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Single-Cell Epigenomics Reveals Mechanisms of Cancer Progression

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Abstract

Cancer initiation is driven by the cooperation between genetic and epigenetic aberrations that disrupt gene regulatory programs critical to maintaining specialized cellular functions. After initiation, cells acquire additional genetic and epigenetic alterations influenced by tumor-intrinsic and -extrinsic mechanisms, which increase intratumoral heterogeneity, reshape the cell's underlying gene regulatory networks and promote cancer evolution. Furthermore, environmental or therapeutic insults drive the selection of heterogeneous cell states, with implications for cancer initiation, maintenance, and drug resistance. The advancement of single-cell genomics has begun to uncover the full repertoire of chromatin and gene expression states (cell states) that exist within individual tumors. These single-cell analyses suggest that cells diversify in their regulatory states upon transformation by co-opting damage-induced and nonlineage regulatory programs that can lead to epigenomic plasticity. Here, we review these recent studies related to regulatory state changes in cancer progression and highlight the growing single-cell epigenomics toolkit poised to address unresolved questions in the field.

1. INTRODUCTION

Diverse regulatory mechanisms establish cell-type-specific and context-specific chromatin states that facilitate the activation of functional gene expression programs. Gene regulatory programs, comprising a combined repertoire of transcription factors (TFs) and chromatin-modifying proteins, shape this chromatin landscape and maintain cellular identity (Lambert et al. 2018, Long et al. 2016). In diseases such as cancer, these gene regulatory programs become altered, leading to the aberrant gene expression that promotes cancer initiation, progression, and metastasis (Massagué & Ganesh 2021, Valencia & Kadoch 2019). Disruption of regulatory programs is driven by oncogenic mutations in a presumed cell of origin, resulting in a cascade of cellular phenotypic changes leading to altered cell function (Blanpain 2013, Ferone et al. 2020) (**Figure 1**). In recent years, the rapid development of single-cell genomic technologies has begun to uncover the diversity of cell states that emerge during tumor initiation, tumor maintenance, and drug resistance in cancer (Castro et al. 2021). Studies suggest that these cellular phenotypes are mediated by increased cellular plasticity, which results in gradually evolving and asynchronous changes to the chromatin landscape reminiscent of gene regulation during development and leads to increased intratumoral heterogeneity (Gola & Fuchs 2021). However, this apparent intratumoral heterogeneity has impaired our capacity to use bulk assays to profile the diverse gene regulatory processes that drive acquired phenotypes of cancer cells during tumor progression.

Single-cell epigenomics has expanded our capabilities to profile these heterogeneous cell states in cancer (Buenrostro et al. 2015, Cusanovich et al. 2015, Kaya-Okur et al. 2019, Kelsey et al. 2017, Rotem et al. 2015, Shema et al. 2019). In this review, we aim to summarize the recent advances in these technologies, including (*a*) efforts to integrate diverse single-cell measurements (multimodal analyses) highlighting chromatin accessibility profiling, (*b*) computational strategies to disentangle the complexities of gene regulation, and (*c*) lineage-tracing approaches to delineate the unique paths cancer cells navigate from initiation to metastasis. We place emphasis on scATAC-seq [single-cell ATAC-seq (sequencing assay for transposase-accessible chromatin)] approaches, which use hyperactive Tn5 transposition to measure chromatin accessibility (Buenrostro et al. 2013, 2015), as these methods have become commercialized and are now widely available for cancer research, facilitating studies that seek to map heterogeneous epigenomic cells states in cancer and

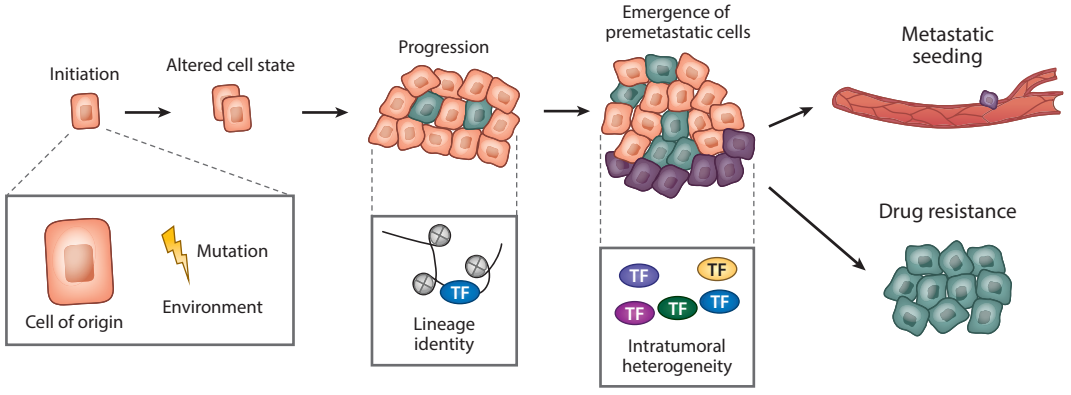

Figure 1

Cascade of cell state transitions during cancer progression. These steps may include initiation in a cell of origin in a permissive environmental context, progression toward increased intratumoral heterogeneity and dissociation from lineage identity, and ultimately metastatic seeding or cell state selection (such as in response to therapy or inflammation). Abbreviation: TF, transcription factor.

normal cells that comprise the tumor microenvironment. In addition, we move beyond these approaches to discuss single-cell technologies to characterize histone modifications, TF localization, and DNA methylation, as well as strategies that provide spatial context for chromatin-mediated regulatory programs. We describe our growing understanding of cancer evolution through the lens of single-cell epigenomics and propose how developing technologies might contribute to a greater understanding of the regulatory mechanisms driving cancer.

