcription binding motifs [31]. A saturating-mutagenesis screen targeting ~700 kb region surrounding drug resistance genes has uncovered regulatory elements in a melanoma model [32]. Another study utilized a saturating-mutagenesis library to examine ~300 kb region in *HBS1L-MYB* intergenic region and identified putative enhancer elements that regulates *MYB* expression, which in turn regulates fetal hemoglobin levels [33]. Multi-guide screens have also been used to search for loss-of-function gene interactions or cooperative regulatory networks [34].

In addition to different effectors, CRISPR screens can benefit from the abundance and diversity of CRIPSR-based DNA-targeting/gene editing systems found in different microbial species. Recent work on the CRISPR effector Cpf1, which recognizes T-rich PAMs [35, 36], suggests a new screening option for targeting T-rich,

NGG-poor regions. Since Cpf1 processes its own repeat array through its ribonuclease activity, it may be easier to multiplex guide RNAs [37] for examining cooperative regulation and deletions. To further expand the screening target from genome to transcriptome, the recently discovered RNA editing Cas9-C2c2 [38, 39] could be deployed to discover functional elements in regulatory RNAs or perform strand-specific screens.

7.3 CRISPR Screen Applications: Genetic Mechanisms of Human Disease and Therapeutic Development

7.3.1 CRISPR Screens in Cancer for Synthetic Lethality and Drug Resistance

Over the past few years there has been tremendous excitement surrounding precision medicine approaches for the treatment of diverse cancers [40, 41]. Despite this excitement, there are still many aspects of cancer genetics and therapeutic resistance that are poorly understood. CRISPR screens for cancer functional genomics fall broadly into three major categories: (1) understanding synthetic lethality and identifying potential new therapeutic targets through screening for cancer- and stage-specific dependencies; (2) finding genes that drive resistance or sensitivity to existing targeted therapies; (3) identifying noncoding regulatory elements that influence oncogene expression to provide alternative targeting options in cases where the oncogene itself may not be druggable.

7.3.1.1 Identifying Cancer-Specific Vulnerabilities

Due to different underlying mutational processes and genome instability, cancer cells often evolve different genomic signatures during cancer progression. Characterizing cancer-specific vulnerabilities requires finding mutated proteins or gene expression programs that are essential to proliferation. These identified targets can be candidates for developing targeted therapy.

By applying genome-scale CRISPRn to multiple cancer cell lines, several groups have identified shared essential (core) genes across different cancer types [42, 43]. For each tumor cell line, we can define context-specific fitness genes by subtracting shared essential (core) genes from all essential genes for that tumor. One recent study comparing four cancer types discovered several context-specific fitness genes in glioblastoma, colorectal carcinoma, cervical carcinoma and melanoma [43]. Intriguingly, two different colorectal carcinomas displayed distinct vulnerabilities, highlighting the potential for using a genome sequencing and/or functional genomic screens to stratify patients.

For rare tumors, combining CRISPR screens with patient-derived *in vitro* models can be helpful for correlating functional genomic data with known pathological features and specific genetic mutations (germline or somatic). In a recently established patient-derived cell line for a rare undifferentiated sarcoma, multiple screening approaches (CRISPRn, RNA interference and pharmacologic screens) converged on CDK4 (a cyclin dependent kinase) and XPO1 (a protein involved in nuclear transport) as potential therapeutic targets [44]. One powerful aspect of this study was that the intersection of all three different screen modalities was used to build greater confidence in the genetic hits, suggesting a novel approach to pooled screen validation. In addition to patient-derived *in vitro* models, *in vivo* mouse models have also been employed to understand specific mutations and to characterize multi-cell interactions, such as primary tumor growth and distal organ metastasis. In one type of *in vivo* CRISPRn screen, tumor cells are transduced *ex vivo* with a lentiviral sgRNA library and then the mutant cell pool is transplanted into immunocompromised (or syngenic) mice. Using this approach, a study identified loss-of-function mutations that contribute to primary tumor growth and cancer metastasis *in vivo* by separately analyzing enriched sgRNA targets in different organs [45]. The identified mutations included both well-established tumor suppressor genes, microRNAs (miRNAs) and several novel drivers of metastasis. It was shown that mutations that drive lung metastasis also stimulate primary tumor growth, suggesting that these events are tightly linked for many genetic driver mutations [45]. Another type of *in vivo* CRIPSRn screen delivered a sgRNA library using the piggyBac transposase and identified novel tumor suppressor genes associated with liver tumorigenesis [46]. Since it can be challenging with non-virally delivered transposase to limit genomic integration to only a single sgRNA per cell, secondary validation of screen hits is essential to confirm their roles in tumorigenesis.

7.3.1.2 Understanding Mechanisms of Drug Resistance

A major obstacle for targeted therapy is drug resistance: When patients are treated with drugs targeting specific oncogenes (such as *BRAF* in melanoma or *EGFR* in non-small cell lung cancer), they often develop resistance to treatment [47]. Genome-wide CRISPRa and CRISPRn screens identified gain-of-function and loss-of-function mutations in BRAF inhibitor-resistant melanoma, and loss-of-function mutations in etoposide-, cytosine arabinoside (Ara-C)- or ATR kinase inhibitor-resistant myeloid leukemias [6, 7, 48, 49]. A genome-wide CRISPRa screen for BRAF inhibitor resistance in melanoma identified potential targets for direct pharmacological inhibition [24]. This highlights a key difference between CRISPRa (gain of function) and CRISPRn (loss of function) approaches. For gain-of-function hits from a CRISPRa screen, it is possible to test established target-specific drugs. In cases where a direct inhibitor is not available, cell lines containing the mutation (or engineered to carry it) can be challenged using a high-throughput drug screen of novel compounds.

For loss-of-function CRISPRn screens, it can be more challenging to translate screen hits into drug targets/strategies. For example, a CRISPRn screen identified *CDC25A* loss-of-function as driver of resistance to ATR kinase inhibition in acute myeloid leukemia (AML) [49]. A WEE1 (G2 checkpoint kinase) inhibitor could restore the ATR inhibitor's efficacy in the resistant cells by forcing mitotic entry in CDC25A-deficient cells [49]. Another approach for overcoming drug resistance is to identify multi-gene synthetic-lethal interactions, where resistance stemming from a single loss-of-function mutation is reversed by a second loss-of-function mutation (synthetic lethality). One recent CRISPRn screen evaluated synthetic lethality by delivering two sgRNAs to mutate two genes simultaneously [50]. The study attempted to test 1.4 million possible synthetic-lethal interactions among 73 cancer genes and identified a total of 152 successful pairs demonstrating synthetic lethality. In subsequent combinatorial drug validation studies, the researchers validated roughly 75% of the synthetic lethal combinations discovered. Synergistic cytotoxicity identified in CRISPRn screens can be quite informative and can provide a roadmap for downstream combinatorial drug studies. Similarly, CRISPRa screens can also capitalize on multi-gene targeting to identify resistance genes for combinatorial inhibition.

7.3.1.3 Examining Noncoding Regulators of Cancer Gene Expression

In addition to protein-coding genes themselves, there are many regions of the noncoding genome involved in the regulation of protein-coding gene expression. CRISPRi was used to identify nine distal enhancers within 1 megabase of sequences near *MYC* and *GATA1* oncogenes [17]. *MYC* is a common oncogenic driver in many different cancers [51] and thus mapping enhancer elements that might increase *MYC* expression is important for identifying potential therapeutic targets. Additionally, noncoding regulators in T-cell exhaustion was studied with a CRISPRn saturating mutagenesis screen [52]. The study mutated all possible sgRNA sites of nine regulatory sequences near the *Pdcd1* gene which codes for programmed cell death protein 1 (PD-1). In the context of cancer immunotherapy, PD-1 inhibition has been approved for a wide variety of different malignancies [53]. By correlating functional regions with putative transcription factor binding motifs, the study suggests possible upstream therapeutic interventions to inhibit immune checkpoint pathways. In general, CRISPR screens can be adapted to detect immune checkpoints or regulatory elements of those checkpoints, providing immunotherapeutic strategies to block T cells from being deactivated by tumor cells. Besides targeting enhancer binding sites, CRISPR screens utilizing saturating mutagenesis or deletion can also detect various other types of oncogenic regulators including long noncoding RNAs (lncRNAs) [30], microRNAs (miRNAs) [54], and other important noncoding regions such as introns and untranslated exons [55].

