Cancer Evolution: A Multifaceted Affair

Giovanni Ciriello[1,2], Luca Magnani[3,4,5], Sarah J. Aitken[6,7], Leila Akkari[8], Sam Behjati[9,10,11], Douglas Hanahan[1,12], Dan A. Landau[13,14,15], Nuria Lopez-Bigas[16,17,18], Darío G. Lupiáñez[19], Jean-Christophe Marine[20,21], Ana Martin-Villalba[22], Gioacchino Natoli[23], Anna C. Obenauf[24], Elisa Oricchio[1,25], Paola Scaffidi[23,26], Andrea Sottoriva[27], Alexander Swarbrick[28,29], Giovanni Tonon[30,31], Sakari Vanharanta[32,33], and Johannes Zuber[24,34]

ABSTRACT

Cancer cells adapt and survive through the acquisition and selection of molecular modifications. This process defines cancer evolution. Building on a theoretical framework based on heritable genetic changes has provided insights into the mechanisms supporting cancer evolution. However, cancer hallmarks also emerge via heritable nongenetic mechanisms, including epigenetic and chromatin topological changes, and interactions between tumor cells and the tumor microenvironment. Recent findings on tumor evolutionary mechanisms draw a multifaceted picture where heterogeneous forces interact and influence each other while shaping tumor progression. A comprehensive characterization of the cancer evolutionary toolkit is required to improve personalized medicine and biomarker discovery.

Significance: Tumor evolution is fueled by multiple enabling mechanisms. Importantly, genetic instability, epigenetic reprogramming, and interactions with the tumor microenvironment are neither alternative nor independent evolutionary mechanisms. As demonstrated by findings highlighted in this perspective, experimental and theoretical approaches must account for multiple evolutionary mechanisms and their interactions to ultimately understand, predict, and steer tumor evolution.

INTRODUCTION

Tumorigenesis and malignant progression embody a Darwinian process of dynamic evolution, involving selective pressures encountered by proliferatively expansive "outlaw cells" facing barriers and limitations intended to preserve tissue homeostasis and prevent the emergence of inappropriate phenotypes. The stepwise evolution that ultimately circumvents these protective mechanisms is apparent in the epidemiology

[1]Swiss Cancer Center Leman, Lausanne, Switzerland. [2]Department of Computational Biology, University of Lausanne, Lausanne, Switzerland. [3]The Breast Cancer Now Toby Robins Research Centre, The Institute of Cancer Research, London, United Kingdom. [4]Breast Epigenetic Plasticity and Evolution Laboratory, Division of Breast Cancer Research, The Institute of Cancer Research, London, United Kingdom. [5]Department of Surgery and Cancer, Imperial College London, London, United Kingdom. [6]Medical Research Council Toxicology Unit, University of Cambridge, Cambridge, United Kingdom. [7]Department of Histopathology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, United Kingdom. [8]Division of Tumor Biology and Immunology, Oncode Institute, The Netherlands Cancer Institute, Amsterdam, the Netherlands. [9]Wellcome Sanger Institute, Hinxton, United Kingdom. [10]Cambridge University Hospitals NHS Foundation Trust, Cambridge, United Kingdom. [11]Department of Paediatrics, University of Cambridge, Cambridge, United Kingdom. [12]Ludwig Institute for Cancer Research, Lausanne, Switzerland. [13]New York Genome Center, New York, New York. [14]Division of Hematology and Medical Oncology, Department of Medicine and Meyer Cancer Center, Weill Cornell Medicine, New York, New York. [15]Institute for Computational Biomedicine, Weill Cornell Medicine, New York, New York. [16]Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Barcelona, Spain. [17]Centro de Investigación Biomédica en Red en Cáncer (CIBERONC), Instituto de Salud Carlos III, Madrid, Spain. [18]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain. [19]Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin Institute for Medical Systems Biology, Berlin, Germany. [20]Laboratory for Molecular Cancer Biology, Center for Cancer Biology, VIB, Leuven, Belgium. [21]Laboratory for Molecular Cancer Biology, Department of Oncology, KULeuven, Leuven, Belgium. [22]Department of Molecular Neurobiology, German Cancer Research Center (DFKZ), Heidelberg, Germany. [23]Department of Experimental Oncology, European Institute of Oncology IRCCS, Milan, Italy. [24]Research Institute of Molecular Pathology, Vienna Biocenter, Vienna, Austria. [25]Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, Swiss Federal Institute of Technology Lausanne (EPFL), Lausanne, Switzerland. [26]Cancer Epigenetic Laboratory, The Francis Crick Institute, London, United Kingdom. [27]Computational Biology Research Centre, Human Technopole, Milan, Italy. [28]Cancer Ecosystems Program, Garvan Institute of Medical Research, Darlinghurst, Australia. [29]School of Clinical Medicine, Faculty of Medicine and Health, UNSW Sydney, Sydney, Australia. [30]Vita-Salute San Raffaele University, Milan, Italy. [31]Center for Omics Sciences, IRCCS San Raffaele Scientific Institute, Milan, Italy. [32]Translational Cancer Medicine Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland. [33]Department of Biochemistry and Developmental Biology, Faculty of Medicine, University of Helsinki, Helsinki, Finland. [34]Medical University of Vienna, Vienna BioCenter (VBC), Vienna, Austria.

G. Ciriello and L. Magnani contributed equally to this article.

Corresponding Authors: Giovanni Ciriello, Computational Systems Oncology lab, Genopode (Office #2025.2), Quartier Sorge, Lausanne, Vaud 1015, Switzerland. E-mail: giovanni.ciriello@unil.ch; and Luca Magnani, Breast Epigenetic Plasticity and Evolution lab, 1st Floor, CBL, 1S6, 237 Fulham Road, London SW7 3RP, United Kingdom. E-mail: luca.magnani@icr.ac.uk