2. SINGLE-CELL EPIGENOMICS PROVIDES AN OPPORTUNITY TO BETTER DEFINE THE CELL OF ORIGIN

Cancer commonly initiates with the acquisition of a genetic mutation in a specific cell of origin. The cell of origin is defined as a cell type that can tolerate oncogenic mutations and transform to initiate cancer progression (Haigis et al. 2019, Visvader 2011). The induction of oncogenic mutations in distinct cell types, such as in genetically engineered mouse models (GEMMs) or correlative analyses of cell identity in human tumors (Ferone et al. 2020, Hoadley et al. 2018), has been used to define the cell of origin for various cancer types. Classically, our definition of cell types, including the cell of origin, has largely been based on the expression of a small subset of uniquely expressed marker genes. In contrast, single-cell genomic studies are uncovering a diverse collection of cell states based on broader regulatory features that exist within otherwise well-defined cell types. Variations in a cell's state reflect diverse cellular programs, such as the cell cycle and inflammatory signaling, as well as the stochastic fluctuations of gene regulation within a cell (Shema et al. 2019) (**Figure 2*a***). Importantly, cell states can be distinguished using single-cell epigenomic and transcriptomic approaches and can be defined by the distinct activity of TFs. Here we describe the importance of these higher-resolution cell-of-origin studies and their relevance to epigenetic poising and memory.

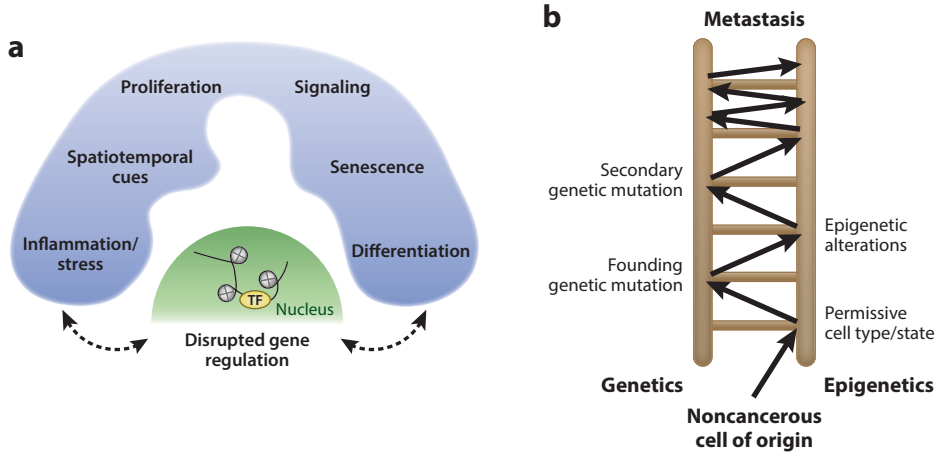

Figure 2

Genetic and epigenetic alterations cooperate to transform and initiate cancer. (*a*) Disrupted gene regulation is mediated by altered activity of transcription factors (TFs) [*yellow oval*, shown in the nucleus (*green*)], which can be caused by or lead to phenotypes that promote cancer progression (*blue*). (*b*) Oncogenic transformation of a cell requires an appropriate cell state that is suitable for cancer initiation via genetic and epigenetic aberrations. This process is depicted here as a noncancerous cell of origin climbing a ladder of genetic and epigenetic changes toward a rare metastatic cell state.

We propose that our definitions of the cell of origin should include the oncogenic potential of cell states. Demonstrating the importance of a revised model, researchers have linked the appearance of sustained wound healing–like programs such as inflammation to persistent cellular states required for oncogenic transformation (MacCarthy-Morrogh & Martin 2020). For example, *KRAS* genetic mutations alone cannot promote permanent transformation of pancreatic cells (Guerra et al. 2007). Rather, *KRAS*-induced transformation must occur following injury, as defined by altered chromatin and gene expression programs following pancreatic inflammation, causing cells to freeze in an oncogenic cell state (Alonso-Curbelo et al. 2021, Li et al. 2021). Researchers utilized scATAC-seq to find key inflammation-induced epigenetic drivers and identified a role for AP-1/FOS TFs (Bejjani et al. 2019), which have been broadly implicated in cancer, and the chromatin reader BRD4 in activating this cell state (Alonso-Curbelo et al. 2021). Further use of single-cell sequencing tools and mouse models is likely to uncover additional epigenetically defined permissive cell states across cancer types, emphasizing the need for an improved understanding of the regulatory mechanisms driving cells into distinct cellular states.

The above example demonstrates how epigenetic mechanisms can poise cells for transformation. In contrast, we also expect genetic mutations may alter epigenomic regulation and position cells to enter permissive cell states. For example, genetic mutations in nonmalignant cells can promote clonality prior to cancer initiation, as demonstrated by clonal hematopoiesis (Bowman et al. 2018). Here, hallmark genetic mutations occur in chromatin-modifying genes that regulate DNA methylation (*DNMT3A* and *TET2*), polycomb silencing (*ASXL1*), and genome structure (genes encoding the cohesin protein complex) (Jaiswal et al. 2014). While mechanisms remain to be fully elucidated, many of these mutations have been implicated in increased inflammatory signaling (Jaiswal & Ebert 2019). Together, this suggests that genetic and epigenetic alterations, which lead to changes in cell state, cooperate over time to initiate cancer (**Figure 2*b***).