7.3.2 CRISPR Screens in Infectious Disease

Pathogenic organisms such as bacteria, parasites, and viruses present a major problem for human health around the globe [56]. Pooled CRISPR screens have provided insight into host-pathogen interactions by identifying host factors that facilitate or resist pathogen infections and intrinsic pathogen factors that enhance infection.

Identifying host factors that contribute to pathogenicity is an important step in understanding toxicity and treating bacterial infections. CRISPR screens for host-bacterial interactions tend to focus around two key areas: resistance and sensitizing factors. By treating gene-edited cell pools with bacterial toxins or infectious pathogens, researchers can identify resistance and sensitizing factors through analysis of significantly enriched or depleted genes, respectively. For instance, to study host resistance factors against diphtheria and anthrax toxin, a targeted screen of ~300 genes (including cell surface proteins, and proteins involved in endocytosis, trafficking and cell death) identified four enriched cell-surface receptor genes (*PLXNA1*, *FZD10*, *PECR* and *CD81*) that confer resistance [57]. Upregulation of genes involved in resistance might protect cells from intoxication. On the other hand, sensitizing factors that facilitate infection can also provide mechanistic insight to pathogenesis. For example, studies have shown that *Vibrio parahaemolyticus* employs two type III secretion systems (T3SS) to inject its payload [58]. A genome-wide CRISPRn screen in human intestinal epithelial cells used a modified *Vibrio* pathogen where either T3SS was removed to identify protein modification pathways for pathogen entry that are specific to each T3SS [59]. Down regulation of host factors might provide alternative paths to mitigate cytotoxicity in pathogen infections.

Similarly, to understand specificity of viral-host interaction, multiple CRISPR screens have been used to identify receptors for viral entry and necessary cellular components for viral replication in host cells. Host interactions with flaviviruses and retroviruses are two key examples. Flaviviruses are a family of arboviruses that includes West Nile, Dengue, Zika, and Hepatitis C virus [60–62]. A genome-wide CRISPRn screen revealed seven protective genes in the endoplasmic reticulum associated protein degradation (ERAD) pathway, where loss-of-function confers resistance to West Nile virus-induced cell death but does not block viral replication [63]. To look for shared replication facilitators in host cells, a second genome-wide screen identified and validated signal peptidase complex 1 (SPCS1) as key requirement for flavivirus replication [64]. For viral specific host factors facilitating viral replication, a third genome-wide screen discovered distinct host-dependency factors required for Dengue or hepatitis C virus [65]. Identification of these novel host factors provides new avenues for developing specific antiviral therapies. In addition to flaviviruses, CRISPR screens have also provided insight into retroviruses, such as human immunodeficiency virus (HIV). Although the entry receptors for HIV have been well-characterized (e.g. CCR5 and CXCR4), a genome-wide CRISPRn screen discovered several new dependencies, including tyrosylprotein sulfotransferase 2 (TPST2) and solute carrier family 35 member B2 (SLC35B2) [66]. These two

proteins function in a common pathway to sulfate CCR5 so that it can be recognized by HIV. Loss of either of these proteins and the modifications they impart to CCR5 results in strong protection against HIV, suggesting further targets for controlling viral load.

Relatively few CRISPR screens have been performed in pathogens themselves compared to screens in host organisms. Intrinsic pathogen factors contribute to severity of infections and a classic example is the acquisition of antibiotic resistance. Studies have shown that carbapenem-resistant *Enterobacteriaceae* [67] and methicillin-resistant *Staphylococcus aureus* [68] are resistant to nearly all available antibiotics, suggesting that novel antibiotics or treatment options are urgently needed for combating antibiotic-resistant bacterial infections. CRISPR screens can be implemented to characterize new antibiotics and their mechanisms of action. For example, to test a novel antibiotic MAC-0170636, a CRISPRi screen analyzed all essential genes in *Bacillus subtilis,* and identified undecaprenyl pyrophosphate synthetase (*uppS*), an essential molecule in construction of the bacterial peptidoglycan cell wall, as a key target for the antibiotic [69]. In addition to antibiotic resistance in bacteria, CRISPR screens have been extended to examine intrinsic factors in other types of pathogens, such as parasites. Apicomplexan parasites are one of the leading causes of human parasite infections such as malaria and toxoplasmosis [70]. A recent study used a CRISPRn screen to target all ~8000 protein-coding genes in *Toxoplasma gondii* [70]. The study defined roughly 200 previously uncharacterized fitness genes and identified the claudin-like apicomplexan microneme protein (CLAMP) as an invasion factor in the initiation of infection [70]. CLAMP is essential for parasite infection in fibroblast cells. In malaria, CLAMP knockdown blocks the asexual cycle of the parasite, indicating that insights from the pooled screen could potentially transfer to other pathogens in the Apicomplexan phylum [70].

7.3.3 CRISPR Screens for Understanding and Treating Inborn Genetic Disorders

Inborn genetic disorders are diseases caused by inherited or *de novo* mutations that affect early development. In this area, CRISPR screens have been used to find regulators of hemoglobin switching and novel treatments for mitochondrial disorders.

Hemoglobin disorders, such as beta-thalassemia and sickle-cell anemia, are relatively common. There are >300,000 births each year with severe forms of these diseases, which result from defects in the adult form of hemoglobin (β-globin) [71]. In early development, an alternative, fetal form of hemoglobin is the dominant oxygen carrier. In patients with β-globin defects, it has been shown that natural variants that result in expression of fetal hemoglobin (HbF) prevent severe forms of the disease [72]. Through human genetics association studies, the transcriptional repressor BCL11A was found to block expression of HbF. Using a CRISPRn screen in an intron of BCL11A, an erythroid-specific enhancer region was identified [31].

Mutagenesis of the enhancer phenocopies knock-out of BCL11A and results in re-activation of HbF. For therapeutic gene editing, this erythroid-specific enhancer might be a preferred target since it only reduces BCL11A expression in erythroid lineages. A second study by the same group targeted a noncoding region surrounding *HBS1L-MYB*, which contains single-nucleotide polymorphisms associated with HbF levels and other red blood cell traits. They identified several regulatory elements in this region that control *MYB* expression, which also regulates HbF [33]. Taken together, these screens have identified several different regulatory elements that are essential to the expression of different forms of hemoglobin. For patients with hemoglobin diseases, these studies suggest specific noncoding targets for therapeutic gene editing and also specific regulatory genes that could be inhibited with small-molecule drugs.

Mitochondrial disorders encompass a set of diseases that stem from dysfunctions of the mitochondrial respiratory chain [73]. Over 150 genes have been identified in mitochondrial disease, making it the largest class of inborn errors of metabolism. Despite this genetic diversity, most of the current therapeutic strategies utilize broad vitamin supplementation with limited efficacy [74]. A genome-wide CRISPRn screen used death screening (actively selecting dead cells via Annexin V staining) to identify genes linked to mitochondrial disorders [75]. The study identified 191 genes that already known to play a role in oxidative phosphorylation as well as a handful of previously uncharacterized genes (*NGRN, RPUSD3, RPUSD4, TRUB2, WBSCR16, PYURF, METTL17, TMEM261, N6AMT1*) [75]. Other studies have focused on identifying specific targets in the oxidative phosphorylation pathway to find new therapeutic approaches. A genome-wide CRISPRn screen in a cell line where respiratory chain function was impaired (either by antimycin or pyruvate removal) identified the Von Hippel-Lindau (VHL) factor as a potential suppressor of mitochondrial disease. VHL was previously described as a key regulator of cellular hypoxic response, linking the hypoxia pathway with mitochondrial metabolism [76]. The protective effects of VHL knock-out was further validated *in vivo* in zebrafish. In a mouse model of Leigh syndrome, hypoxia treatment ameliorated a respiratory chain defect in which complex I is disrupted and extended lifespan by over threefold [74]. A separate study combined a chemical screen with a genome-wide CRISPRn screen to identify factors that could rescue defects in complex I of the mitochondrial respiratory chain. The chemical screen identified I-BET 525762 as a bromodomain protein inhibitor, and the CRISPRn screen revealed that the target of the inhibitor was the bromodomain containing protein 4 (BRD4) [77]. Ablating BRD4 increases oxidative phosphorylation and, here, the complementary drug screen provided additional support for this hit. Both screens suggest that inhibiting the activity of BRD4 might help the mitochondria compensate for defects in complex I. Overall these studies highlight the potential for new therapeutic approaches and demonstrate that mitochondrial disorders require treatments to be tailored for specific genetic lesions or specific impairments to respiratory chain complexes.