Cancer Discov 2024;14:1–13

doi: 10.1158/2159-8290.CD-23-0530

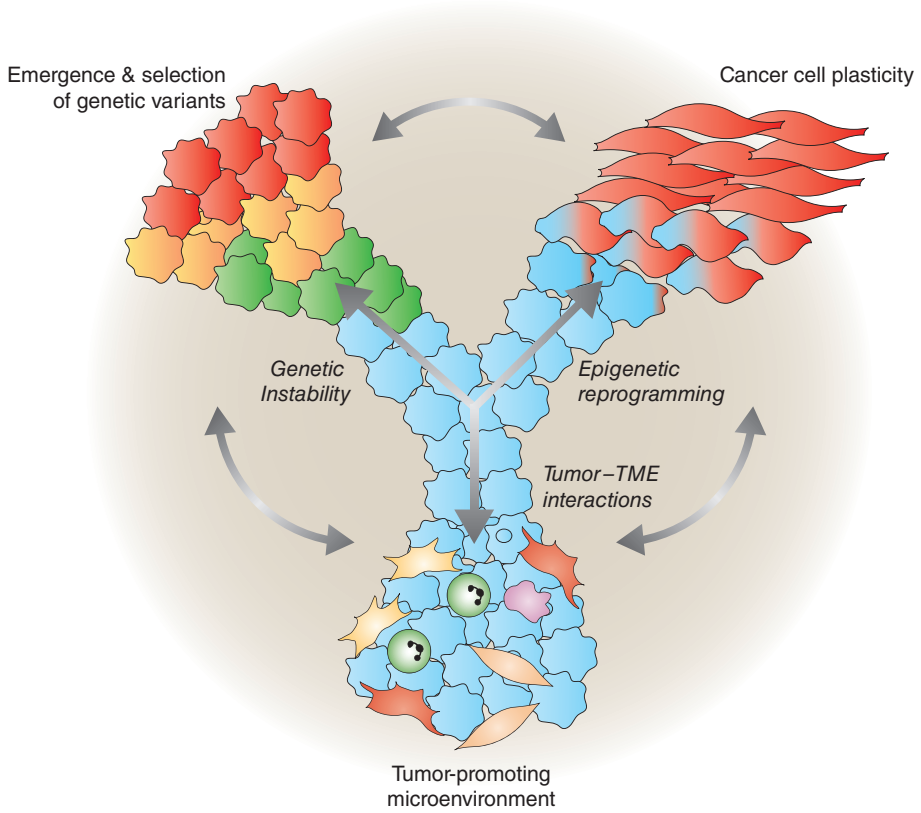

Figure 1. The many faces of cancer evolution. Enabling characteristics such as genetic instability, epigenetic reprogramming, and interactions between tumor cells and the TME enable cancer evolutionary mechanisms such as the emergence and selection of genetic variants, cancer cell plasticity, and re-education of the TME.

and histopathology characteristics of human cancer, and in the realization that distinctive capabilities dubbed the hallmarks of cancer (1, 2) underlay the process of tumor progression. The appearance of such hallmarks can be seen as the result of an evolutionary process: Tumor phenotypes that can be considered as "enabling characteristics," such as genomic instability or epigenetic reprogramming, facilitate tumor cell diversification and the Darwinian selection of the set of hallmark traits instrumental for tumorigenesis and malignant progression. At the DNA level, genome instability leads to driver (and passenger) mutations, here intended as changes in the DNA sequence, that convey functional capabilities that are fundamental to the disease. Beyond mutations altering the activities of classic oncogenes and tumor suppressors, mutations in chromatin remodeling factors mechanistically contribute to tumor evolution by broadly affecting gene expression, contributing to the variation among which hallmark-enabling changes can be phenotypically selected during tumor evolution. Even in the absence of mutated chromatin remodeling factors, nonmutational epigenetic reprogramming can diversify the cancer cell populations (3). We should note that whereas epigenetic alterations broadly encompass any heritable molecular alteration that leads to a phenotypic change without modifying the DNA sequence, in this review, the term will largely refer to chromatin-based changes including modifications of DNA methylation, histone posttranslational modifications, and chromatin accessibility, which are directly associated with altered transcriptional programs. Cellular plasticity and epigenetic reprogramming guide the ordered phenotypical events underlying embryogenesis, organogenesis, mammalian development, and tissue homeostasis. As such, these processes are expected to play a role in cancer, by promoting lineage plasticity and adaptive responses to external stimuli. Nongenetic triggers of cellular plasticity can often be found in cell–cell interactions, in particular, those among cancer cells and the tumor microenvironment (TME). Cancer evolution is indeed also enabled by interactions with surrounding and infiltrating stromal and immune cells, which themselves change and evolve in response to tumor–TME interactions. Genetic mutations, epigenetic reprogramming, and cell interactions within the TME are general promoters of change, although arguably not the only ones that enable tumor evolution (Fig. 1). Herein we review recent findings illuminating the role of these enabling characteristics in tumorigenesis, malignant progression, and adaptive resistance to therapy, and the mechanisms through which alterations confer selective advantages to the tumors they create.

Mutagenesis and Genetic Cancer Drivers

Dividing cells accumulate mutations throughout a lifetime. Whereas most somatic mutations have neutral phenotypic effects, occasionally, mutations confer a selective advantage to the cell, potentially leading to clonal expansion of the mutated lineage and, eventually, mutated cells may develop into a tumor. The interplay between mutagenesis and selection has allowed us to model cancer evolution according to neo-Darwinian principles of species evolution.

Mutations emerge and are distributed across the genome as the result of DNA damage and inaccuracies in replication and repair processes. DNA lesions induced by damaging

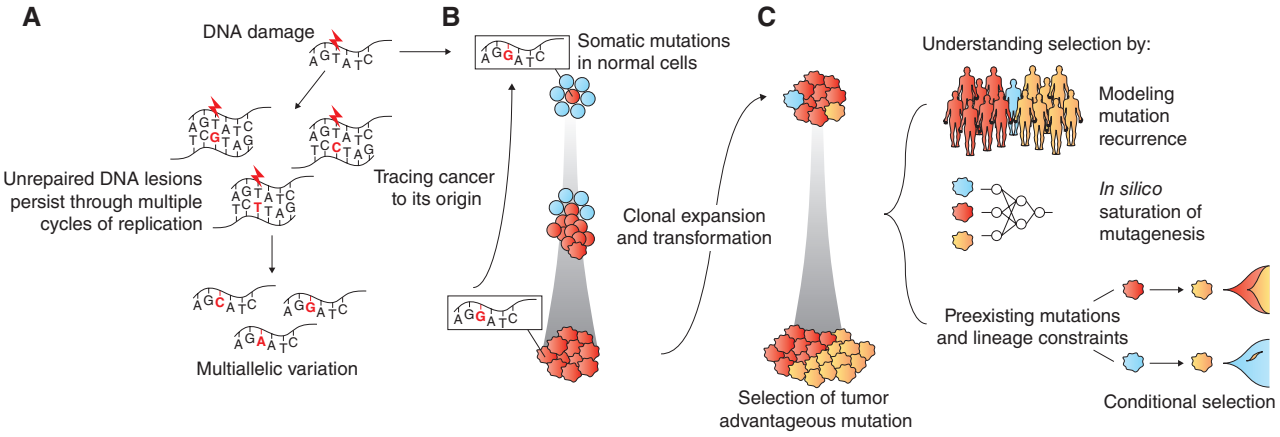

Figure 2. Emergence and selection of somatic mutations. **A,** The propagation of DNA lesions that are not resolved into mutations within one cell cycle leads to lesion segregation and multiallelic variation. **B,** Initiating genetic mutations observed in cancer cells can be found in normal cells at the tumor site allowing us to trace the origin of the tumor. **C,** Genetic instability during tumor progression leads to the acquisition and selection of tumor-advantageous variants. Variant selection can be studied by modeling the recurrence of somatic mutations across large tumor cohorts, developing machine learning approaches to predict variant oncogenicity, and investigating whether the selection of specific variants depends on either preexisting alterations or the cell of origin of the tumor.

