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

R.K. Delker, Ph.D. (✉) • R.S. Mann, Ph.D.
Department of Biochemistry and Molecular Biophysics and Systems Biology, Mortimer B. Zuckerman Mind Brain Behavior Institute, Columbia University, 612 West 130th Street, 9th Floor, New York, NY 10027, USA
e-mail: rd2643@columbia.edu; rsm10@columbia.edu

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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

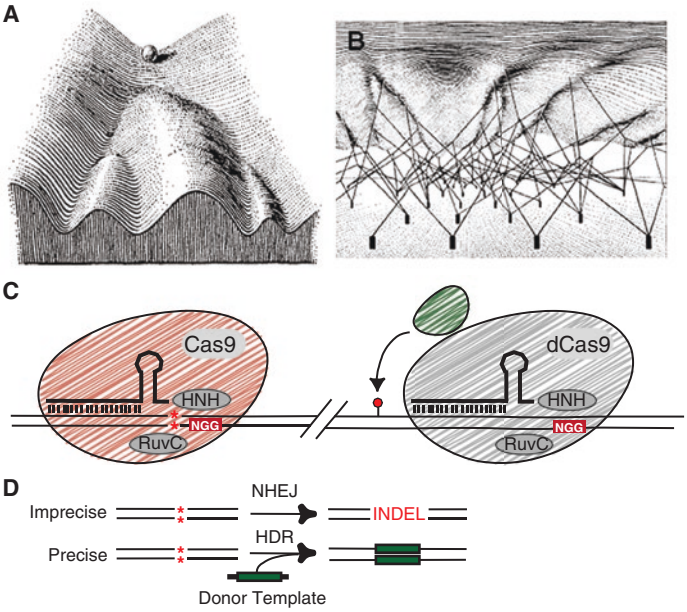

Fig. 3.1 An old idea meets a new technology. (**a**) Waddington's Epigenetic Landscape. A ball, representative of a developing cell, is pulled through one of many developmental pathways to reach the bottom of the hill as a mature, differentiated cell. (**b**) Waddington envisioned that networks of genes 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].

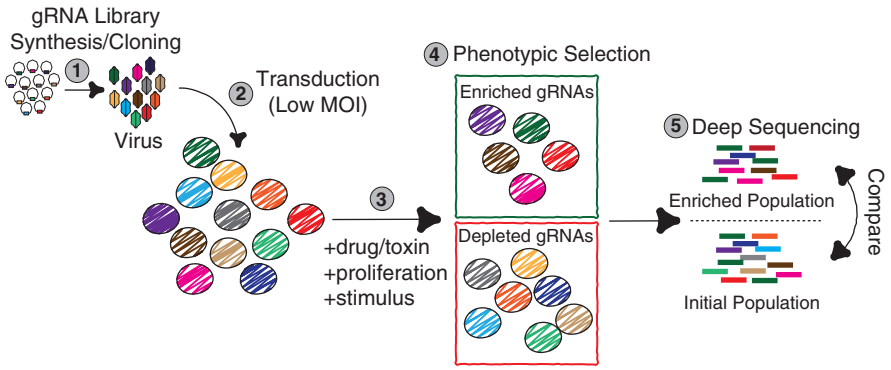

Fig. 3.2 Pooled high-throughput CRISPR GE screens. A schematic details the steps involved in pooled, high-throughput CRISPR GE screens. (*1*) Large-scale production of guide RNAs *in situ* is followed by bulk cloning into a desired vector to generate a gRNA library. (*2*) The library is packaged in virus 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

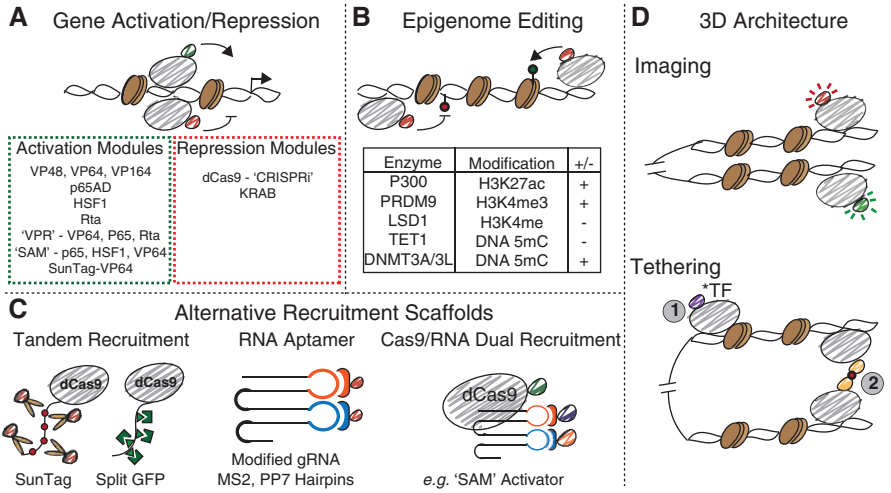

Fig. 3.3 Epigenome modifications with CRISPR GE. (**a**) A schematic depicts the recruitment of dCas9 fused to activation or repression domains to effect changes in gene expression. The activating and repressing modules that have been used are displayed. (**b**) A schematic depicts the recruitment of 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-

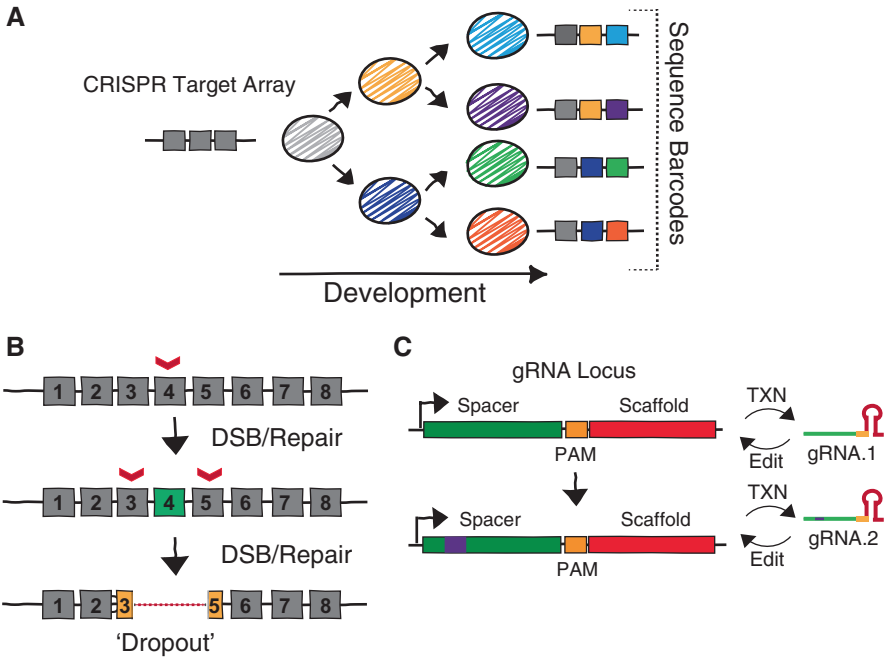

Fig. 3.4 Lineage tracing with CRISPR GE. (**a**) A schematic depicts an idealized example of lineage tracing with Cas9. An array of CRISPR targets is inserted into the genome and subject to the activity of the introduced Cas9/gRNA complex. Mutations induced by Cas9 within the array are replicated and 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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