Towards a systems-level probing of tumor clonality

Emanuelle I. Grody[1,2,3,5], Ajay Abraham[1,3,4,5], Vipul Shukla[1,3,4,*], Yogesh Goyal[1,2,3,*,#]

[1]Department of Cell and Developmental Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA
[2]Center for Synthetic Biology, Northwestern University, Chicago, IL 60208, USA
[3]Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA
[4]Center for Human Immunobiology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA
[5]These authors contributed equally
*Authors for correspondence: vipul.shukla@northwestern.edu and yogesh.goyal@northwestern.edu
[#]Lead contact: yogesh.goyal@northwestern.edu

Summary

Cancer has been described as a genetic disease that clonally evolves in the face of selective pressures imposed by cell-intrinsic and extrinsic factors. Although classical models based on genetic data predominantly propose Darwinian mechanisms of cancer evolution, recent single-cell profiling of cancers has described unprecedented heterogeneity in tumors providing support for alternative models of branched and neutral evolution through both genetic and non-genetic mechanisms. Emerging evidence points to a complex interplay between genetic, non-genetic, and extrinsic environmental factors in shaping the evolution of tumors (**Figure 1**). In this perspective, we briefly discuss the role of cell-intrinsic and extrinsic factors that shape clonal behaviors during tumor progression, metastasis, and drug resistance. Taking examples of pre-malignant states associated with hematological malignancies and esophageal cancer, we discuss recent paradigms of tumor evolution and prospective approaches to further enhance our understanding of this spatiotemporally regulated process.

Clonality as a cancer cell-intrinsic property

Cell-intrinsic differences between cancer cells can emerge from a range of genetic sources including point mutations, copy number changes, and large structural variations [1]. Differences can also result from several non-genetic mechanisms, including transcriptional fluctuations [2], epigenetic reprogramming [3], metabolic plasticity [4], and altered protein conformations [5]. Unlike the duality (presence or absence) of genetic alterations that could justify classical models of selection, non-genetic mechanisms can exist on multiple continua, further providing conceptually rich frameworks for newer models of tumor evolution, metastases, and responses to anti-cancer therapies[6–24].

Cell-intrinsic non-genetic differences can either be pre-existing within a population or can be induced by the selective pressures (e.g., anti-cancer therapies) [6–8,25–28]. These modes are not necessarily mutually exclusive and are often attributed to phenotypic plasticity, such as the well-established paradigm of epithelial-mesenchymal transition [29]. Collectively, the genetic and non-genetic variabilities can impart fitness advantage to a cell, enhancing its survival, proliferation,

and dominance over other cells in both premalignant lesions and cancerous tissues [30–36]. Cell-intrinsic differences driving the clonal expansion of cancer cells are shown to impact clinically relevant outcomes, including tumor progression, metastasis, and drug resistance [1,9,12–14,37–52]. The wide breadth of cell-intrinsic factors driving diverse clonal architectures have been investigated in great depth in hematological cancers, which we discuss later.

Cell-intrinsic differences have been particularly implicated in driving differential responses in single cancer cells within a tumor when exposed to anti-cancer therapies, leading to drug resistance. Although few during early stages of treatment, the treatment-resistant clones escape therapy, ultimately leading to clinical relapse in patients. Response to therapy is heterogeneous within and across cancers and treatments, and several models have been proposed as potential resistance mechanisms [47,51,53,54] (**Figure 1**). Indeed, several studies have highlighted the role of both genetic alterations and non-genetic heterogeneity in enabling a rare subset of tumor cell clones to bypass therapies and develop resistance [1,9,12–14,37–48,50,55,56].