agents or defective endogenous processes are for the most part efficiently removed or repaired. However, occasionally damage leads to mutations, such as single-base substitutions (SBS). SBS may not be resolved within a single-cell cycle. Consequently, DNA lesions in individual DNA strands can segregate unrepaired into daughter cells for multiple cellular generations, resulting in chromosome-scale strand asymmetry, termed lesion segregation (ref. 4; Fig. 2A). This novel mechanism has recently been shown to be a feature of DNA-damaging agents and shed new light into the mechanisms of action of known mutagens (4–6), including ultraviolet radiation and chemotherapy agents, and the etiology of multiple tumor types. The propagation of strand-specific lesions through multiple cell generations provides an engine for genetic diversity. Pervasive lesions can act as a template in successive rounds of replication, meaning that different incorrectly (or correctly) paired nucleotides can be incorporated opposite an individual lesion. This results in the observation of multiple alleles at the same position in the resultant cell population. For example, diethylnitrosamine (DEN) is known to produce the long-lived thymine adduct O^4-ethyldeoxythymidine on the sugar-phosphate backbone of DNA. These small mutagenic ethyl-adducts most commonly result in T>A SBS, but T>C and T>G SBS are also observed. This phenomenon is referred to as multiallelic variation (ref. 4; Fig. 2A). Multiallelic sites, which are often and mistakenly filtered out during variant calling, provide the opportunity to disentangle DNA damage and DNA repair mechanisms, and reveal, for example, that DNA substitution patterns across the genome are largely shaped by the influence of DNA accessibility and repair efficiency, rather than gradients of DNA damage (bioRxiv: 2022.06.10.495644). From an evolutionary perspective, multiallelic sites violate the infinite site assumption, a commonly held assumption in genetics and evolutionary models that forbids recurrent mutations at the same site (7), and, thus, require to rethink of traditional cancer evolutionary models that rely on such assumption.

DNA damage and, therefore, mutagenesis are fostered by endogenous mutational processes, for example, associated with aging and faulty DNA-repair mechanisms, and by exogenous mutagens, such as UV radiation, tobacco consumption, or dietary habits. In cancer cells, the resulting mutations accumulate over time, sometimes gradually, and others in a more punctuated or even catastrophic manner (8, 9), especially in stress responses (10, 11). Mutagens often act on specific nucleotide contexts, thus generating specific mutational signatures. It is therefore possible to infer the causes of DNA mutations from mutational signatures, which has become a powerful tool in the investigation of human cancers. Somatic mutations can also be used as a "barcode" to trace the developmental history of a cell and, in the case of cancer, to trace cancer evolution to its origins (ref. 8; Fig. 2B). By extending this search from cancer cells to surrounding normal tissues, one may be able to trace the earliest origin of cancers and its relationship to human development. This approach has been applied to some childhood cancers, revealing, for example, that the commonest childhood kidney cancer, Wilms tumor, often originates in clonal expansions residing in normal kidney tissue (12). This discovery, replicated in other tumor types (13–16), indicates that there may be a window of opportunity for preventing tumor formation, by interfering with the process that connects the earliest clonal expansion with malignant transformation. Ultimately, to faithfully recapitulate the series of multistep clonal expansions that led to the tumor and intervene in this dynamic process, it is critical to understand what triggered a clonal expansion, i.e., determine which mutations provided a selective advantage (Fig. 2C).

Mutations providing a selective advantage to the tumor cell are typically referred to as "driver mutations" or "cancer drivers." Cancer driver mutations not only alter the function of the corresponding protein, but they do so in a way that promotes cancer-enabling features such as tumor initiation, progression, invasion, or resistance to therapy. Whereas the identification of cancer drivers requires the possibility of

monitoring the dynamics of an evolutionary process, the initial discoveries of driver mutations and cancer genes relied on targeted functional approaches. In the past two decades, significant advances in genomics technologies have allowed us to unbiasedly assess the mutational status of the entire genome of thousands of tumors, and with that came the challenge of disentangling between driver mutations and mutations that do not contribute to tumor phenotypes, so-called neutral or "passengers." When analyzing mutation occurrences across multiple tumors, the task is to determine whether the mutational pattern observed in a gene or DNA region can be explained by neutral mutagenesis; hence, the mutation would be classified as passenger, or if, by contrast, it constitutes a signal of positive selection, as would be expected by a driver mutation (17). Approaches based on this principle largely relied on estimating the expected frequency of a given mutation or mutated gene under the hypothesis of neutral evolution, to then statistically assess when observed frequencies exceed expectation (Fig. 2C). A critical challenge for these approaches is the correct estimation of the mutational probability of each nucleotide under neutral mutagenesis (17–19). As already mentioned, DNA accessibility and repair efficiency have been shown to be key determinants of local mutation rates in our genome (refs. 20, 21; bioRxiv: 2022.09.14.507952), but additional studies are required to improve our models of the background mutation rate under neutrality, especially in the noncoding genome (22). That notwithstanding, a general approach, even without using prior knowledge, can effectively rediscover well-known cancer genes, and it has been adopted to identify new cancer genes in several large-scale cancer genomics studies, eventually leading to the generation of multiple catalogs of cancer drivers (17, 22, 23). Recently, with increasing data availability, new machine-learning approaches have emerged to predict the pathogenicity of individual mutations independently of their actual occurrence (refs. 24, 25; Fig. 2C). Overall, these approaches have shown that only a minor fraction of the mutations observed in a tumor can be classified as drivers, typically less than 10 per tumor (18). However, these estimates should be taken with caution, as the phenotypic consequences of a mutation are unlikely to be binary (driver or passenger), but they rather describe a gradient, and they may be transient and/or dependent on the emergence of specific conditions.

Even with a well-annotated map of mutational processes and cancer drivers, the route to tumor initiation and progression remains sometimes elusive. Indeed, the discovery of ubiquitous clonal selection of cancer drivers in normal tissue mosaicism (26–28) confirmed that human tumorigenesis requires specific combinations of genetic and, possibly, nongenetic alterations (29). Analyses of large tumor cohorts have shown that these combinations are often nonrandom (30, 31): certain sets of mutations are more frequently observed in the same tumor than expected by chance (cooccurring mutations), while others are rarely or never found together (mutually exclusive mutations). Nonrandom comutation patterns could help draw evolutionary trajectories, from the emergence and accumulation of mutations in normal cells to tumor initiation, progression, and metastatic invasion (Fig. 2C). Intriguingly, comutation patterns only emerged among driver mutations, whereas no significant cooccurrence or mutual exclusivity was observed among synonymous SBS or mutations of unknown significance (32). These results indicated that comutation patterns provide evidence of selection. Importantly, such evidence is independent of individual mutation frequencies and, thus, could be used as orthogonal features in the search for new cancer drivers.

Overall, mutagenesis and selection are not only key mechanisms of cancer evolution but have become critical ingredients in modern cancer therapy. The discovery of driver mutations has fueled the development of targeted treatments to selectively kill only cells harboring specific oncogenic mutations and, as a consequence, introduced the need for accurate DNA mutation sequencing in the clinic. In this context, unraveling tumor evolutionary dynamics will be critical to predict treatment response based on the combination of mutations observed in a patient, and anticipate and monitor the emergence of new mutations leading to treatment resistance.