7.4 Conclusion and Future Perspectives

In order to develop new therapies for complex diseases, a key challenge is to identify genes and other functional elements in the genome involved in pathogenesis. With new targeted gene editing technologies, large-scale, pooled genetic screens in human cells are significantly easier than with alternative approaches (e.g. transposons, retroviral insertion, chemical mutagenesis). To date, most pooled screens have focused on probing one target per cell but future screens can take advantage of multiplexing to probe multiple genome targets in a combinatorial fashion. Combinatorial approaches can be useful in cancer and infectious disease in the context of synthetic lethality to identify optimal multi-drug cocktails, and also in inborn genetic disorders to identify background-specific modifiers for disease severity and therapeutic efficiency. With respect to precision medicine, future CRISPR screens could be performed in patient-derived cell lines to identify targets specific to the patient genetic background or to perturb specific gene variants.

In addition to gene targets, there is tremendous interest in understanding how noncoding regulatory regions influence gene expression, given that most common-disease-associated variants are in noncoding regions [78]. A key problem going forward for high-throughput pooled screens is to find screenable (cell autonomous) phenotypes for complex diseases. Traditionally, pooled screens have employed survival phenotypes (e.g. resistance to a drug or a pathogen) but many disease-relevant phenotypes are subtle or difficult to analyze in a pooled format. Despite these challenges, new advances in CRISPR pooled screening, such as recent work to combine pooled editing with single-cell readouts of RNA, DNA or genome state [79–83], deletions to perturb larger regions of the genome [30, 84], and new effector domains for manipulating epigenetic states [11, 27], will improve our understanding of the genetic basis of disease and help identify new therapeutic targets for treating these diseases.

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Chapter 8
CRISPR in the Retina: Evaluation of Future Potential

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Abstract Clustered regularly interspaced short palindromic repeats (CRISPR) has been gaining widespread attention for its ability for targeted genome surgery. In treating inherited retinal degenerations, gene therapies have had varied results; the ones effective in restoring eye sight are limited by transiency in its effect. Genome surgery, however, is a solution that could potentially provide the eye

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with permanent healthy cells. As retinal degenerations are irreversible and the retina has little regenerative potential, permanent healthy cells are vital for vision. Since the retina is anatomically accessible and capable of being monitored *in vivo*, the retina is a prime location for novel therapies. CRISPR technology can be used to make corrections directly *in vivo* as well as *ex vivo* of stem cells for transplantation. Current standard of care includes genetic testing for causative mutations in expectation of this potential. This chapter explores future potential and strategies for retinal degenerative disease correction via CRISPR and its limitations.

Keywords CRISPR • Genome surgery • Retinal degeneration

8.1 Introduction: CRISPR and the Retina

Treatment of inherited retinal degenerations has been elusive due to the retina's lack of regenerative potential. Once the retinal cells degenerate, photoreceptor death renders the eye irreversibly blind [1]. Thus, transplantation of retinal pigment epithelial (RPE) cells, in an attempt to replace the degenerate cells, has been the goal of many [2–8]. Transplantation though an attractive solution, has many considerations. Non-autologous transplantation requires lifelong immunosuppression [4, 7, 9]. In order to avoid rejection, research has moved toward utilizing autologous induced pluripotent stem cells (iPSCs), which has recently become possible [9–11]. However, the shortfall of this technique is that autologous iPSCs contain the same genetic error which caused the disease.

For this reason, there is great anticipation for the potential of the clustered regularly interspaced short palindromic repeats (CRISPR) system [12]. The CRISPR system, derived from bacterial immune system, has the ability to make precise modifications to the genome [13]. The RNA-guided endonuclease Cas9 of the CRISPR system is able to edit, activate, repress, and epigenetically modify DNA *in vivo* [13]. With CRISPR-Cas systems, the therapeutic potential of iPSCs have never been greater. Transplantation in combination with genome surgery could be the long-awaited cure for inherited retinal degenerations.

The retina is perhaps the best clinical research model for genome surgery. RPE cells are able to undergo *in vitro* treatments for genome surgery prior to transplantation, and do not make synaptic neuron connections to other retinal neurons; meaning simply survival in the proper location is sufficient for functionality [14]. The retina enjoys relative immune privilege from the blood-retina barrier and is easily accessible for monitoring without invasive techniques [15]. Though all stem cell transplantations carry the risk of teratoma formation, in such an event the eye can be removed without damaging other organ systems [15].

8.2 "Is There Anything You Can Do for Me?"

Perhaps what we hear the most from our patients with inherited retinal disorders is: "Is there anything you can do for me?" And perhaps it is this question that drives and motivates us the most to continue to search for new better treatment options; blindness or low vision impairs mobility and activities of daily living, and as a result has been linked to depression and anxiety—it is one of the most feared illnesses in America [16]. Unfortunately, with no proven cure, current standard of care for our patients can only be described as management. Patients are followed long-term in intervals of 6 months to a year to assess changes, direct patients to resources that improve use of residual vision, treat complications that arise, and continue genetic counseling.

In the past decade, a new component of the standard of care emerged: genetic testing. The Human Genome Project was completed only recently in 2003, cost 2.7 billion US dollars to complete over the course of 12.5 years; its completion signaled a new era of genetic medicine [17–19]. The completion of the Human Genome Project also brought acceleration of gene identification and faster more powerful genome sequencing tools [19–21]. Sequencing an individual patient's exome was unimaginable before; but now with next-generation sequencing technologies, it can be completed in a matter of months and at a thousandth of the cost from 10 years prior [20–23]. Diagnosis is no longer symptom-based alone but gene-based [24, 25]. Beyond the diagnosis, identifying the causal gene is the first step towards treatment [25–27]. Though all in the clinical trial phase and not without limitations, gene therapy can treat patients with recessive conditions or haploinsufficiencies by inserting therapeutic genes using viral vectors [1]. It is necessary to identify the faulty gene through genetic testing and understand disease etiology in order to determine whether gene therapy could be helpful.

We reported in January of 2017 that there are 31 gene therapy trials for retinal diseases. The diseases currently under trial are: Leber's congenital amaurosis, choroideremia, achromatopsia, X-linked retinoschisis, Leber hereditary optic neuropathy, retinits pigmentosa, Usher syndrome, Stargardt disease, and neovascular age-related macular degeneration [28]. Put into perspective however, there are over 250 retinal disease causing genes and loci that have been identified to date [29]. The diverse group of gene therapy trials have had varying degrees of success, some with the therapeutic effect limited by transiency [1, 30–36]. Which leads us back to CRISPR and its potentials: CRISPR genome surgery of iPSCs allows corrections for both dominant and recessive mutations; corrected iPSCs developed into RPE could be transplanted into retina giving the eye permanent healthy cells essentially curing blindness [1, 37, 38]. CRISPR currently has the greatest potential to change standard of care from management to treatment [1, 13, 28].

8.3 Research Highlights

Driven by a desire to improve patient care, applications of CRISPR is evolving at an accelerated rate [12, 13]. In January 2016, Bassuk et al. reported genetic repair of retinitis pigmentosa (RP) in patient-derived iPSCs [37]. The correction was made in skin-punch biopsy generated iPSCs [11, 39] from a patient with X-linked RP (XLRP) caused by a mutation in the retinitis pigmentosa GTPase regulator (RPGR) gene [37, 40]. Homology-directed gene repair (HDR) was completed at a correction rate of 13% in the ORF15 exon by transfecting the patient-derived iPSCs with CRISPR/Cas9 along with guide RNA (gRNA) g58 and RPGR anti-sense single-stranded donor oligonucleotide (ssODN) template [37]. Of note, the RPGR gene in the ORF15 region is a high GC content repeat-rich region spanning 59,000-bp [41]—homology-directed CRISPR repair is capable of precisely targeting DNA sequences that are challenging to manipulate [37]. But mostly importantly, Bassuk et al.'s work concretely demonstrates CRISPR/Cas9's capability to make precise correction in retinal degeneration causing mutations and supports the development of iPSC transplantation strategies for inherited retinal diseases [37].