Finally, we might expect that cancer cells maintain epigenetic memory reflecting their cell of origin. For example, cell-of-origin studies using bulk ATAC-seq in leukemia have shown that cancer cells maintain epigenomic signatures of their cell of origin (George et al. 2016). By extension of this principle, DNA methylation patterns best identify tumors within the same tissue of origin (Hoadley et al. 2018) and can be used to exquisitely classify subtypes of tumors (Capper et al. 2018). These data suggest that cancer cells preserve epigenetic memory of the cell of origin at the level of DNA methylation. Therefore, studies of epigenetic memory may improve disease prognosis, serve as a valuable correlative tool to identify the cell of origin in primary human tumors, and motivate the development of preventative therapies.

Advances in single-cell epigenomic technologies will broadly refine our understanding of cellular states (Sinha et al. 2021) (**Table 1**); this includes droplet-based scATAC-seq approaches (Lareau et al. 2019, Satpathy et al. 2019). Furthermore, microfluidics-free combinatorial indexing approaches (Cusanovich et al. 2015, Ma et al. 2020), which enable sample multiplexing by barcoding during transposition, expand the number of samples or tumors that can be analyzed in one experiment (LaFave et al. 2020). Together, this diverse family of scATAC-seq methods has facilitated the discovery of heterogeneous chromatin features in normal cells and of the expansive complexity of the epigenome in tumors and the surrounding tumor microenvironment (Alonso-Curbelo et al. 2021, LaFave et al. 2020, Satpathy et al. 2019). These methods continue to evolve, and now incorporate new measurement modalities, such as RNA, protein epitopes, and adaptation to pooled CRISPR screening to study gene regulatory networks (GRNs) (Sinha et al. 2021).

Multimodal analyses that integrate single-cell epigenomic and transcriptomic analyses will enable the construction of GRNs with deeper insights into regulatory programs and dynamics across cancer progression (Kamimoto et al. 2020). As one specific example highlighting the utility of this approach, multiomic analyses enable the study of epigenetic latency or chromatin potential,

Table 1 Principle areas of technological development in epigenomics

Approach	Description
Chromatin and RNA multiomics	Chromatin accessibility (ATAC-seq) and gene expression (RNA-seq) can be profiled within the same single cell at high throughput. Methods are either droplet based (Chen et al. 2019) or plate based and mediated by combinatorial barcoding (Ma et al. 2020).
Lineage tracing	Single-cell genomics enables new opportunities for tracing cell lineages (Woodworth et al. 2017). These methods can be divided into three categories: methods that leverage natural genetic variation (Ludwig et al. 2019), DNA methylation methods (Gaiti et al. 2019), and engineered barcoding strategies (Bowling et al. 2020, Weinreb et al. 2020).
Single-cell ChIP-seq	Technological advances have enabled low-input or single-cell analyses of DNA-bound proteins (Grosselin et al. 2019). New methods utilize antibodies tethered to the protein micrococcal nuclease (Skene & Henikoff 2017) or Tn5 (Kaya-Okur et al. 2019).
Spatial analyses	Genomic methods to spatially visualize epigenomic features within cells are emerging. These methods can be categorized by their resolution, either subcellular (Chen et al. 2015, Payne et al. 2021, Wang et al. 2018) or cellular (Rodriques et al. 2019, Stickels et al. 2021, Zhao et al. 2021).
Data integration	Computational methods have advanced our capacity to integrate single-cell genomic data types. This includes the integration of epigenomic, transcriptomic, protein expression, and spatial data (Granja et al. 2021, Satija et al. 2015).
Deep learning	Deep learning methods are rapidly improving the quality and efficiency of computational tools for epigenomics. These methods have broad applicability, enabling data integration (Lopez et al. 2018), data denoising (Lal et al. 2021), and the prediction of epigenomic features based on DNA sequence (Avsec et al. 2021).

Abbreviations: ATAC-seq, sequencing assay for transposase-accessible chromatin; ChIP-seq, chromatin immunoprecipitation and sequencing; RNA-seq, RNA sequencing.

which is defined as chromatin changes that happen prior to gene expression activation (Ma et al. 2020). These observations build from the well-established stepwise mechanism of gene activation, whereby enhancers transition between silenced, poised, and active states as cell type–specific TFs recruit specific histone readers and writers (Rada-Iglesias et al. 2011). While current single-cell studies use chromatin accessibility to identify poised enhancer states, histone modifications reflecting primed (H3K4me1) and active (H3K27ac) enhancers have been demonstrated to be powerful markers of poised regulatory elements. Excitingly, single-cell cleavage under targets and tagmentation (scCUT&Tag) approaches may now enable the study of histone modifications in single cells (Bartosovic et al. 2021, Wu et al. 2021). While these methods have largely been used to understand cell fates in development and normal differentiation, they also hold tremendous promise for providing a clearer understanding of epigenetic poising that contributes to permissive cellular states for oncogenic transformation.

3. LINEAGE INFIDELITY IS A COMMON FEATURE OF CANCER PROGRESSION

Single-cell analysis enables the study of lineage identity by comparing gene regulatory programs in normal cells to those dysregulated in cancer. In addition to TFs, chromatin-modifying enzymes modulate the activity of regulatory elements, such as promoters, enhancers, and insulators to fine-tune chromatin structure and gene expression. Under normal tissue homeostatic conditions, cell type–specific regulators must activate the appropriate programs, and the mechanisms by which cells access non-lineage-specifying programs in cancer are not well understood. Loss of tissue homeostatic gene regulation in stem cells of the skin leads to lineage infidelity during

cancer progression (Ge et al. 2017). In this context, lineage infidelity occurs by the activation of stress-associated programs that lead cells to decouple from their identity and transdifferentiate into aberrant lineages. As a result, some researchers have described cancer as a "wound incapable of healing" (MacCarthy-Morrogh & Martin 2020). In this section we explore the epigenomic determinants of cell identity, their dysregulation in cancer, and the use of single-cell tools to define these altered regulatory programs.