Lineage Identity: Hardwired Constrains on Tumor Evolution

Somatic mutations in most cancer-driver genes are detected only in a small fraction of tumor types (23), and inherited genetic cancer predisposition variants typically affect only a limited set of tissues (33). Hence, cell identity, defined by tissue-specific transcriptional programs, has a profound impact on the phenotypic output of oncogenic mutations (Fig. 2C). For example, the transcription factor SOX10 specifies a developmental stage in the neural crest–melanoblast–melanocyte trajectory in which *BRAF*, an oncogene mutated in 50% of cutaneous melanomas, is capable of cellular transformation and tumor initiation (34). Interestingly, acral melanomas, which develop on the palms and feet, depend on a different set of lineage transcription factors and mutations (35). Lineage factors can thus establish a permissive cellular state that facilitates oncogenesis by some genetically activated pathways but not others. In line with this, the renal lineage factor PAX8 is required for oncogenic signaling by common kidney cancer-associated genetic alterations (36). Inactivation of the VHL tumor suppressor is the initiating genetic event in 90% of clear cell renal cell carcinomas (ccRCC). VHL loss leads to HIF2A stabilization, which supports tumor development and metastatic progression through tissue-specific expression of oncogenic drivers, such as the cell-cycle regulator CCND1 (37, 38). The ability of the VHL–HIF2A pathway to regulate *CCND1* mRNA expression is dependent on PAX8, suggesting a possible explanation for the tissue-restricted tumor-suppressive role of VHL. A PAX8-dependent lineage factor program is also required for *MYC* expression from ccRCC metastasis-associated genetic amplicons (36). This functional convergence of lineage factors and oncogenic programs in the regulation of universally important cancer driver genes such as *CCND1* and *MYC* may open opportunities for inhibiting canonical oncogenic programs in a tissue- and cancer type–specific manner.

Indeed, cancer cells are often exquisitely sensitive to the inhibition of lineage transcription factors (39). Although transcription factors have long been viewed as "undruggable," their gene-regulatory activities typically depend on specific cofactor interactions that can be exploited for drug development (40, 41). In addition, pathways controlling the dynamic

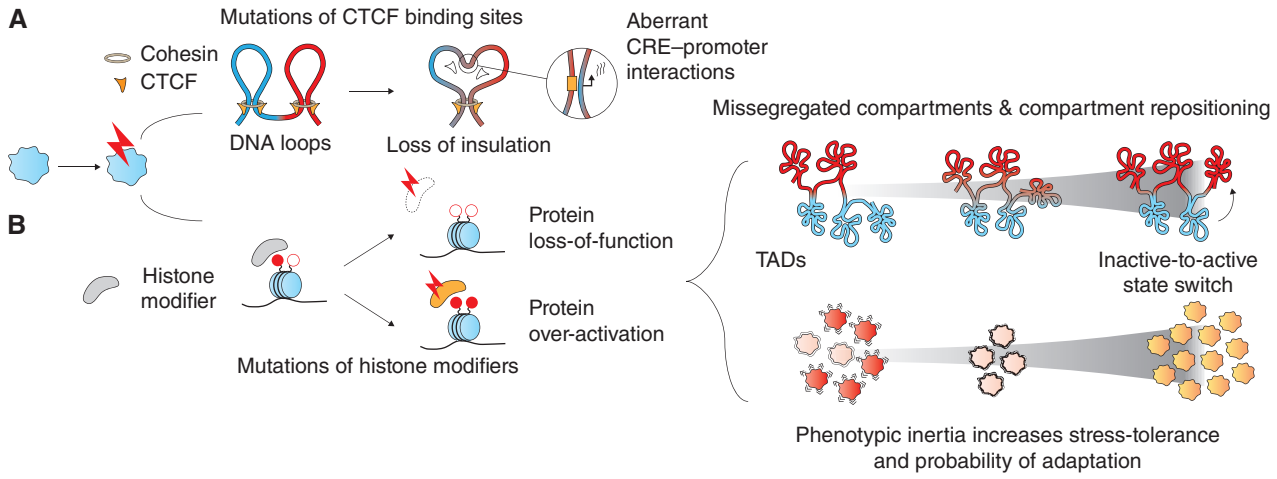

Figure 3. From somatic mutations to epigenetic reprogramming. **A,** Somatic mutations targeting CTCF binding sites can impair CTCF binding and insulation between chromatin loops or topologically associating domains (TAD). Loss of insulation leads to aberrant interactions between gene promoters and cis-regulatory elements (CRE). **B,** Somatic mutations of histone modifiers may lead to altered histone modification (e.g., methylation and acetylation). Aberrant histone marks have been associated with missegregated chromatin compartments and compartment repositioning, as well as the emergence of a state of "phenotypic inertia," which allows tumor cells to tolerate oncogenic stress and ultimately adapt.

transcriptional, posttranscriptional, and posttranslational regulation of transcription factors provide alternative entry points for pharmacologic interference. For example, the transcription of *MYC* is exquisitely sensitive to BET bromodomain inhibition in various hematopoietic malignancies (42, 43), while PROTACs and molecular glues enable the rapid elimination of specific transcription factors through proteasomal degradation (44, 45). To systematically explore such alternative molecular targets, a more detailed understanding of the pathways that control the expression and turnover of specific transcription factors is highly desirable. Time-resolved FACS-based CRISPR/Cas9 mutagenesis screens have recently been used to systematically characterize regulators of the essential transcription factor MYC in diverse cellular contexts (46). Besides context-specific regulators of MYC, these screens uncovered a conserved pathway that controls the nuclear import of proteasomes, and thereby the proteasomal turnover of transcription factors and other nuclear proteins. Extending this screening approach to other oncogenic transcription factors will further help to distinguish broadly relevant from factor- and context-specific regulators and cofactors.

Somatic Mutations Through the Lens of Chromatin 3D Structures

The development of chromosome conformation capture technologies has allowed us to study cancer somatic point mutations and structural variants in a new light, by exploring their effect on the three-dimensional (3D) structure of the chromatin in the nucleus as well as the impact of 3D organization on the functional consequences of the mutation. Chromosome conformation capture technologies determine the frequency with which two genomic loci are found in close 3D proximity, also referred to as contact or interaction frequency. Using high-throughput chromosome conformation capture (Hi-C), it is possible to derive genome-wide maps of contact frequencies to estimate features of chromatin folding and segregation (47). These approaches have revealed that, during the interphase, chromatin is organized into a hierarchy of structural elements, ranging from a few kilobase-long loops to submegabase topologically associating domains (TAD) and compartment domains, which, at a broader scale, cluster into a handful of compartments, each characterized by specific epigenetic features (48–51).