Soon after in August 2016, Wu et al. achieved rescue of retinitis pigmentosa in a preclinical rodless (*rd1*) mouse model [42]. Wu et al. targeted the Y347X mutation in exon 7 of the *Pde6β* subunit on chromosome 5 in the *rd1* mouse using CRISPR-mediated HDR [42, 43]. CRISPR/Cas9 with single guide RNA (sgRNA) and ssODN donor template was injected into the pronuclei and cytoplasm of FVB/N (RP phenotype *rd1* mouse model) inbred zygotes [42]. Zygotes were carried to term; retina analysis via electroretinograms (ERGs), optical coherence tomography (OCT), and fundus autofluorescence (FAF) showed mosaic correction with dose-dependent rescue, dependent on percentage of cells corrected [42, 44–46]. At 35.7% and 18.8% correction, although not equal to wild-type, restoration of function was still achieved [42, 44, 45]. Most importantly, Wu et al.'s work is significant because CRISPR repair was achieved *in vivo*, affirming clinical applications. Further, though not in the retina, another *in vivo* model by Long et al. had genetically mosaic rescue (2–100% correction) with partial phenotypic rescue in mdx mice, Duchenne muscular dystrophy (DMD) model, with CRISPR/Cas9 mediated repair applied at the zygotic level carried to term [47]; Long et al. then applied CRISPR/Cas9 to postnatal mdx mice which also saw partial phenotypic rescue [48]. Long et al.'s mdx model suggests that postnatal CRISPR delivery could be developed into a protocol that can restore function in adults, which could be theoretically applied in the same manner in the retina [42].

8.3.1 Limitations and Imprecision Medicine

Although the potential to cure blindness through stem cells, especially in conjunction with CRISPR, has created much excitement and shows much promise [12, 13], limitations should be acknowledged. Especially as public expectations for stem

cells to be a "regenerative cure-all" has grown, exploitation of patients has also become a real concern [49, 50]. We reported recently a complication from such practice; a patient with slow progressive bilateral peripheral vision loss, expected to maintain useful central vision for many years, suddenly experienced severe vision loss from acute central retinal artery occlusion after unregulated autologous stem cell injection [51]. Autologous stem cell line does not mean lack of complications and their role in retinal degeneration still needs further investigation [52, 53].

Arguably, the biggest limitation of CRISPR-mediated genome surgery is also its biggest strength: precision. CRISPR/Cas9 is able to make precise corrections by homology between CRISPR RNA (crRNA) and target DNA strand [13, 54]. Use of experimentally verified single guide RNA (sgRNA) to direct Cas9 endonuclease to the target DNA strand is imperative to avoid off-target mutagenesis as well as achieve correction [40, 42, 55, 56]. With over 250 retinal dystrophy genes [29] and multiple disease-causing allele alterations for each condition, personalizing to each patient, identifying, targeting, and correcting each mutation may not be the most efficient endeavor [57]. For this reason, although further developments of CRISPR methods may create more realistic faster deliverable personalized cell therapy, and should absolutely be further studied, development of therapies that can be generalized to a wider patient group, i.e. Imprecision Medicine, should be developed in conjunction.

A potential way to approach the concept of Imprecision Medicine is addressed by Zhang et al. in their paper published on November 2016: "Reprogramming metabolism by targeting sirtuin 6 attenuates retinal degeneration" [58]. One mechanism by which retinitis pigmentosa (RP) induced rod cell death is theorized to occur is from defective metabolism [59, 60]. Photoreceptors convert 80–96% of glucose into lactic acid via aerobic glycolysis [59], and sirtuin 6 (SIRT6) is a transcriptional repressor of glycolytic enzymes [61]. Zhang et al. hypothesized that SIRT6 ablation in a preclinical RP model could rescue degeneration by pushing the cell's metabolism toward anabolism [58, 62]. SIRT6 ablation promoted photoreceptor survival slowed the rate of disease progression but did not completely halt cell death [58]. As metabolism is a complex process with multiple enzymatic pathways [59] and other mammalian sirtuins have been identified [61], the exact reason that SIRT6 ablation counteracted but could not stop disease progression is unclear [58]. Despite limitations, the finding alongside other studies [63, 64] does indicate the role for investigating metabolism reprogramming as a non-gene-specific rescue strategy [58].

8.3.2 Future Strategies

Needless to say, there is still much to be accomplished before curing blindness, whether it be precise or imprecise medicine, becomes a realization. One of the most important roles that CRISPR has in this process is acceleration of the generation of animal models and untangling the effects of genetic differences [13, 42, 65, 66]. Adding to this functionality of CRISPR is the development of CRISPR-X which has the ability for directed evolution via targeted hypermutation adding to the diversity

of preclinical model library [67]. CRISPR-X harnesses the antibody maturation process which creates point mutations in immunoglobin (Ig) regions through somatic hypermutation (SHM); SHM in the antibody maturation process is mediated by activation-induced cytidine deaminase (AID) which initiates DNA repair response that causes errors in the Ig locus generating point mutations to create diverse immunoglobulins [68–70]. In the CRISPR-X system, catalytically dead Cas9 (dCas9) targets AID with guide RNA to induce localized diverse point mutations; CRISPR-X creates mutations at rate of ~1/2000 bp^{-1}, similar to that of SHM [67, 69]. Adding to the currently existing CRISPR recombination, insertion, or deletion strategies [71, 72], CRISPR-X's ability to create point mutations allows even greater diversity and further investigation of RNA and protein function [67]. This technique could prove instrumental in evaluating the vast array of retinal dystrophy mutations *in vivo* [29].

Continual development of genetic engineering techniques has changed the way we view inherited retinal degenerations. When a mere 20 years ago acceptance of a lack of a cure was the only option, inherited retinal degenerations can now be approached with growing optimism and hope for treatment even despite limitations. We expect to see continued accelerated development for treatment in the upcoming years.

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Chapter 9
The Future of CRISPR Applications in the Lab, the Clinic and Society

Soren H. Hough and Ayokunmi Ajetunmobi

Abstract CRISPR (clustered regularly interspaced short palindromic repeats) has emerged as one of the premiere biological tools of the century. Even more so than older genome editing techniques such as TALENs and ZFNs, CRISPR provides speed and ease-of-use heretofore unheard of in agriculture, the environment and human health. The ability to map the function of virtually every component of the genome in a scalable, multiplexed manner is unprecedented. Once those regions have been explored, CRISPR also presents an opportunity to take advantage of endogenous cellular repair pathways to change and precisely edit the genome [1–3]. In the case of human health, CRISPR operates as both a tool of discovery and a solution to fundamental problems behind disease and undesirable mutations.

Keywords CRISPR • Bioethics • Biodiversity • Agriculture • Medicine • Next-generation sequencing • Germline • Regulation

CRISPR (clustered regularly interspaced short palindromic repeats) has emerged as one of the premiere biological tools of the century. Even more so than older genome editing techniques such as TALENs and ZFNs, CRISPR provides speed and ease-of-use heretofore unheard of in agriculture, the environment and human health. The ability to map the function of virtually every component of the genome in a scalable, multiplexed manner is unprecedented. Once those regions have been explored, CRISPR also presents an opportunity to take advantage of endogenous cellular

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repair pathways to change and precisely edit the genome [1–3]. In the case of human health, CRISPR operates as both a tool of discovery and a solution to fundamental problems behind disease and undesirable mutations.

CRISPR arrived at just the right time. Following the conclusion of the Human Genome Project in 2003 [4], interest in genomics exploded. The cost of genomic sequencing has dropped precipitously to an affordable consumer-level rate [5]. Major national initiatives have begun in countries across the world to sequence their populations. These programs aim to provide better understanding of the variation and subpopulations of genetic disposition, particularly as it relates to clinical information.

The advent of CRISPR in 2013 coincides with this flood of new sequencing data. Researchers quickly realized that genomic information needs to be better understood before it can be used to drive breakthrough clinical advances. Until just a few years ago, most (98%) of the human genome [6] was considered "junk DNA" because it does not code for protein. As researchers have since learned, these regions are not only highly important for regulating coding regions of DNA [7], but are also a source of tremendous variability between both species [8] and individuals within a species [9].

Older approaches to large scale genetic interrogation were either difficult to develop, such as TALENs and ZFNs, or unable to target the noncoding (untranscribed) genome, as is the case with RNA interference (RNAi) [10]. These limitations sufficiently stymied the potential for robust clinical application, although there has been some success using the aforementioned techniques [11–14].

CRISPR, on the other hand, seems to show significant promise for both in vitro and *in vivo* genome editing due to its cost effectiveness and relative simplicity. Advances in CRISPR research have illuminated myriad disease pathways. Basic and therapeutic research subjects thus far include liver cancer [15, 16], lung cancer [17], neurodegenerative diseases [18], heart disease [19], immune pathways [20] and more [21, 22].