The ability of cancer cells to occupy altered differentiation states has been well described across various cancers. As one example, single-cell studies of human lung adenocarcinoma have shown substantial differences in alveolar identity within tumors (Laughney et al. 2020). Extending this observation, researchers have demonstrated using single-cell analyses of lung adenocarcinoma tumors in mouse models that cells become detached from the identity of the transformed cell type [alveolar type 2 (AT2) cells], acquire developmental programs (endoderm) (Tata et al. 2018), and adopt altered fates (AT1-like and gastric-like cell states) (LaFave et al. 2020, Marjanovic et al. 2020). Interestingly, these transitions of cell identity contribute to intratumoral heterogeneity and appear continuous, without clear subpopulations within the tumor (LaFave et al. 2020). Coincidentally, within normal cells, injury-associated programs have been described to induce AT2-to-AT1 differentiation, implicating damage (wounding) in loss of lineage identity (Nabhan et al. 2018). Similar mechanisms of trans- or dedifferentiation have been described across many tumor types, including pancreatic adenocarcinomas, small-cell lung cancers, breast tumors, and basal cell carcinomas (Biehs et al. 2018, Ge et al. 2017, Ireland et al. 2020, Storz 2017, Van Keymeulen et al. 2015). Beyond the lung, these data support the hypothesis that loss of lineage restriction, paired with stress responses, are important mechanisms by which cells acquire epigenetic plasticity in cancer, potentially allowing cells to enter states similar to those important in the developmental history of the cell.

Single-cell epigenomic methods can provide insights into the regulatory factors that govern these trans- and dedifferentiation fate transitions, such as paired single-cell epigenomic and transcriptomic measurements to define regulatory dynamics of TFs. Computational approaches utilizing multiomic single-cell methods [scATAC-seq and scRNA-seq (single-cell RNA sequencing)] can connect distal enhancers to genes by utilizing the correlation of chromatin accessibility with gene expression across single cells (Buenrostro et al. 2018, Granja et al. 2021, Ma et al. 2020). With this approach, single-cell methods can identify domains of regulatory chromatin (DORCs) (Ma et al. 2020) that describe chromatin regions with a high density of enhancer-gene interactions (10–50 distal enhancers). DORCs commonly regulate lineage-determining genes—which are likely a result of cells requiring exquisite control of transcription at these genes—and are highly concordant with regulatory regions known as superenhancers (defined by the association of H3K27ac, MED1, and BRD4) (Hnisz et al. 2013). The emergence of single-cell tools measuring chromatin accessibility or histone modifications promises to uncover aberrant TFs that promote the activation of lineage-altering regulatory elements, thereby revealing mechanisms that govern lineage infidelity in cancer.

Several studies have demonstrated that single-cell tools may be used to characterize cell fate dynamics within cancer cells by pseudo-temporal ordering (pseudo-time) of single-cell profiles (Marjanovic et al. 2020, Young et al. 2018). As an example, single-cell tools have identified TF-mediated trajectories in Wilms' tumor progression (Young et al. 2018). Single-cell methods may be used to track diverse cell fates as cells progress to metastasis; however, the true trajectories by which cells move between cell states are complex and may not be well suited for pseudo-time models. True trajectories may include (*a*) dead ends that are not compatible with metastatic progression (i.e., senescent or dormant cell populations), (*b*) reversible paths that are consistent with trans- and dedifferentiation, and (*c*) mixtures of these dynamics (**Figure 3**). As such, this conceptual

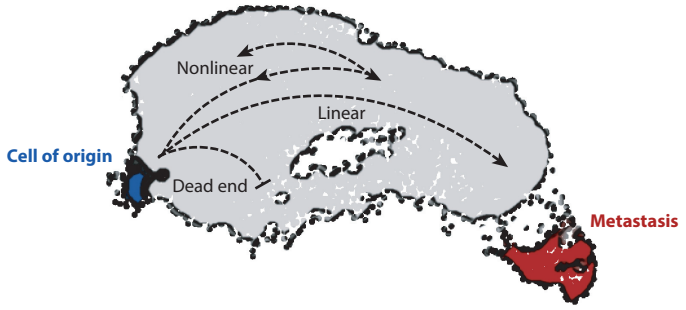

Figure 3

Potential routes of cancer progression. Stylized uniform manifold approximation and projection (UMAP) from continuous epigenomic cell states isolated from murine lung adenocarcinoma cells. Several potential trajectories toward metastasis include linear paths, nonlinear (reversible) paths, and dead ends. Figure adapted from LaFave et al. (2020).

understanding of pseudo-time and its associated computational tools may not generalize to all the complex lineage trajectories expected in cancer.

Lineage-tracing methods (**Table 1**) will enable the dissection of more complex cell fate trajectories (Woodworth et al. 2017). Diverse strategies leveraging engineered barcodes, including in GEMMs, can be utilized to enable tracking of cell states in cancer, including DNA barcodes, transposon-based methods, or CRISPR-mediated evolutionary barcodes (Bowling et al. 2020, Wagner & Klein 2020). In metastasis, lineage tracing in cancer xenografts has shown that there are several routes to metastatic progression and has uncovered important regulators at specific nodes of progression, such as the suppressive role of *KRT17* (Quinn et al. 2021). Overall, lineage-tracing strategies will be helpful to further understand cancer progression, including the complex trajectories exemplified by partial epithelial-to-mesenchymal transition (EMT) (Puram et al. 2017, Simeonov et al. 2021).