While classical experiments have largely relied on measuring the spectrum of changes after the tumors have progressed, recent advent of quantitative imaging, single-cell profiling, and barcode-based lineage tracing techniques are facilitating longitudinal tracking of clones at an unprecedented resolution. For example, quantitative studies of therapy resistance in cancers have identified a new class of cell-intrinsic properties, i.e. transient, yet heritable non-genetic fluctuations in a rare subset of cancer cells, that enable them to escape therapies and adopt diverse drug-resistant fates [2,9,11–13,42,52,54,57,58]. Such rare and heritable cell-intrinsic states have been associated with the emergence of dominant clones underpinning resistance to a variety of treatment regimens (e.g., targeted therapy, cytotoxic chemotherapy, and immunotherapy) and across cancers including melanoma, acute myeloid leukemia, triple-negative breast cancer, and lung adenocarcinoma [9,12,13,43,57]. In metastasis, recent studies have integrated lineage tracing and computational algorithms in mouse models to quantitatively track properties of metastatic clones [59–61]. Similar to the findings reported in therapy resistance, pre-existing, heritable cell-intrinsic states faithfully predicted the metastatic potential of individual clones [59,60]. The clonal differences driving resistance and metastasis likely originate as a consequence of interplay between genetic alterations and non-genetic mechanisms, but the precise sequence of events remain to be elucidated.

Mathematical models of clonal evolutionary dynamics across spatial and temporal scales have played a major role in rationalizing experimental and clinical observations and, importantly, providing *in situ* predictions of tumor dynamics [27,62–69]. For instance, seminal data-inspired mathematical models of somatic evolution in colon cancer provided an important insight that selective advantage plays a more important role than either the cell population size or the mutation rate in driving malignancy [66]. Other, more recent, computational frameworks are facilitating a finer and robust inference of cancer (sub)clone phylogenies from careful integration of spatial, bulk, and single-cell sequencing datasets. Similarly, dynamical systems models—both at molecular (e.g gene regulation and signaling dynamics) and population scale—have revealed the origins and dynamics of relatively shorter timescale cell state transitions underlying phenotypic plasticity in cancer drug resistance and metastasis [2,7,24,70–73]. Collectively, as our computational capabilities continue to increase, both theoretical and data-driven modeling of clonal dynamics will play a pivotal role in informing rational experimental design and making testable predictions.

Together, a plethora of experimental and computational studies have established roles of cell-intrinsic factors in regulating clonal trajectories during therapy resistance, metastases, and more broadly tumor progression. However, several fundamental questions remain unanswered and stall progress toward durable cures. Yet to be answered are the origins of intrinsic determinants, their precise nature, interactions between determinants often separated by timescales, and how they bidirectionally communicate with their environment. These parameters remain critical for developing predictive computational models of cancer origin, progression, and responses to treatments.

Clonality influenced by tumor cell-extrinsic factors

Cancer cells evolve in intricate association with many non-cancer cell types that constitute the tumor microenvironment (**Figure 1**). In the past decade, while we have developed a greater appreciation of microenvironmental cues that could shape tumor cell evolutionary dynamics, studies have often relied on static snapshots which reveal little about how tumors genetically and non-genetically co-evolve with its micro-environment over space and time. Developing a comprehensive understanding of the co-evolution of tumors with their microenvironment necessitates performing longitudinal studies. Spatial transcriptomic and epigenomic measurements in clinical samples and animal models over time can identify the organization and relative contributions of non-cancer cells to altered tumor cell dynamics. Defining such dynamics over the course of tumor progression or during metastases or response to therapies can further highlight mechanisms that contribute to macro-evolutionary, saltatory events often thought to encompass the emergence of tumor heterogeneity [22] (**Figure 1**).

Among various parameters that contribute to the tumor microenvironment, changes in immune system activity have been one of the best characterized, and linked to critical steps during oncogenesis, such as initiation, progression, metastasis and therapy resistance [74–77]. The immune system by itself represents a diverse array of cell types, often with contrasting functions. For instance, while the infiltration of tumors by cytotoxic T cells and natural killer cells is associated with efficient anti-tumor responses and smaller tumor sizes, presence of regulatory T cells and myeloid-derived suppressor cells correlates with an immunosuppressive milieu and enhanced tumor burden [78]. Besides, the role of other immune cell subsets such as B cells, macrophages, neutrophils, and T-helper cell subsets, is less well-characterized [78]. Clinical and pre-clinical models have predominantly taken a “cell-centric” approach to describe the roles of distinct immune cell populations in cancers; however, a systems-level view to study the immune cell dynamics and their co-evolution with cancers is largely missing. Recent studies have begun to unravel systems level changes in the immune compartment in response to cancers [79–82]. Application of contemporary lineage tracing approaches [9,13,42,59,60,83,84] to simultaneously label both immune and cancer cells in pre-clinical models will be critical in examining how peripheral and tumor-associated immune cell populations change over time and may influence clonal dynamics within tumors.