The potential for chromatin 3D structures to explain or even determine the oncogenic capacity of genetic alterations is probably most evident in TADs. TADs represent genomic regions that display increased interaction frequencies and where loops between cis-regulatory elements (CRE) and their target genes are usually contained (52). As such, TADs facilitate proper CRE–promoter and promoter–promoter interactions, and insulation between adjacent TADs reduces improper or disease-promoting interactions. In their role of scaffolds for regulatory interactions, TADs shed new light on the impact of hotspot mutations of the PRC2 catalytic unit *EZH2*, which generally lead to increased histone-3 lysine 27 trimethylation (H3K27me3) genome wide. Interestingly, the spreading of H3K27me3 in tumors with EZH2 gain-of-function mutations was found frequently confined within the boundaries of selected TADs, resulting in the downregulation of all genes in the domain. When multiple tumor suppressors were contained in the same domain, this downregulation synergistically drove tumorigenesis (53). Structural variants affecting TAD boundaries can induce novel interactions between CREs and genes that were previously isolated (refs. 54, 55; Fig. 3A). Such aberrant interactions have been associated with developmental disorders (56, 57) and cancer (58–61). Additionally, alteration of TAD boundaries may occur by preventing or reducing the binding of the chromatin architectural protein CTCF, which is essential for TAD formation and maintenance (62, 63). Indeed, high-density CTCF binding at TAD boundaries ensures insulations between adjacent TADs. DNA hypermethylation of

CTCF binding sites in *IDH*-mutant gliomas or *SDH*-mutant gastrointestinal stromal tumors reduced protein binding and TAD insulation leading to spurious activation of oncogene expression (64, 65). Similarly, lower levels of CTCF in childhood acute lymphoblastic leukemia (ALL) led to reduced TAD insulation and consequent gene misregulation (66), consistent with CTCF being a quantitative modulator of gene activation across TADs (67).

More recently, CTCF levels were shown to be significantly reduced in cells undergoing whole-genome doubling (WGD), causing loss of insulation at TAD boundaries (68). Interestingly, CTCF downregulation was a result of a more general inability of upscale protein synthesis in WGD cells (69). Indeed, beyond CTCF, WGD cells exhibited reduced histone-3 lysine 9 trimethylation (H3K9me3), a marker of heterochromatin and a driver of chromatin compartmentalization. Lower H3K9me3 led to reduced segregation of chromatin compartments in WGD cells and longitudinal analyses of tumors originating from WGD cells showed that regions losing compartment segregation ultimately were ultimately found associated with a different compartment, having altered their overall spectrum of chromatin contacts, and exhibiting new histone posttranslational modifications and CRE–promoter loops associated with oncogene activation (68). Compartment repositioning of genomic regions has been more frequently observed across cell types and cell states than changes in TAD boundaries (70, 71) and has been reported in cancer in association with altered histone modifications (refs. 72, 73; Fig. 3B). Given the high prevalence of cancer mutations targeting chromatin remodeling factors (74), it will be interesting to explore the dynamic interplay between mutated histone modifiers, epigenetic changes, and subcompartment repositioning, and whether it can further explain the selective advantage provided by these mutations. Overall, these results suggest that modifications of chromatin 3D features are not simple bystanders or consequences of epigenetic alterations but could themselves initiate epigenetic and transcriptional reprogramming. Longitudinal analyses tracing chromatin structural changes in single cells will shed new light on the role of chromatin plasticity in tumor evolution.

Beyond their effect on chromatin 3D structures, recurrent mutations of chromatin remodeling factors induce genome-wide epigenetic changes that could favor cancer cell plasticity. This hypothesis was recently functionally tested via large-scale CRISPR-based mutagenesis (75). Inactivation of over 100 epigenetic regulators of diverse functions converged into a common phenotype characterized by increased tolerance to environmental stress, which is selected during tumor growth. In this particular case, disruption of epigenetic control does not appear to enhance the phenotypic plasticity of cancer cells but rather prevents cells from mounting an efficient stress response at the transcriptional level. The resulting cell state was thus characterized by phenotypic inertia (ref. 75; Fig. 3B), defined as the inability of the cell to halt proliferation and activate an apoptotic program in response to unfavorable environments.

Overall, cell epigenetic features, such as DNA methylation and histone posttranslational modifications, characterize physiologic states during normal cell differentiation and can be co-opted to promote malignant cell transformation (76). Interestingly, the oncogenic capacity of somatic mutations has been shown to depend on such cell states, as exemplified by differential oncogenic competence of melanocytes, neural crest, and melanoblasts in melanoma development (34, 77). Whether driven by genetic variants or nongenetic mechanisms, the emergence of heterogeneous phenotypes in a cell population enables tumor adaptation and evolution.

From Genetic Mutations to Cancer Cell States

Intratumor heterogeneity and its relationship with cancer evolution have been often investigated in terms of genetic mutations, which can be ubiquitously observed across all cancer cells or present only on a subset of them, determining distinct subpopulations. In recent years, the notion of heterogeneous "cancer cell states" in otherwise genetically identical cells has gained traction. Evolving cancer cell states characterize the progression of several tumor types and can emerge independently of specific genetic mutations through cancer cell plasticity. Cell plasticity often ensues in response to epigenetic and transcriptional reprogramming associated with the progression of the disease, tumor–TME interactions, micro-RNA (miRNA) regulation, and treatment administration (Fig. 4A). In contrast to lineage-defined cell states, which characterize developmental stages of normal cell lineages, cancer cell states have been associated with phenotypic properties of cancer cells, which can be transient, induced, and reversible (78). Investigating functional diversity within transformed cells is a complex and possibly unfeasible endeavor. Therefore, the existence of heterogeneous states is routinely inferred from the transcriptional and/or epigenetic diversity of individual cells within a tumor, with different "states" potentially integrating static (genetic) and dynamic and reversible (epigenetic and transcriptional) determinants (79–81). Cancer cell states have been largely inferred algorithmically from single-cell RNA-sequencing (scRNA-seq) data sets by describing mRNA expression of ~20,000 genes through a much smaller number of nonredundant transcriptional programs (82, 83). Such transcriptional programs were either themselves defined as cell states, or clustered into cell states, consistent with the notion that cell phenotypes are the result of concurrent activation of different biological processes. The notion that cancer phenotypes are encoded by combinations of genetic and nongenetic modifications motivated the further development of new single-cell multiomics technologies that integrate genotypes, transcriptomes, and epigenetic profiling (refs. 84–87; Fig. 4A). The integration of transcriptional activity and chromatin states and accessibility in single cells provide a more comprehensive description of cell states allowing to infer a fingerprint for transcription factor activity and cell state transitions (88–90). Joint high-throughput capture of genotypes and transcriptomes revealed a complex interplay between somatic mutations and cell states (91) with somatic mutations associated with cell progenitor-type specific aberrations, years before malignant transformation (92, 93). Stochastic, heritable DNA methylation changes can also be leveraged as native lineage barcoding marks for high-resolution phylogenetic reconstruction of single cells in primary human cancer samples (86). Excitingly, the ability to obtain high-resolution evolutionary trees together with the concomitant transcriptional