In human tumors, single-cell tools may utilize naturally occurring genetic and epigenetic barcodes. Beyond copy number variants and single-nucleotide variants (Navin et al. 2011), tantalizing proof-of-principle experiments have used DNA methylation (Gaiti et al. 2019) and mitochondrial variants (Ludwig et al. 2019) to provide new means by which to trace cell lineages in the absence of engineered barcode sequences. We anticipate that applications of these tools, in concert with single-cell epigenomic or multiomic approaches, will help identify the regulatory determinants of increased lineage plasticity leading to altered cell fates. Together, the incorporation of lineage-tracing strategies, in combination with single-cell epigenomics, will be essential for understanding the exact trajectories of tumor cells in otherwise unstructured epigenomic data.

4. EFFECTS OF CODING AND NONCODING MUTATIONS ARE CANCER CELL STATE SPECIFIC

Large-scale genome sequencing efforts, such as The Cancer Genome Atlas (TCGA), have provided comprehensive maps of the genomic alterations within coding regions of the human genome (Weinstein et al. 2013). These driver mutational patterns are relatively distinct for different cancer types and cells of origin, motivating the study of how genetic mutations cooperate with the epigenome to promote cancer initiation and progression. However, most studies are predominantly restricted to mapping mutations in protein-coding genes, and as a result, the role of noncoding mutations and how they disrupt regulatory elements across the genome are not well

understood. Given the utility of genome-wide association studies (GWAS) in elucidating disease-associated predisposition genes, regulatory maps linking somatic variation across cancer subtypes with downstream gene targets are critical to understanding context-specific gene regulation in cancer. In this section we describe efforts to extend the study of noncoding variation across cancers and recent endeavors to map regulatory elements in normal and cancer cells.

The regulatory architectures that govern GRNs are highly context specific, as evidenced by the fact that a distinct subset of enhancers regulate the same gene in different cell types (Corces et al. 2016), and these architectures provide enough detail to serve as markers of prognosis in patient samples (Cejas et al. 2019). Without a greater understanding of GRNs in normal cells and cancer, the implications of noncoding variation remain relatively unclear. Mutations in noncoding regulatory elements have been studied previously in cancer, including the identification of TERT promoter mutations (Huang et al. 2013) and enhancer hijacking through fusions, as with the activation of *MYC* via its fusion with predicted superenhancer regions such as those in immunoglobulin-associated genes (Affer et al. 2014). Other examples of noncoding alterations include polymorphisms in superenhancer regions of *LMO1* in neuroblastomas and *TAL1* noncoding mutations (Mansour et al. 2014, Oldridge et al. 2015). While these examples demonstrate that noncoding variations can function as drivers of cancer progression, the identification and validation of noncoding alterations have been relatively rare in cancer to date.

Prior consortia-based efforts, such as the Encyclopedia of DNA Elements (ENCODE) Project Consortium, have integrated a variety of assays including chromatin accessibility, DNA methylation, chromatin looping, and histone modifications in an effort to discover human regulatory elements (ENCODE Proj. Consort. et al. 2020). These large-scale data can be utilized to annotate regulatory elements by their function (Boix et al. 2021) or their association to target genes (Nasser et al. 2021) to help identify the genetic basis of human diseases, including loci associated with cancer susceptibility (Sud et al. 2017). In addition, chromatin accessibility data from TCGA has been useful in identifying noncoding variation in human tumors (Corces et al. 2018). However, these studies have largely been performed using bulk assays; hence, the dynamics of cell type–specific interactions between regulatory elements and their target genes during cell differentiation, damage, etc. are not well understood. Single-cell tools, including computational methodologies, are now revolutionizing our capability to identify noncoding regulatory elements within emergent rare cell types or transient cell states.

Computational pairing of scATAC-seq and scRNA-seq methods is broadly employed and has been demonstrated across diverse contexts (Buenrostro et al. 2015, Granja et al. 2021, Satija et al. 2015). These developments have motivated machine learning–based methods to predict GRNs from single-cell data (Kamimoto et al. 2020). Deep learning methods promise to extend the power of these single-cell tools, enabling the analysis of nuanced interactions between TFs and chromatin (Avsec et al. 2021). Despite the apparent success of these approaches, major challenges still limit the ability of epigenomic profiling to infer clear and concise gene regulatory interaction maps. Bulk and single-cell methods for functionally annotating regulatory elements rely on prior knowledge of gene regulatory mechanisms, are largely correlative, and rely on markers of activity such as chromatin accessibility, DNA methylation, and histone modification data. Approaches utilizing high-throughput perturbations with single-cell analysis have already significantly improved our understanding of noncoding gene regulatory elements and their impact on gene expression. To this end, reports have suggested that only a small fraction (10–15%) of accessible noncoding regions are indeed functional (Gasperini et al. 2019). By leveraging single-cell data, high-throughput functional studies will significantly improve predictive models of gene regulation. The impact of these tools as they improve in accuracy will be broad and will enable new insights into cancer susceptibility and noncoding drivers of tumor progression.