As CRISPR begins to move toward the clinic, serious questions have begun to emerge around the safety and ethical considerations of genome editing. The ubiquity and seemingly endless potential of the new technique are simultaneously exciting and worrisome for researchers, bioethicists and the public around the world. Experts have maintained an ongoing discussion on how best to address the debate around genome editing technology.

These concerns are wide-ranging. Some are concerned with the moral implications of editing both humans and nonhuman animals for clinical and nonclinical purposes. Others have discussed the ramifications of permanently changing the trajectory of genetic evolution across species. Still more are worried about the potential for genomic editing to go awry, leading to unintended genetic manipulation with potentially debilitating or fatal consequences. Navigating these concerns will require a parsing of terms, a clear understanding of the benefits and risks of genome editing per the limits of modern technology, a broad effort to educate the public and legislators and an open conversation between biologists, bioethicists and the public.

9.1 The Scientific Community Debates Somatic and Germline Genome Modification

Government regulators [23–26] and many scientists and ethicists [27, 28] believe the primary concern in genome editing comes in the distinction between somatic and germline cell editing. In general, government bodies have until recently been reluctant to give approval to genome editing therapeutics that target germline cells. This is due to a general aversion to editing DNA which can be passed onto offspring; ostensibly, somatic cells cannot pass on their genetics to subsequent generations whereas germ cell modifications are heritable.

Some question the value of editing the germ line at all. Specifically, a 2015 editorial by Lanphier et al. entitled "Don't edit the human germ line" asserts that the risks associated with germline editing are too high to warrant consideration. Current methods of evaluating the downstream effects of genome editing on the individual and population scale remain unclear. The authors suggest ethical concerns are a significant barrier to this type of genome modification, but even that debate cannot be addressed until safety has been assured "over multiple generations" [28].

The authors in [28] also state they "cannot imagine a situation in which [CRISPR's] use in human embryos would offer a therapeutic benefit over existing and developing methods" [28]. In response, geneticist George Church, Ph.D., stated that alternative methods such as pre-implantation genetic diagnosis are in fact insufficient; researchers are finding that the interconnectedness of genetic pathways mean that in vitro fertilization (IVF) clinics have to discard most embryos. Genome editing could, conceivably, alleviate this inefficiency. Still, Dr. Church feels a moratorium on germline editing should halt research in this area until consensus in the community has been reached [29].

A separate report by Baltimore et al. in 2015, co-authored by prominent genetic researchers and pioneers, further emphasized reluctance to edit the germ line. The piece tentatively states that more research in the "efficacy and specificity" of CRISPR would be a crucial first step in deciding whether future germline applications of genome editing would be warranted. Still, the authors worry that going down this path is a "slippery slope" toward non-therapeutic applications of the technology. In their conclusions, they "strongly discourage" germline editing around the world regardless of whether individual countries permit such work [27].

Baltimore et al. also speak to safety concerns of genome editing and express worry that unintended off-target effects may have an impact on the environment. The authors go further to state that targeting one gene may not have straightforward consequences as many genetic pathway relationships remain ambiguous. They assert that there is a clear need to standardize detection methods for off-target editing events as the field moves toward practical use of CRISPR outside of basic science [27]. A report by the Nuffield Council on Bioethics reiterates the need for more research on CRISPR safety, stating that "efficacy of the genome editing technique has not been demonstrated sufficiently through research" [30].

Francis S. Collins, M.D., Ph.D., current director of the United States National Institutes of Health, released a statement on the use of CRISPR in human embryos. He stated that the NIH is hesitant to permit this kind of research or therapeutic development because it would modify future generations "without their consent." He goes further in his letter to say that there is "a current lack of compelling medical applications justifying the use of CRISPR/Cas9 in embryos" [23].

Dr. Collins cited the Dickey-Wicker amendment to state that clinical research in human embryos faces tough regulation. He also pointed to the NIH's Recombinant DNA Advisory Committee which refuses to approve research proposals which would edit the human genome in germline cells [23]. Indeed, the direct quote from the April 2016 report states categorically that the government will not "entertain proposals for germ line alterations but will consider proposals involving somatic cell gene transfer" [25].

Hesitating to edit the human germ line is not new. A 1982 statement from the President's Commission under the Carter administration, "Splicing Life," discusses the "serious ethical concerns" of editing human embryos. The report suggests that eliminating "bad traits" from the population is a form of eugenics. The Commission points to another 1982 report by the Council of Europe Parliamentary Assembly which outlines the human "right to a genetic inheritance which has not been interfered with, except in accordance with certain principles which are recognized as being fully compatible with respect for human rights" [26]. Once again, concern for the consent of future generations is paramount.

"Splicing Life" goes on to outline the safety concerns associated with gene therapy. Medicine is designed and approved with a consideration for the risk-to-benefit ratio of the therapeutic. In the case of genome editing, CRISPR included, there is always potential for unintended off-target editing events—a subject which will be discussed later. As the report points out, if there is a limited chance of inheritance of a given genetic disorder, it may not be worth the risk to edit the embryo. It is because of these "technical uncertainties" and "ethical implications" that the report suggests there "are strong contraindications against therapy of fertilized eggs or embryos" [26]. This sentiment was echoed in 1997 when UNESCO released the "Universal Declaration on the Human Genome and Human Rights: From Theory to Practice," stating that germline modification "could be contrary to human dignity" [31].

Twenty years following UNESCO's declaration, the Food and Drug Administration released a statement addressing the regulation of genome edited food and drugs. The letter was written by the Commissioner of the FDA, Robert Califf, M.D., and a senior policy advisor in the FDA's Office of Policy, Ritu Nalubola, Ph.D. In this address, they confirm that all regulation of CRISPR and related genome editing technology in humans pertains solely to somatic cells [32]. They suggest that human germline edits are expressly forbidden by H.R.3049 (Agriculture, Rural Development, Food and Drug Administration, and Related Agencies Appropriations Act 2016). This bill states that funds may not be used to approve or evaluate proposals for "research in which a human embryo is intentionally created or modified to include a heritable genetic modification" [33].

The FDA statement goes on to describe any portion of an animal's DNA that is modified through some form of recombinant or genome editing technology as a "drug." Therefore, these modified sequences fall under the purview of FDA regulation [32]. This raises significant questions as researchers explore not only editing the DNA of livestock, but using them as a means to grow human cells and organs as well.

Unsurprisingly, emerging applications of genome editing are moving quicker than regulations. A 2017 study by Wu et al. demonstrates the successful use of CRISPR to generate interspecies mammalian chimeras. Mice were injected with pluripotent stem cells from rats, while pigs and cattle were injected with human iPSCs [34]. The study builds on previous work which demonstrated similar results in primate-mouse [35] and human-mouse [36] chimeras. In the future, this kind of research may allow researchers to grow human organs in closely related animals for use in transplantation and other medical applications. Where these modified organisms would fall under current FDA regulations remains to be seen.

Deciding which genetic disorders should be treated using germline or somatic genome editing raises additional worries. Bioethicist Silvia Camporesi, Ph.D., discussed the activism of various communities at a 2016 talk, "CRISPR Genome Editing Technologies: Bioethics and Biopolitics," at Santa Clara Univeristy. As an example, she pointed to some in the UK autism, deaf and Down's syndrome communities who worry about preserving their culture and identities as they face the prospect of genome editing. She says some have also argued they "don't want to live in a society that gets rid of people with those traits" [37].

These arguments are extensions of older debates within these groups. For example, the deaf community has hotly debated the merits of procedures like cochlear implants, suggesting their deafness is not something needs to be "fixed" [38]. According to Dr. Camporesi, leaders in these activist communities are being included in the discussion around prenatal genetic screening and, in tangent, genome editing [37].

9.2 The Somatic-Germ Line Barrier

Although government regulators have drawn hard distinctions between germline and somatic cells for decades, these categories are not so easily delineated in biology. Dr. Camporesi points out that somatic cells have been used to generate stem cells, termed induced pluripotent stem cells (iPSCs). These undifferentiated lines have enormous therapeutic potential, particularly in conjunction with ex vivo CRISPR procedures [37], some of which are already entering clinical space [39].

In one 2015 study, CRISPR-edited patient-derived iPSCs were shown to effectively treat hemophilia A in mice following transplantation [1]. However, Dr. Camporesi notes that iPSCs have also been developed into healthy mouse embryos, a point reflected in the 2016 Cambridge Public Policy Strategic Research Initiative

(SRI) report, “The Future of Gene Editing.” These mouse embryos featured iPSCs in every tissue including germline cells [40]. Thus, if the iPSCs are edited before developing into embryos, those modifications may also carry over across all differentiated tissue types.