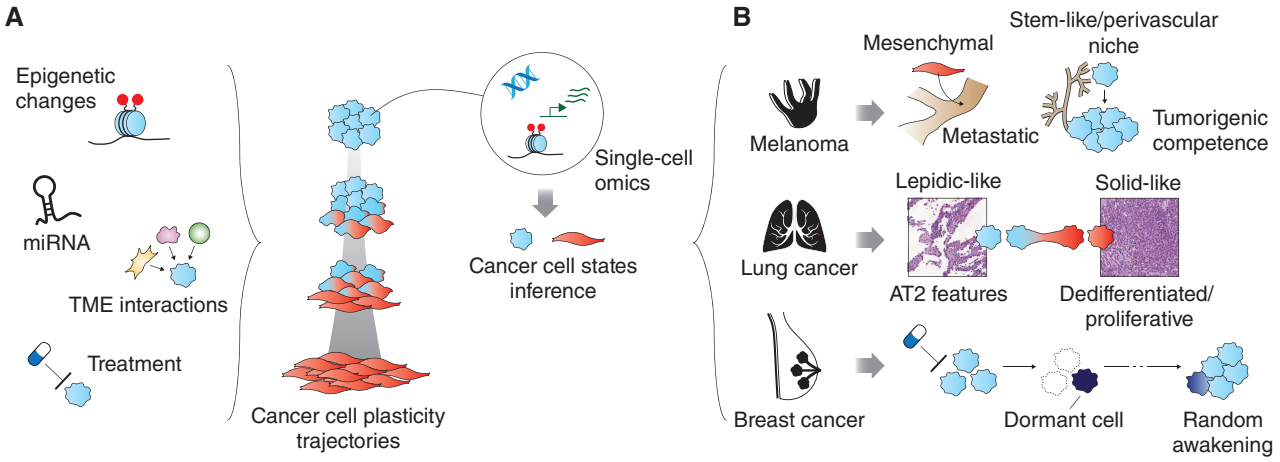

Figure 4. Cancer cell plasticity and cancer cell states. **A,** Histone modifications, miRNA, and interactions with the TME induce epigenetic and transcriptional reprogramming that alters cancer cell identity. This process defines cancer cell plasticity transitions among phenotypically heterogeneous cell states that can be inferred from single-cell molecular profiles. **B,** Examples of such cell state transitions have been reported in skin melanoma, where mesenchymal and stem-like states have been associated with metastatic and tumorigenic capacity, respectively, in lung adenocarcinoma, where disease progression was associated with morphologic differences and transition from an alveolar-type 2-like state (AT2-like) to a dedifferentiated and proliferative cell state; and in breast cancer, where resistance to therapy was shown associated with cell dormancy and random awakening mediated by epigenetic reprogramming.

annotation of leaves allowed the direct measurement in human tissues of the heritability of cell states (94).

Cancer Cell States in Tumor Progression and Treatment

The existence of cancer cell states has many implications: (i) genetically identical cells may be phenotypically heterogeneous and thus implement different functions to foster disease progression and treatment resistance; (ii) treatment and microenvironmental conditions are likely to impact cancer cell properties and, thus, drive state transitions; (iii) opportunities exist to reprogram cells to a therapy-sensitive state. To test these hypotheses, there is a need to develop models that recapitulate not only genetic diversity in the tumor but also the presence and transitions of dynamic states.

Examples of cell plasticity transitions have been recently reported in several tumor types. In this context, micro-RNA regulation of gene expression has been shown to determine extensive transcriptional reprogramming in the development and progression of pancreatic neuroendocrine tumors (PNET; Fig. 4A). The RIP-Tag mouse models undergo stereotypic development of PNET through distinctive histologic stages, arising from the insulin-producing beta cells of the pancreatic islets in response to the targeted expression of the SV40 large T antigen (Tag) oncoprotein, which abrogates the functions of the RB and TP53 tumor suppressor protein (95). Leveraging the possibility to physically isolate and individually molecularly profile premalignant lesions as well as solid tumors and liver metastases, the micro-RNA transcriptome revealed that sets of microRNAs were up- and downregulated during each of the stepwise transitions in this tumorigenesis pathway (96). Additionally, pancreatic tumors could be subclassified by micro-RNA (miR) signatures into comparatively benign (noninvasive) islets tumors (IT) and invasive and metastasis-like tumors (MLP). Focusing on the miR signature that defines malignant progression from IT to MLP, functional perturbation has revealed that many of the miRNAs in the signature of malignant progression from IT to MLP enable hallmark capabilities: upregulation of miR137 stimulates invasive growth (97), whereas the miR23b cluster enables metastatic seeding, in part by suppressing expression of the ALK7 receptor (98). Additionally, one facet of cellular plasticity—dedifferentiation—has been exceptionally revealed to be a distinctive step, separable from increases in aberrant proliferation, in the course of malignant progression. Upregulation of miR181cd, a component of the MLP signature, instructs dedifferentiation along the pathway by which endocrine progenitor cells normally differentiate into mature insulin-producing beta cells, by altering the expression of developmental transcription factors that orchestrate a progenitor-like phenotype in MLP tumors (99). Although the mechanisms of this dynamic regulation of dozens of microRNAs during tumorigenesis and malignant progression remain to be elucidated, there is no evidence to ascribe its basis to mutational evolution of the cancer cell genome, which rather most logically involves nonmutational epigenetic reprogramming, fertile ground for continuing investigation.

Similarly to PNET, lung adenocarcinoma progression also involves a transition through histologically defined stages, with the lepidic and solid histology associated with the most indolent and most aggressive manifestations of the disease, respectively (100). Histopathology-guided multiregion sampling of lung adenocarcinoma has shown that histologically distinct regions are predominantly characterized by epigenetic and transcriptional differences (101). These differences trace a transition from a lepidic-like cell state, characterized by retained expression of alveolar-type 2 (AT2) lineage markers and transcription factors (*NKX2-1* and *FOXA2*), toward a less differentiated state that no longer expresses AT2 markers and activating proliferation and stemness factors such as *FOXM1* and *MYBL2* (Fig. 4B). When enhanced proliferation is not the major selective force, tumors may evolve in unexpected

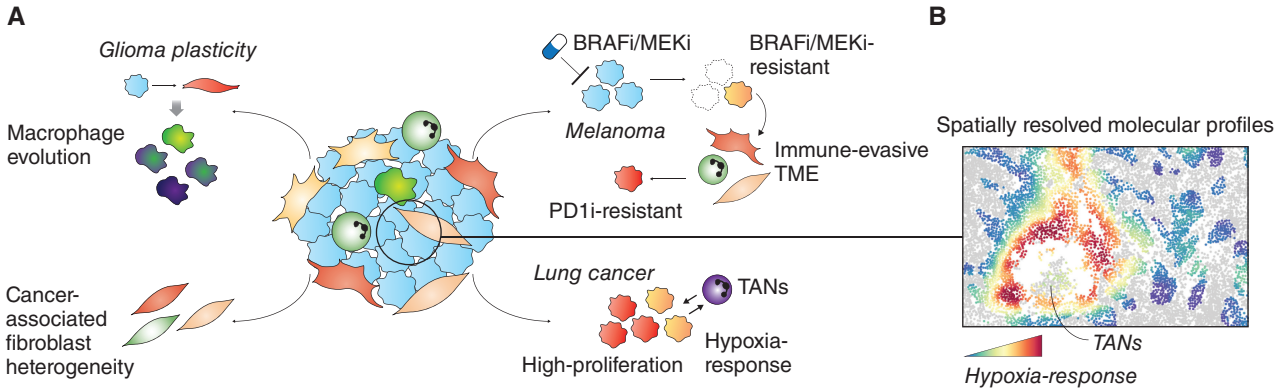

Figure 5. Interactions with an evolving TME. **A,** Along with tumor cells, the surrounding TME also evolves often acquiring protumor features. Examples include reeducation of macrophages in treatment-resistant glioma, phenotypically heterogeneous populations of cancer-associated fibroblasts, treatment-induced generation of a tumor-evasive TME, which is associated with dual resistance to targeted and immunotherapies in melanoma, and interactions between tumor-associated neutrophils (TAN) and lung cancer cells, which toward a less proliferative, but hypoxia responsive cell state. **B,** Spatially resolved molecular profiles provide a critical tool to investigate tumor–TME interactions.