5. DNA METHYLATION AND HISTONE MODIFICATIONS REGULATE CANCER PROGRESSION

Cancer evolution unfolds in part through a series of genetic and nongenetic alterations. However, connections between genetic mutations and emergent downstream phenotypic landmarks in cancer evolution, as well as their roles in associated intratumoral heterogeneity, have been less obvious than expected (Schwenger & Steidl 2021). Reproducible, sequential acquisition of hallmark genomic events, such as in the Vogelstein model of colon cancer progression, does not fully explain the complexities of genomic and epigenomic variation across cancer evolution (Bailey et al. 2021, Fearon & Vogelstein 1990, Reiter et al. 2019). In addition, descriptions of cancer progression solely through the lens of increasing genomic variation ignore the importance of GRN-associated events implicated in cancer progression, such as altered DNA methylation patterns and dysregulation of chromatin-modifying enzymes with corresponding histone modifications (Timp & Feinberg 2013). Furthermore, associations between aneuploidy and enhancer activity are correlated across cancers, adding to the intermingled genomic and epigenomic phenomena in cancer (Chen et al. 2018). In this section we explore the need to study the intersection of genetic and nongenetic mechanisms, with a focus on DNA methylation, in cancer evolution and describe new strategies to disentangle ambiguities in the field.

Varied methylation patterns across regulatory elements have been attributed as a nongenetic driver of cancer (Black & McGranahan 2021). However, the function of DNA methylation in cancer progression more broadly, and at precise regulatory elements specifically, has not been well resolved. Additionally, the mechanistic connection between DNA methylation and histone modifications associated with open, poised, or closed chromatin at various regulatory elements has been challenging to dissect using bulk methodologies (**Figure 4*a***). Tightly coordinated DNA methylation patterns maintain cell type–specific and context-specific GRNs and preserve the imprinting and silencing of repetitive elements across the genome (Ishak & De Carvalho 2020). In hematologic malignancies, mutations in chromatin-modifying enzymes associated with

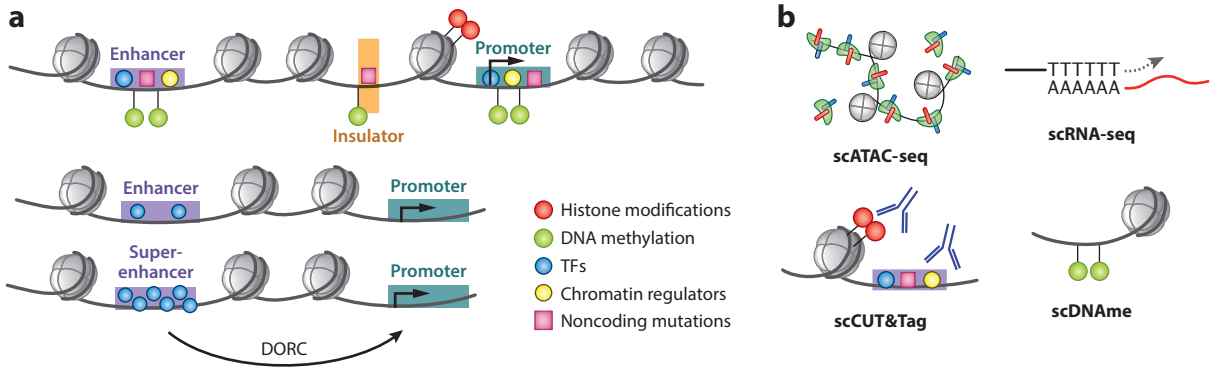

Figure 4

Regulatory elements are disrupted by diverse mechanisms across cancer progression. (*a*) Aberrant gene regulation at enhancers (*purple*), promoters (*teal*), and insulators (*orange*) contributes to cancer evolution. DNA methylation (*green*) and histone modifications (*red*) are placed and bound by chromatin regulators (*yellow*) and TFs (*blue*). Noncoding mutations or nongenetic epigenomic mechanisms have been shown to perturb gene regulation in cancer. Importantly, superenhancers and DORCs have increased regulatory connections and have been associated with the maintenance of cellular identity. (*b*) Various approaches to mechanistically dissect altered gene regulation can now be performed at single-cell resolution. Abbreviations: ATAC-seq, sequencing assay for transposase-accessible chromatin; CUT&Tag, cleavage under targets and tagmentation; DNAme, DNA methylation; DORC, domain of regulatory chromatin; RNA-seq, RNA sequencing; sc, single-cell; TFs, transcription factors.

methylation lead to wide-ranging disruption of methylation landscapes (Glass et al. 2017). While overt reorganization of methylation patterning is a feature of these cancers, and while therapeutic agents specifically targeting aberrant methylation are used clinically with varied success, the exact mechanisms by which methylation disrupts GRNs are still elusive. Furthermore, in cancer a majority of aberrant methylation occurs in repetitive regions of the genome, namely in Alu and LINE-1 elements, suggesting that derepression of silenced elements might co-opt GRNs (Cajuso et al. 2019, Ewing et al. 2020, Gu et al. 2021). The demonstration that extrachromosomal DNA can function as a mobile enhancer has added to the increased potential of context-specific epigenomic dysregulation at regulatory elements in cancer (Zhu et al. 2021).

While much work remains to be done to decipher these mechanisms, some headway has been made in interrogating differentially methylated regions. For example, altered methylation at CTCF binding sites in gastrointestinal stromal tumors and in IDH-mutant gliomas leads to hyperactivation of *FGF4/FGF5* and *PDGFA*, respectively (Flavahan et al. 2016, 2019). Furthermore, in glioblastomas, methylation heterogeneity corresponds with tumor subtype classification (Klughammer et al. 2018). Interestingly, the DNA hypo/hypermethylation loci and histone modification patterns are structured into compartments in normal nuclei, with heterochromatin around the periphery, yet this compartmental structure collapses in tumor nuclei (Johnstone et al. 2020). Elucidating the mechanisms by which altered methylation and histone modification landscapes cooperate with rewired TF-mediated GRNs in cancer will require the use of additional multimodal single-cell approaches.