A 2017 paper by Cohen et al. further discussed the potential of in vitro gametogenesis. In this case, iPSCs were derived from skin cells and then differentiated into eggs [41]. This presents an unprecedented opportunity for IVF to provide access to women beyond the limits of their natural egg production. On the other hand, this once again confuses the line between somatic (skin) and germline (egg) cells. Editing either skin or iPSCs prior to differentiation would arguably generate the same effect as editing the germ line directly.

Epigenetic modification of the genome remains a new and understudied phenomenon that further confuses the barrier between germline and somatic editing [40]. Nevertheless, current studies suggest that there may be multigenerational inheritance of epigenetic traits [42]. If true, then humanity may already be affecting future generations without consent through multiple means of inheritance such as smoking [43] and pollution [44].

Regulators currently use somatic and germline categories as if they are rigidly agreed upon. The rate of technological innovation and biological understanding moves faster than legislation, and older terms do not always apply to a rapidly changing scientific landscape. As an example, “genetically modified organism” or “GMO” refers specifically to transgenic species—that is, species modified with foreign DNA. Therefore, simple CRISPR edits (genomic deletions) do not officially fall under the auspices of GMO regulation.

Some groups, such as Dupont Pioneer, have made use of this flexible terminology to bypass the confines of the United States Department of Agriculture (USDA) [45]. In a similar fashion, the same loopholes may develop for somatic and germline editing. Dr. Camporesi therefore addresses the need to more rigorously define these terms as the limits of genomic editing research are established [37].

Although many governmental organizations have called for a moratorium on germline editing, experts suggest that opinion may continue to shift in coming years. Biomedical ethicist Tim Lewins, Ph.D., captured this in his 2015 essay, saying, “Every month the ethics of the germline become more blurry.” He goes on to say that “if the evaluation of risk is equally favourable there will be pressure to legalise other forms of germline intervention—including genome editing techniques—in the near future” [46].

Taking a more direct stance, ethicist Julian Savulescu, Ph.D., and colleagues makes the case for editing the germ line. They state that arguments for banning germline application of CRISPR modification for the sake of future offspring are illogical. Savulescu et al. state that many technologies we use today that could impact future generations already, both within the biomedical world and outside of it, and that the potential benefits to genome editing make the research by Liang and Kang et al. a “moral imperative” [47].

In a 2015 joint report in the United Kingdom by the Academy of Medical Sciences, the Wellcome Trust and other major research organizations and charities, the authors leave the door open on germline edits. They state "We also recognise … that there may be future potential to apply genome editing in a clinical context using human germ cells or embryos." As a caveat, they point out that legal barriers would prevent any work in the immediate term [48].

A 2017 report from the National Academies of Sciences, Engineering and Medicine in the United States took a similar stance. The Academies state that while caution should be used around germline editing for therapeutic use, that "does not mean prohibition." They recommend that the issue be addressed once researchers have compellingly investigated the risk/benefit of implementing these technologies in the clinic [49].

The Academies go further to set specific standards for germline therapeutic use. They recommend this approach only be considered in scenarios where no other option is available and where the disease in question has been well-studied and linked to the disease phenotype. This should be followed up with multigenerational monitoring and "rigorous oversight" [49].

Part of the urgency for more conversation on genome editing of somatic and germline cells comes in the wake of a 2015 paper detailing the first ever use of CRISPR in human embryos. The paper, written by Liang et al., used nonviable embryos to demonstrate the utility and specificity of CRISPR in an *in vivo* human model [50]. The paper has since been criticized for not bringing novel insight to the scientific community despite the ethical dubiousness of its premise [51].

A subsequent paper in 2016 was the second demonstration of CRISPR use in humans. There, Kang et al. showed successful use of precise homology-dependent editing to introduce the *CCR5Δ32* allele into nonviable tripronuclear embryos. Despite their work, the authors still caution further research in human germline editing until "after a rigorous and thorough evaluation and discussion are undertaken by the global research and ethics communities" [52].

In 2017, Tang et al. took the unprecedented step of editing normal human embryos with CRISPR-Cas9. The authors once again used homology-dependent repair pathways to correct mutant alleles in *Gpd6* and *HBB* by delivering complexed ribonucleoprotein to the cells. Tang et al. indicated that they did not find convincing off-target editing events using whole-genome sequencing, but that there was notable mosaicism in at least one edited embryo. In their conclusion, the authors indicate that therapeutic use of CRISPR is "not a current option" due to "safety, mosaicism, and other factors" [53].

This was followed by a paper by Ma et al. in 2017 where CRISPR was once again used to edit viable human embryos. The authors corrected a mutant allele associated with hypertrophic cardiomyopathy and, importantly, avoided mosaicism by editing the embryos just after fertilization. Ma et al. found that based on their data, there is a case to be made for germline-level editing of heritable pathogenic mutations as they did in *MYBPC3*. However, as with other studies involving CRISPR and human embryos, the research team cautions against moving toward the clinic without further study [54].

9.3 A New Paradigm for Genomic Medicine

Leaving the germline debate to one side, CRISPR shows immense potential in medical research. The low cost and potentially one-time treatment options of CRISPR make it an appealing tool for researchers and clinicians. The advent of this genome editing technology promises an efficient path beyond the symptoms toward treating the root cause of disease. CRISPR could serve as a relief to millions seeking an affordable, single-use alternative to current pharmaceutical options around the world.

The first CRISPR-based therapy entered human trials in China [55]. Meanwhile, several more programs are progressing into Phase I clinical trials internationally [56]. The excitement of clinical applications of CRISPR is in part driven by the numerous cellular and animal studies demonstrating their powerful clinical utility [57].

For example, in an in vitro CRISPR study, researchers edited the cystic fibrosis transmembrane regulator (*CFTR*) gene in patient-derived iPSCs. This corrected the mutation in the iPSC population which then differentiated into healthy mature epithelial cells [58]. Another investigation illustrated similar curative potential in Fanconi anemia where gene-correction was observed in patient-derived fibroblasts [59].

A breakthrough animal study by Yin and Xue et al. in 2014 used CRISPR genome editing to treat disease for the first time in an adult animal. In their paper, CRISPR was delivered to adult mice affected by hereditary fumarylacetoacetate hydrolase (*Fah*)-related tyrosinemia type 1. Excitingly, the mice demonstrated a curative phenotype despite only 6% gene correction in hepatocytes [3].

Further illustrations of CRISPR-based therapies include a series of studies by three separate groups showing successful correction of a faulty dystrophin exon using CRISPR generating functional protein [60–62]. In another study, a group administered CRISPR as a single subretinal injection in rat models of severe autosomal dominant retinitis pigmentosa. Selective disruption of the rhodopsin gene prevented retinal degeneration and improved visual function [63].

These outcomes are accelerating CRISPR into the clinic, driven by companies with ambitions to provide mainstream cell and gene therapies [64]. CRISPR approaches include the use of engineered chimeric antigen receptor T (CAR T) cell therapy to target liquid tumors [39]. In these *ex vivo* studies, immune cells are edited with CRISPR and introduced into the patient. Ex vivo editing provides a more controlled environment to introducing editing agents for *in vivo* applications as the edited cells can be analyzed and characterized before patient delivery.

9.4 Converging Technologies to Democratize CRISPR

Beyond CRISPR-based medication, the progress of technology also suggests significant steps forward in genomic medicine. Specifically, whole genome sequencing (WGS) is decreasing in cost by the year. In a 2016 statement, the United States National Human Genome Research Institute reported that the cost per

genome is decreasing at a much faster rate than Moore's Law predicts. In 1 year alone, WGS dropped from 4000 dollars in mid-2015 to 1500 dollars in late 2015 [5]. In 2017, Illumina announced plans for an individual sequencing service that would cost just 100 dollars per person [65]. This price, which is bound to drop even further, presents access to personal clinically-relevant data to individuals around the world.

The reduction in sequencing costs dovetails with national initiatives to sequence the population for the purposes of clinical diagnosis, research and medical care. The 100,000 Genomes project in England is already well underway under the auspices of Genomics England [66]. The Precision Medicine Initiative in the United States, announced in 2015, will sequence one million individuals in accordance with recommendations from the National Research Council [67, 68].