manners. This is often the case when therapeutic intervention leads to the formation of persister cells, a cell state characterized by treatment tolerance and transient inability to proliferate. In a hormone-dependent breast cancer model, dormant persister cells are directly induced by cytostatic treatment (102). A long-term evolutionary experiment has now revealed that the dormant persister pool is stochastically generated (bioRxiv: 2021.04.21.440779, 2022.02.15.480537) via the accumulation of heterochromatin modifications. The epigenome of dormant cells is then reversed in awakening clones, leading to tumor relapse and overt drug resistance (Fig. 4B). In skin melanoma, drug-tolerant persister cells harboring a neural crest stem cell phenotype can emerge through drug-induced reprogramming (103), rather than selection of a preexisting cell population, and that this population promotes emergence of drug resistance through a nongenetic mechanism. These findings further support epigenetic mechanisms as major drivers of tumor evolution in these tumor types (104–106).

Cell states, just like mutations, are potential therapeutic targets (79) and may become the main targets of tumors with currently no actionable mutations. An interesting example is a high mesenchymal cell state driven by increased expression of a specific master regulator, *ZEB1*, which, on the one hand, is associated with aggressiveness and resistance to therapy and, on the other, generates unique therapeutic vulnerabilities (107). Along the same line, the high transcriptional diversity of pancreatic ductal adenocarcinoma has been linked to microenvironmental changes, which affect sensitivity to therapy (ref. 108; bioRxiv: 2022.12.12.520054). Because cell states are also driven by local microenvironmental conditions, it follows the need to integrate such state diversity with spatial information within the tumor, a task now made possible by advances in spatial omics approaches (109, 110). A recent study leveraged time-resolved single-cell analyses and spatial transcriptomics, to establish a high-resolution four-dimensional (space and time) map of the drug-naïve melanoma ecosystem and study its evolution under therapy (111). This map revealed the presence of 6 evolutionarily conserved melanoma transcriptional states: melanocytic, mesenchymal-like, neural crest-like, antigen presentation, and stress, associated either with a p53 or hypoxia response. These cell states exhibited distinctive tissue localization and their presence was associated with divergent responses to immune-checkpoint blockade. Moreover, these cell states decoupled the proliferative and metastatic potential of melanoma cells. Indeed, single-cell tracing revealed that the mesenchymal population are responsible for metastasis initiation while they do not support primary tumor growth, which is instead associated with a stem-like population forming a perivascular niche (ref. 111; bioRxiv: 2022.08.11.502598; Fig. 4B). The observation that oncogenic competence can depend on specific microenvironmental signals warrants the development of therapeutic strategies that could interfere with such signals and their potential for cancer cell plastic reprogramming.

Overall, multiple mechanisms can induce epigenetic and transcriptional reprogramming within tumor cells, independently of genetic mutations. Transcriptional reprogramming in turn leads to the emergence of heterogeneous cell states which may undergo Darwinian selection. This heterogeneity appears associated with the tumor's spatial architecture, temporal progression, and resistance to therapy.

Coevolution in the TME

Detailed analyses of cancer and noncancerous cell populations that compose the tumor ecosystem revealed that heterogeneity and evolution are not concepts restricted to cancer cells. As already alluded to in the previous sections, mutation-induced cancer cell signaling and plastic cancer cell state transition inevitably affect and are affected by the TME. Indeed, a plethora of factors directly modulate immune and stromal cell features and, with evolutionary changes occurring in tumor cells, the TME and interactions between the TME and cancer cells also change, often contributing critically to therapy resistance (112–114).

Examples of coevolving tumor cells and TME under treatment have been recently shown in melanoma and glioblastoma (Fig. 5A). Indeed, when BRAF-mutated melanomas acquire resistance to MAPK pathway-targeted therapies (RAFi/MEKi), tumors simultaneously acquire cross-resistance to immunotherapy (115). This cross-resistance is surprising given the entirely different mode of action of these therapies. Functional analyses on immune-competent mouse models revealed that

cross-resistance was mediated by an immune-evasive TME lacking antigen-presenting cells. This immune-evasive TME was cancer cell–instructed, and it evolved from an increased transcriptional output of the reactivated MAPK pathway in tumors that bypassed MAPK-signaling inhibition, rather than from selective pressure to overcome immune response. Similarly, adapting cancer cells posttreatment can shape the evolution of subpopulations of tumor-associated macrophages (TAM; refs. 116–118). In treatment-resistant glioblastoma, glioma cells shifted their subtype toward a mesenchymal-like state and induced transcriptional reprogramming of TAMs, turning them into "food providers." These timed and regulated alterations of TAM metabolic capacities reactivated developmental properties of brain scavenger cells (119) leading to the coevolution of TAMs and glioblastoma cells to enable proliferation and immunosuppression fueling relapse posttreatment (120).

Systematic and unbiased interrogations of heterotypic cellular interactions within the TME have been enabled by scRNA-seq and, more recently, spatial transcriptomics and proteomics approaches. In particular, the recent application of image mass cytometry and other spatial proteomics or transcriptomics technologies has allowed to spatially resolve cell type admixing within the TME and, thus, to define TME niches as markers of disease progression and response to therapy (121–126). Granular subclassification of cell types determined phenotypic heterogeneity for multiple immune cell types (127, 128). For instance, recent studies have shown that cancer-associated fibroblasts diversify into states with distinct functions, ranging from immunomodulatory fibroblasts (iCAF) that can suppress lymphocyte activation, to myofibroblasts that remodel extracellular collagen matrices (129–132). Importantly, spatial "omics" technologies can now bridge the gap between the identification of cancer cell states and spatial TME niches, to determine when an association and interactions exist between the two (Fig. 5B). For example, macrophages and lymphocytes were found associated with the acquisition of an interferon-activated phenotype characterized by noncanonical expression of antigen presentation pathways in nearby cancer cells (83). In lung adenocarcinoma, cancer cell states emerging with the progression of the disease interacted with vastly different TMEs, with transitions between immune cold, inflamed, and excluded environments being observed within the same tumor, in correspondence with distinct cancer cell states (101). In particular, tumor cells exhibiting a response-to-hypoxia and migratory phenotype were found colocalizing with tumor-associated neutrophils (bioRxiv: 2023.01.10.523386; Fig. 5A–B). Consistently, cells in this state with expression of neutrophil recruiting chemokines, such as CXCL1 and CXCL3, further indicating that interactions between tumor cells and the TME shape cancer cell state emergence and transition (bioRxiv: 2023.01.10.523386). Intriguingly, the physical properties of the TME can themselves determine epigenetic adaption. Indeed, interstitial pressure-driven fluid flow was shown to induce an autocrine signaling circuit comprised of secreted glutamate that activates the NMDA receptor, stimulating invasive tumor growth (133, 134).