The expanding repertoire of single-cell methods beyond scATAC-seq and scRNA-seq to include single-cell DNA methylation (scDNAme), single-cell ChIP-seq (chromatin immunoprecipitation and sequencing), and scCUT&Tag studies will facilitate research into how DNA methylation and histone modifications, together and separately, contribute to disruption of GRNs (**Figure 4*b***). Many variations of scDNAme technologies have been effectively applied to various normal and cancer types to both confirm known DNA methylation heterogeneity and discover novel single-cell variation (Karemaker & Vermeulen 2018). Additionally, integrated multiomic analyses that pair DNA methylation with TF and histone modification landscapes, gene expression data, and chromatin accessibility assays have more power to distill the relationships within this multifaceted regulatory landscape (Angermueller et al. 2016, Cheow et al. 2016, Clark et al. 2018, Guo et al. 2017, Hou et al. 2016, Hu et al. 2019, Pott 2017), and expanding these multiomic approaches to include scDNAme will be useful to determine the kinetics of DNA methylation, which has been challenging to decipher previously. Further studies using single-cell DNA topology profiling methods such as in situ genome sequencing and single-cell Hi-C, coupled with insights from scDNAme, will produce further insights into how aberrant methylation in cancer modifies genomic topology and contributes to oncogenic phenotypes (Gravina et al. 2016, Payne et al. 2021, Ramani et al. 2020). Additionally, the development of dCas9 (dead CRISPR-associated protein 9) systems that can be designed to target the epigenome, such as a dCas9-TET fusion system (Liu et al. 2016), will be useful for iterative validation studies. Together, the pairing of multimodal strategies with functional validation will provide insights into the varied epigenomic mechanisms that initiate cancer-associated chromatin states and cooperate with genetic variation to promote cancer.

6. EPIGENOMIC PLASTICITY AND CANCER THERAPEUTICS

The study of cancer evolution requires an understanding of not only how cell states evolve naturally but also how these populations respond to selective pressures, including in response to

cancer therapy. In the past, the field predominantly viewed drug resistance through the lens of emergent genetic mutations leading to overt selection and permanence of resistant cell populations (Hendricks et al. 2017). However, while genetic mutations in response to cancer therapeutics constitute one important mechanism of drug resistance, the acquisition of aberrant cell states that do not harbor obvious genetic variation is another mechanism by which this resistance can occur (Schwenger & Steidl 2021). With more sophisticated approaches to trace cellular responses to cancer therapeutics, there are immense opportunities to study mechanisms of epigenomic plasticity, both in a treatment-naive setting and posttreatment, and to assess whether these resultant cell states are irreversible or reversible. Furthermore, there are still unresolved questions regarding whether treatment-resistant cells exist in the tumor pool prior to therapy or whether they arise in response to treatment. In this section we highlight some recent studies investigating drug resistance within perceived clonal populations, along with strategies that leverage single-cell epigenomics to investigate drug resistance.

Mechanisms of drug resistance in response to cancer therapeutics are likely multifaceted, and it remains unclear if resistant cells arise from cellular adaptation or selection for a rare preexisting cell population. Intriguingly, chromatin state variations in rare cancer cell populations, which lead to persistently high expression of several genes driving unique phenotypes, have been shown to arise stochastically in cell populations perceived to be homogeneous (Sharma et al. 2010). In addition, cell populations sorted by specific phenotypic markers can reachieve a phenotypic equilibrium by interconverting between different cell states, suggesting that there is substantial epigenomic plasticity in cancer cells (Gupta et al. 2011). To identify these rare populations that may exist in a preexisting pool of cancer cells, researchers have developed new strategies such as Rewind to decipher cell states that arise posttreatment (Emert et al. 2021, Shaffer et al. 2017). Rewind allows for the retrospective identification of cell states that give rise to drug-resistant populations by coupling lentiviral barcoding with the synthesis of barcodes from resistant populations to label untreated cells via RNA FISH (fluorescence in situ hybridization). Using this strategy in $BRAF^{V600E}$ melanoma, researchers identified rare cell populations in the untreated context that evaded cell killing by the BRAF inhibitor vemurafenib due in part to sustained activation of the MAPK signaling pathway. Furthermore, scRNA-seq has identified gene programs in preexisting cell states that led to distinct cell fates following drug treatment (Emert et al. 2021). Further study of specific gene programs that are activated in these preexisting populations is important. For example, in glioblastoma, kinase inhibitor treatment leads to a rare population of slow-cycling cells that upregulate developmental programs, raising the possibility that resistant cells enter a more dedifferentiated cell state (Liau et al. 2017).

Single-cell tools are advancing our capacity to measure diverse perturbations across cancer cells, including the interrogation of chromatin- or transcription-targeting drugs. New methods such as sci-Plex use single-cell sequencing of transcriptional responses to perform high-throughput drug screening (Srivatsan et al. 2020). Advances in single-cell lineage tracing and in temporally resolved gene expression and chromatin analyses (Cao et al. 2020, Emert et al. 2021, Qiu et al. 2020) will provide additional clarity to selection in response to cancer therapeutics. Additionally, developments in dCas9-based epigenome editing systems will provide mechanistic insights into the heritability of gene expression states that are relevant to drug resistance in tumors. For example, CRISPRoff/CRISPRon strategies allow for the deposition of DNA and histone methylation at targeted genomic loci, resulting in gene expression alterations that are inherited over multiple cell divisions (Nuñez et al. 2021). These strategies will enable greater understanding of chromatin-mediated drug resistance and strategies to interfere with epigenomic programs that poise cells for therapeutic selection.