In Ireland, Genomics Medicine Ireland is looking to sequence 45,000 people [69], while France plans to sequence 235,000 genomes per year [70]. The Beijing Genomics Institute (BGI) in China has ambitions across species; they plan to sequence one million humans, one million plants/animals and one million microbial ecosystems [71]. These programs will give more citizens direct access to their own medical data, medical professionals more precise information about their patients and the research community a better understanding of clear risk-associated alleles and variants across the population.

This influx of data will provide medical professionals with unprecedented understanding of patient backgrounds on a personal scale, a necessary step as CRISPR moves toward the clinic [72]. However, to best utilize this information, scientists will need to employ CRISPR both as a basic research tool (to functionally interrogate population-specific alleles) and as a therapeutic tool to edit and treat those suffering from genetic diseases. CRISPR may even serve as a means to preemptively treat at-risk populations for known disease-causing variants as determined by genome-wide association studies [27].

One paper by Stuart Orkin, MD, discusses the difficulty in treating hemoglobin disorders, such as β-thalassemias and sickle cell disease, in developing countries. He states that invasive treatments like bone marrow transplantation have been successful, but access to that kind of care can be difficult "where medical resources are limited." He therefore points to novel therapeutic approaches such as CRISPR as potential solutions to these disorders, a threat which he calls "global in scope." Ultimately, he believes this will provide access to those with "meager or modest medical resources" and thereby realize "the full promise of molecular medicine" [73].

It is worth noting that cost and simplicity are not the only factors affecting therapeutic democratization. Governmental bodies around the world can also stifle access to major therapeutic advances [74]. As Dr. Camporesi noted in her talk, some countries banned germline editing a decade before it was feasible, and since then, 40 countries have expressly forbidden or discouraged the practice. At present, 15 of 22 nations in Western Europe have instituted legal bans on germline editing altogether [37]. It is not infeasible to predict that some countries, for religious, ethical or other reasons, may choose to restrict access even to somatic genome editing, thereby reducing or eliminating the spread of CRISPR as a therapeutic option in that nation.

The potential for CRISPR to treat currently incurable diseases has captured the imagination of the scientific community. Despite these advances, drawbacks to CRISPR intervention remain, including many unanswered questions around safety [75]. The challenge for genome editing therapies is to maintain high efficiency to produce a phenotypic outcome while ensuring that other edits are not unintentionally introduced elsewhere. The specter of off-target editing events arises from sequence similarity between the target site and other regions in the genome [76]. Unintended editing events at these off-target loci may cause mutations that lead to harmful phenotypes such as tumorigenesis or cell death.

As such, genome editing assessment standards require continuous evaluation. Inadequately addressing safety concerns could lead to unintended impacts on current and, potentially, future generations. In their 2015 paper, Baltimore et al. stated this standardization was a "critical" step forward; they outline the need for consistent "benchmarking methods to determine the frequency of off-target effects" and protocols to "assess the physiology of cells and tissues that have undergone genome editing" [27]. Several sequencing methods have emerged to evaluate genome editing outcomes but no single approach has been agreed upon.

While engineered "designer-babies" may dominate the headlines, it misses the most immediate concerns pertaining to genome editing applications. The pressing dangers lie in the unintended consequences of accelerating the clinical applications of genome editing technologies without stringent sequence-level evaluation. As stated by fertility specialist Eric Widra, M.D., in a collection of brief essays, moving hastily in the name of "progress, promise or profit" can lead to causing "harm rather than benefit, and in doing so, prematurely discard a potentially revolutionary technology out of fear and impatience" [77].

9.5 The Impact of CRISPR on Human Biodiversity

Although immediate attention has focused on establishing the safety of CRISPR in cures for existing disorders, some researchers are exploring the potential for CRISPR as a preventative therapy. For example, naturally occurring loss-of-function mutations in *PCSK9*, found in 3% of the human population, not only reduce cholesterol levels in blood but also the risk of myocardial infarction [78]. This variant appears to come with no adverse consequences and has led some researchers to suggest CRISPR as a single shot therapy to reduce blood cholesterol permanently. Indeed, clinical work with RNAi targeting of *PCSK9* is already underway [11].

In one study, high-efficiency editing was observed in adenovirus-delivered CRISPR targeting the *Pcsk9* gene in mouse livers. Disruption of *Pcsk9* alleles in the liver resulted in a 90% reduction of circulating Pcsk9 protein and a 40% reduction in cholesterol levels [79]. A subsequent study was carried out in "humanized" mouse models whereby endogenous mouse hepatocytes were replaced with transplanted human hepatocytes. In this scenario, CRISPR delivery by adeno-associated virus (AAV) resulted in a 50% reduction in circulating human PCSK9 protein levels [80].

These remarkable observations lend credence to the use of CRISPR to administer protective genotypes as preventative measures against complex disorders like cardiovascular disease [81], the leading cause of death in developed countries and a health burden on developing countries [82]. As previously discussed, there is reticence within the scientific community to engage in germline genome editing. In contrast, genetic enhancement to prevent disease or illness in adult genomes remains a possibility [49].

In a 2015 interview, Dr. George Church stated "in addition to common variants of small impact and rare deleterious variants, there are rare protective gene variants of large impact" on human health. He mentions examples of ten genetic enhancements that may be desirable in the wider human population. These include mutations conferring protections against viral infection, cancer and diabetes, as well as more cosmetic changes such as stronger bones, leaner muscles and low odor production [83].

These protective enhancements may initially focus on improving patient health and relieving disease burden. However, the line between therapeutic and cosmetic is easily blurred, as seen in other clinical domains such as cosmetic surgery [84]. While natural selection is predominantly driven by environmental factors, it is conceivable that genome editing could be used to meet nonclinical societal pressures.

As CRISPR use becomes democratized, genome editing for human enhancement may be seen as an inevitable next step in the progression of the technology. One ethical consideration is the consent of minors as parents decide on their children's future for reasons unrelated to health. Another concern lies in the potential for genome editing to further exacerbate inequality as socioeconomic restrictions to genetic enhancements provide further advantage to select members of the global population.

A major issue of contention is the impact of reducing diversity within human population as referenced by Dr. Camporesi. This point is powerfully highlighted in conversations on disability rights. As discussed during the National Convening on Disability Rights and Genetic Technologies, many cautioned that the increasing use of genetic technologies reflects and reinforces societal assumptions that disability is always harmful and should be prevented [85].

Biochemist and disability scholar Gregor Wolbring, Ph.D., speaking at the 2015 International Summit on Human Gene Editing, expressed the history of disagreement between scientific and clinical experts and the disability-rights community over their perception of people with disabilities. He argues that ableism, a view that disability is an abnormality instead of a feature of human diversity, can lead to flawed solutions and disempower those affected [86].

Sociologist Ruha Benjamin, Ph.D., outlines that it is necessary to involve the insights and expertise of those who stand to be most impacted by the pressures of imposed societal norms. She writes that they "offer us a more rigorous foundation by which to democratize science than the current model in which citizens are imagined to be 'We, the patients' waiting for the fruits of science to ripen." Further, she implores the scientific community to acknowledge a need to be as inventive in "addressing social complexity as we are about biological complexity" [87].

9.6 Editing the Ecosystem

The possible impact of CRISPR on biodiversity is not limited to the human genome. In attempts to ease the burden of infectious diseases and limited food sources on society, the concept of engineering ecosystems is gathering steam. Crop genome editing [88], livestock genome editing [89] and gene drive technologies [90, 91] explicitly present the case for re-engineering a species to better human lives. In each of these cases, humans systematically modify the genome of a population allowing desirable variants to become dominantly expressed within the ecosystem.

The first iteration of crop and livestock genome editing, collectively referred to as GMOs, were created by randomly inserting cloned genes into the host using a bacterial vector. This approach often required screening multiple crosses to identify breeds with the desired traits. Conversely, CRISPR enables a targeted and specific approach, drastically reducing the time required to introduce the desired mutation [30].

The regulatory landscape of genome edited agriculture remains unclear [92]. Most jurisdictions define GMOs as the introduction of DNA which may not pertain to certain types of editing such as knockout mutations. In contrast to the FDA report in 2017, the USDA has ruled that certain uses of CRISPR technology will not be regulated as GMOs [45]. There are still outstanding questions about genome modification in plants with regard to the environment, including the potential for "plant pest or noxious weed risks" [32].