The advent of technologies capable of probing single-cell spatial organization and interactions at high resolution and throughput will provide critical clues into TME components at different stages of the disease and how these can determine cancer cell plasticity. Ultimately, charting this evolving landscape of tumor and nontumor cells will enable the development of therapeutic strategies targeting cellular interactions and cancer–TME niches (135). The new frontier lays in identifying the appropriate longitudinal model, especially in the clinical setting, which will allow enough temporal resolution to match these technological advances.

DISCUSSION

Although cancer evolution has mainly been viewed through a genetic lens, mutations are just one face of the complex prism of forces that underpin tumor evolution (8, 136, 137). Nonmutational transcriptional and epigenetic reprogramming and interactions among tumor cells and the TME equally provide numerous opportunities for diversification that may favor cancer phenotypes. Even more, environmental pressures and treatment can induce adaptive responses, establishing non-Darwinian evolutionary routes (10, 138). Examples reviewed in this manuscript highlight how different evolutionary mechanisms are not acting in an independent or, even less, mutually exclusive manner. Genetic variants can induce and modulate cancer cell plasticity, which, in turn, can favor mutagenesis or determine the oncogenic impact of specific mutations. Similarly, interactions among stromal and tumor cells can induce transcriptional reprogramming, leading to the emergence of heterogeneous cancer cell states exhibiting different immunogenicity and, thus, establishing different immune microenvironments. To decipher the interactions among biological processes that have been historically the focus of different scientific communities requires fostering conversations and collaborations crossing the barriers among such communities. As in the old parable of "the blind men and the elephant," the incomplete experience of a single field will not be sufficient to capture the whole picture.

Toward this goal, the increasing complexity of high-throughput technologies and data has enabled to integrate multiple layers of molecular information within each cell, which could allow to directly measure how different evolutionary mechanisms concurrently alter cell phenotypes. These data sets could be used to generate novel hypotheses, but they require rigorous statistical approaches to be correctly interpreted and need to be coupled with appropriate experimental models. Indeed, on the one hand, data-driven approaches have the power to decode the complexity of human tumors but provide limited mechanistic insight. On the other hand, experimental models allow us to decipher the mechanisms generating specific observations but are often limited in the number of variables they can model and remain proxies of human disease.

In this review, we only touched upon some of the many facets of cancer evolutionary mechanisms. We have, for example, not discussed the reprogramming of cellular metabolism that enables tumors to meet their nutrient requirements and opportunistically adapt to nutrient scarcity. Multiple metabolic pathways have been implicated in tumor growth, and metabolic reprogramming has been associated with cell differentiation and interactions with the TME (139–141). Currently, several trials are incorporating metabolic intervention in combination with classic approaches, highlighting the patient benefit of exploring this facet of tumor evolution. Analogously, the composition of the human gut microbiota has been shown

to be a critical effector of response to cancer immunotherapy and possibly influencing response to other treatments. Interactions between cancer cells and the microbiota are mediated by secreted small molecules and have been further implicated in tumorigenesis and progression (142, 143). Metabolic reprogramming and microbiota interactions are just two examples of additional mechanisms that are likely to promote and influence the evolution of the disease. Along with different mechanisms, different evolutionary models should be evaluated to explain experimental and clinical observations. A recent study showed that rather than the emergence and selection of new alterations, air pollution–driven inflammation enables the oncogenic potential of preexisting mutations in the lung driving lung adenocarcinoma in never smokers (144). These observations are reminiscent of genetic studies investigating the role of evolution of preexisting variation in a population (145, 146). In cancer, such "preexisting variation" could be embodied by somatic mutations accumulated before tumor formation, as well as germline variants, and their phenotypic effect being neutral in the absence of specific endogenous (e.g., cancer cell states) or exogenous (e.g., TME interactions) conditions.

As we deepen our understanding of cancer evolution from a fundamental scientific view, we may be able to translate some actionable lessons into clinical interventions. Critical questions in this direction are whether we can monitor, anticipate, and, ultimately, interfere with the evolution of the disease (147–149). To address these questions, large-scale clinical research studies have been implemented by the TRAcking Cancer Evolution through therapy (Rx; TRACERx) consortium, which, for example already provided actionable classifications of renal carcinoma based on evolutionary trajectories detected in patients (150, 151), and reveal unexpected genetic and nongenetic routes of lung cancer evolution (152, 153). A deeper understanding of tumor evolutionary mechanisms and trajectories offers the opportunity to design strategies to interfere with them and optimize the chances of therapeutic success (11, 154–156).

Targeted deep DNA sequencing is now routinely performed in most cancer hospitals and often at multiple timepoints. The scale and resolution of these data sets could represent a critical resource to trace recurrent cancer evolutionary trajectories. In particular, with the advent of modern artificial intelligence approaches in precision medicine, data scale and complexity will become an asset rather than a limitation (157). Initiatives toward increasing the spectrum of molecular data generated in the clinic, standardizing and sharing data collection, and creating cancer registries matching anonymized molecular data and clinical annotation will ultimately transform our ability to predict the evolution of the disease in patients.

Authors' Disclosures

S.J. Aitken reports grants from the Medical Research Council during the conduct of the study. S. Behjati reports grants from Wellcome Trust during the conduct of the study. A.C. Obenauf reports other support from Boehringer Ingelheim outside the submitted work. E. Oricchio reports grants from the Swiss National Foundation and Swiss Cancer League during the conduct of the study. P. Scaffidi reports grants from the Francis Crick Institute during the conduct of the study. G. Tonon reports grants from the Italian Association for Cancer Research (AIRC) during the conduct of the study; personal fees from Adaptive Biotechnologies outside the submitted work; in addition, G. Tonon has a patent for single-cell technology GET-seq licensed to Millipore. S. Vanharanta reports grants from Sigrid Jusélius Foundation during the conduct of the study. J. Zuber reports personal fees from Quantro Therapeutics GmbH, Mirimus Inc., and grants and nonfinancial support from Boehringer Ingelheim outside the submitted work. No disclosures were reported by the other authors.

Acknowledgments

We acknowledge the European Molecular Biology Organization (EMBO) for their support in facilitating the EMBO workshop "The Many Faces of Cancer Evolution" that convened the contributors of this perspective. The discussions from this assembly were instrumental in the development of this piece.

Received May 4, 2023; revised August 29, 2023; accepted October 23, 2023; published first December 6, 2023.

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