7. THE ROLE OF THE TUMOR MICROENVIRONMENT IN GENE REGULATION

While single-cell epigenomics has begun to resolve the heterogeneous cell states that exist across cancer types, the understanding of how these cell states arise and persist in relation to their local tumor microenvironment is not well established. Cancer cells intermingle with diverse immune and stromal cell types, as well as with normal or untransformed cells, in a continuously changing environment in response to aberrant cues from altered proliferation, hypoxia, and signaling pathways (Jerby-Arnon et al. 2018, Sun & Yu 2015). Single-cell profiling studies aim to characterize these cell states; however, the etiology of these states can be challenging to decipher in the absence of spatial information. Spatial context also provides hints related to clonality, both epigenomic and genomic, and allows for the exploration of cell-cell contacts in the unique local neighborhood of individual cancer cells. In this section we describe some current insights of intratumoral heterogeneity positioned within the tumor microenvironment and discuss new single-cell approaches to explore chromatin biology spatially.

Cancer cells exist in a diverse ecosystem and are sustained by cell-cell interactions in the tumor microenvironment. Several examples of codependencies among cancer and noncancer cells have been identified that contribute to cancer cell persistence. For example, in pancreatic ductal adenocarcinoma, single-cell transcriptomics paired with protein analyses identified that stromal cancer-associated fibroblasts alter gene expression programs within cancer cells to promote EMT and proliferation (Ligorio et al. 2019). Other examples of these interdependencies include the development of self-supporting niches in squamous tumors to promote macrophage differentiation (Taniguchi et al. 2020), environmental cues from myofibroblasts in colon cancer to alter stemness (Essex et al. 2019), the exclusion of T cells in melanomas to reduce immune surveillance (Jerby-Arnon et al. 2018), and codependencies of neuroendocrine and non-neuroendocrine cell fates in small-cell lung cancer to provide extracellular matrix organization and trophic support (Lim et al. 2017). Disruption of additional tissue homeostatic factors, such as biophysical forces, can also promote the activation of oncogenic gene programs leading to an altered tumor microenvironment and metastasis (Fiore et al. 2020, Kaur et al. 2019). How the epigenome in cancer cells promotes gene programs that facilitate the interactions with neighboring cells and how neighboring noncancer cells alter the epigenome of cancer cells are still poorly understood and growing areas of interest.

In cancer biology, spatial context is classically inferred by traditional immunohistochemical or fluorescent staining to study the localization of gene expression changes across a tumor. In contrast, single-cell spatial transcriptomics is an unbiased method to identify gene expression across a tumor (Smith & Hodges 2019). For example, the integration of scRNA-seq with array-based spatial transcriptomics in pancreatic tumors delineates a spatially restricted cancer stress response that localizes to inflammatory fibroblasts (Moncada et al. 2020). Rapid advances in spatial transcriptomics technology, such as with Slide-seq, have made it possible to study spatial gene expression at even higher cellular resolution and are ready to be broadly applied in the context of cancer (Rodriques et al. 2019). While spatial transcriptomics is powerful at identifying gene expression patterns, advances in spatial epigenomics are required to understand how chromatin regulation integrates microenvironment stimuli into these gene expression outputs. Spatially resolved chromatin profiling methods, such as sciMAP-ATAC, in situ genome sequencing, and MERFISH (multiplexed error-robust FISH), can provide deeper insights into chromatin changes that underlie heterogeneous gene expression states (Payne et al. 2021, Su et al. 2020, Thornton et al. 2021). Additionally, in situ chromatin protein assays such as CODEX enable the multiplexed labeling of histones and TFs in tumor nuclei (Goltsev et al. 2018). Studying the spatial distribution of different

epigenetic cell states will further refine our understanding of the interplay between the microenvironment and cancer evolution, and will inform drug targets for microenvironment-dependent interventions.

8. CONCLUSIONS

In this review, we have described key biological processes underlying tumor evolution and highlighted relevant opportunities for scientific advances using single-cell epigenomic tools. The examples provided are broad, ranging from identifying the cell of origin to understanding the gene regulatory mechanisms underlying metastatic transitions. Most importantly and most excitingly, single-cell epigenomic tools are rapidly evolving, and now are expanding beyond simple measures of chromatin accessibility or DNA methylation. Given the already apparent utility of these mature scATAC-seq technologies, we anticipate that the evolving repertoire of single-cell epigenomic tools—integrating histone modifications, genome structures, spatial information, lineages, etc. (**Table 1**)—will dramatically advance our understanding of the cellular diversity within tumors and the gene regulatory mechanisms underlying tumor evolution. These technologies are enabled by the synergistic advances in experimental and computational strategies developed in academic and industry labs.

Since tumors are highly heterogeneous in cell type composition, we envision that single-cell analysis of the epigenome will become the standard for cancer cell biology. As such, these technologies will enable large-scale consortium efforts to, for example, understand regulatory diversity within human tumors (Corces et al. 2018), as well as help individual labs to synthesize single-cell and lineage-resolved regulatory maps of tumor evolution. Overall, we contend that the dissection of regulatory programs using single-cell epigenomic technologies will enable new opportunities for scientific discovery and will greatly improve our understanding of cancer evolution with implications for improved therapeutic interventions in cancer.

DISCLOSURE STATEMENT

J.D.B. holds patents related to ATAC-seq and is on the scientific advisory board for CAMP4 Therapeutics, seqWell, and Celsee. L.M.L. and R.E.S. do not have any disclosures to declare.

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Errata

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