In most cases, CRISPR editing of livestock in the United States falls under the same regulatory provisions as apply to plants. Examples of genome editing use in livestock include increased muscle mass in sheep [93] and pigs [94] and the creation of tuberculosis-resistant cattle [95]. The first FDA-approved genome-edited animal intended for human consumption, an Atlantic salmon (*Salmo salar*) modified with sequences from Chinook salmon (*Oncorhynchus tshawytscha*), was approved in 2015. This only came after 20 years and over 50 studies proving its safety as a food; the transgenic salmon still had not made it to market as of January 2017 due to further deliberation on labeling [96].

Researchers have suggested CRISPR as a means of increasing agricultural yield to meet global requirements for food production. Yet a major caveat to this approach is the potential of highly engineered homogeneity within a narrow ecological niche being unable to respond to sudden environmental variation [30]. Other impediments include safety concerns and a poorly informed citizenry which could lead to negative public sentiment.

Negative public sentiment may also influence the adoption gene drive technologies. Gene drives are a species-specific and potentially cost-effective means of controlling the population of disease carrying insects such as mosquitoes [97, 98]. Controlled field tests evaluating the effectiveness of such approaches have already received the approval of the U.S. Academies of Sciences [99]. According to Jonathan Pugh, Ph.D., objections to pursuing this line of research center on two main ethical ideologies, "sanctity of life" and "playing God" [100].

The "sanctity of life" principle argues the intrinsic wrongness of inducing the extinction of another species. This idea conflicts with vaccination programs in the twentieth century which led to the extinction of the variola species of virus responsible for smallpox, widely heralded as one of triumphs of modern medicine. Alternatively, the "playing God" principle criticizes the hubris of humanity in exerting dominion over nature [100].

The challenge of greenlighting gene drive technology lies in the unknown ecological consequences of these actions; opinion on the impact of mosquito eradication on the environment remains divided [101]. Some scientists argue that mosquitoes are a vital food source and pollinator while others claim they have negligible involvement in the ecosystem. Some even question whether gene drives could achieve their intended goal at all. In one study, the authors suggest gene drives may have a limited impact due to potential resistance alleles becoming dominant in the population [102]. This finding since been reported again in other work [103]. Gene drive technology therefore still needs to be refined before ethical questions can be raised.

The philosophical and technical uncertainty around gene drives has presented a challenge to governmental regulators. Kenneth Oye, Ph.D., a political scientist at MIT, and co-authors argue that more action is needed to address the potential unexpected and undesirable outcomes of such a drastic assault on the ecosystem [104]. As researchers and government officials continue to debate this issue, the pace of regulatory oversight may be outstripped by the fervor surrounding its application.

The impact of new technologies is always difficult to assess a priori. Gene drives and agricultural engineering have the potential to alleviate significant human burden. In Florida in the United States, genetically modified mosquitoes have already come up for referendum as a solution to the Zika virus crisis [105]. Reasoned debate and inclusive engagement with stakeholders are pivotal to making informed decisions. This is a key step in addressing scientific progress that may have profound effects on our environment.

9.7 CRISPR Calls for a Conversation

A common theme among statements from researchers, regulators and ethicists is the need for an open discourse on how best to proceed with CRISPR technology. The FDA statement by Drs. Califf and Nalubola suggests a "broader, inclusive public discussion involving multiple constituencies (e.g., scientists, developers, bioethicists, and public interest and community groups)" [32]. Jennifer Doudna, Ph.D., one of the co-discoverers of CRISPR, has similarly recommended improving the dialogue between scientists, the media and the public [106].

Dr. Camporesi makes the case that the conversation around mitochondrial DNA transfer (MDT), a recently litigated and approved embryo editing technique in the United Kingdom, provides a good framework for how these discussions can take

place [37, 107]. This was corroborated in the report by Lanphier et al., which stated that the MDT case set an "excellent precedent" by "involving scientists, bioethicists, regulators and the general public" [28]. This framework is echoed by Savulescu et al. who similarly called for an "ethical debate" over the future of genome editing [47]. The National Academies of Science, Engineering and Medicine similarly recommend ongoing public discourse as questions around genome editing continue to arise, whether that be around germline editing or genetic enhancement [49].

In the assessment by Baltimore et al., the authors concluded that the key to moving forward with genome editing policy is "public trust in science" which "ultimately begins with and requires ongoing transparency and open discussion" [27]. Dr. Baltimore and co-authors then outline how they envision these discussions taking place. First, they point to forums where bioethicists will provide "information and education" about advances in genome editing. They also highlight the need for a diverse set of voices when they suggest a "globally representative" group of experts work with "the public and governmental agencies" to help navigate the ethical and moral issues that may arise as science progresses [27].

The chapter authors also believe science outreach to be the most appropriate way to accommodate a rapidly changing landscape in biomedicine, agriculture and genetics. We believe there is a clear need to improve the interface between the scientific community and the public. This begins with education, which, in addition to genomics and precision medicine initiatives, needs to be invested in by governmental and non-governmental bodies.

The only way genome editing will earn broad acceptance is if the public is invited along in a transparent, inclusive manner. It is important to note that the public in this case extends not only to the citizens of a given country but to the whole world. The ramifications of editing the genome in any one population, human or otherwise, may be transnational. International collaboration on science-based efforts have already borne fruit and set some precedent for similar initiatives moving forward [108].

Moreover, historical precedent with regard to selective genetics and medical experimentation suggests that it is often the most vulnerable who suffer [109]. CRISPR may have the potential to revolutionize health for all people, but it can also be used to only benefit a select few. Therefore, we reiterate the suggestion made by Baltimore et al. that this must be a globally representative body of experts and laypeople of varying socioeconomic backgrounds, ethnicities, beliefs, abilities and other identities.

A successful interface with the public will also require scientists to be upfront and clear about the risks and benefits of genome editing technology. This means a standardized, clearly delineated set of guidelines for evaluating the physiological consequences to editing the genome. Once researchers have agreed on these standards, they can more clearly present their findings both within the scientific community and beyond.

The next step is communicating this data clearly to the public. This means reducing the barriers to the literature, perhaps by eliminating paywalls and offering more

direct access to the science as it has been reported by researchers in peer-reviewed journals [110–112]. The more access the public has to this material, the more familiar they can become with the structure, language and fundamentals of scientific literature and study. This is particularly true in developing nations where institutional subscriptions may be harder to come by for academics and laypeople alike [113]. Literature access is also key for physicians and other experts who directly interact with the public and rely on the literature to keep up to date with the latest in their respective fields [114].

Beyond public forums, we also recommend the government and non-governmental organizations invest in training science communicators to bridge the gap between the lab and the community. Currently, too few scientists or science-literate persons are given a clear path toward science outreach. Celebrity science advocates and academics, such as Neil DeGrasse-Tyson, Ph.D., and Bill Nye, have been successful in popularizing core scientific concepts to a broad audience. Others have called for scientists themselves to receive outreach training as a part of their undergraduate or graduate degrees [115, 116]. Despite these efforts, there is still a clear need for a larger contingency of science communicators to engage with the public on these topics.

This is particularly essential with regard to CRISPR and genome editing. This is a technology which has not yet entered the public consciousness the way other technologies have. It is therefore critical to educate and inform the public before these new technologies come to the clinic, agriculture and the wider ecosystem. Using a multimedia approach [117] to access a broad range of ages and educational backgrounds, we might prevent misinformation from distracting from substantive conversations around the safety and utility of the technology as it has with other essential scientific ideas such as climate change [118]. The effects of misunderstanding science can impact everything from personal choice to national referenda and policymaking [119].

Improving the interface between science and the public will also be necessary as CRISPR reaches patients. We believe that in addition to science communicators, genetic counselors should be trained to help patients understand and cope with the information received from genomic sequencing data [120]. Understanding the risks associated with specific genomic variants, whether to use genome editing to repair those mutations and how best to handle complex health-related knowledge will require expertise and careful discussion. Providing similar training to clinicians and healthcare providers will further help patients adjust to any difficult news.

Ultimately, the future of CRISPR and similar genome editing technologies, some that are yet to be discovered, is in the hands of scientists right now. It is incumbent upon the research community to step forward, as many have, and acknowledge that bringing CRISPR beyond the bench is uncharted territory. There is a fountain of knowledge that these tools are likely to unlock and a mountain of clinical and ecological needs that these technologies may answer. The simplicity of CRISPR promises to herald a democratized, personalized and accessible era of genome editing—a future that remains uncertain if the public are not sufficiently engaged. But if we do, the future of humanity could be brighter than it has ever been.

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