Aging is another microenvironmental variable that naturally affects cancer initiation, progression, metastasis and therapy resistance. Indeed, most cancers are thought to originate from temporal, age-associated accumulation of cellular damage that may in turn promote intrinsic cellular variability to both tumor and neighboring non-cancer cells [74–77,85]. In general, aging can be seen as a multifactorial driver of oncogenesis, which impacts many critical body functions, including changes in metabolism, regenerative capacity, and immune surveillance [86,87]. Each of

these processes, in principle, could impose unique selective pressures. In addition, low-grade chronic inflammation, which is commonly associated with aging in many tissues, may lead to an altered cytokine and chemokine milieu that can itself function as growth stimuli to orchestrate clonal selection [75–77,88]. More broadly, aging can be considered a complex trait that may aggravate underlying cellular diversity to shape the clonal architectures during cancer initiation and progression. Systems level computational synthesis of experimental data offers a great opportunity to curate high-resolution interaction networks [89–91]. These networks may help uncover the precise mechanisms of multifaceted processes such as aging during tumor formation and progression. Measuring how the age-associated local and systemic changes contribute to cancer cell-intrinsic variability is important to delineate the etiologies of age-linked malignancies.

Conceptualizing clonality through hematological cancers and other tumors

In non-human primates and humans, steady state hematopoiesis is estimated to be sustained by 50,000- 200,000 hematopoietic stem cells (HSC) that give rise to many trillions of blood cells over the lifetime of an individual [92]. These estimations mean that at any given time, thousands of peripheral blood cells can be traced back in their lineages to a few bonafide HSCs. Moreover, compared to other systems, the hematopoietic system is readily amenable to granular investigation of evolutionary trajectories in the context of hematological malignancies. In fact, the first evidence of clonality in cancers were described by coarse use of karyotyping methodologies in the hematopoietic compartment (**Figure 2**) [93]. The recent advent of next-generation sequencing technologies and computational approaches has further afforded interrogation of this system at an extraordinary resolution and scale. Notably, several studies in recent years have described that clonal attributes are commonly associated with aging in the hematopoietic compartment and are present in a large proportion of seemingly healthy individuals. This condition, commonly known as Clonal Hematopoiesis of Indeterminate Potential (CHIP), is characterized by over-representation of clones harboring certain somatic mutations in peripheral blood of individuals that otherwise have no overt hematological disorder, and is considered a genuine pre-malignant state [94,95] (**Figure 2, Figure 3A**). Furthermore, CHIP is highly prevalent in aging individuals; when defined with a variant allele fraction of 0.5%, it manifests in ~20% of all individuals at age 50, and further increases to ~50% by 70 years of age [96,97]. More recent studies using very high-depth sequencing have shown that the incidence of age-associated CHIP could be even higher than previously reported. Although there is no current consensus on what variant allele frequency defines CHIP of clinical significance, advanced genomics methodologies could further aid in tracking the origins and progression of mutant clones over extended timescales [98].

CHIP is suspected to emerge when hematopoietic stem cells (HSCs) acquire somatic mutations that confer a fitness advantage, consequently producing clones which account for a disproportionately large fraction of mature peripheral blood cells [99,100]. Mutations identified in CHIP are commonly thought of as drivers of cancer and predominantly include epigenetic modifiers (*DNMT3A, TET2*), and less frequently, splicing factors (*U2AF1, SRSF2, SF3B1*), DNA repair regulators (*TP53, PPM1D*), and signaling pathways (*JAK2, CBL*) [94,95,101–103] (**Figure 3A**). Moreover, recent studies have shown that the somatic mutations associated with CHIP arise at different ages and appear to be biased by gender in some instances [104]. Though not as well-studied, additional non-genetic programs associated with transcriptional control, RNA splicing, and chromatin and DNA states can further diversify the gamut of cell-intrinsic variabilities that

may in turn impart clonal fitness [13,105]. Therefore, the genetic and non-genetic diversity reported in CHIP is a multiscale process, likely operating through a combination of changes on molecular, cellular, and chronological levels (**Figure 3B**). These changes may be a direct consequence of aging or could themselves be drivers of the "aging phenotype"; decoupling these cell-intrinsic and -extrinsic scenarios will be critical to pursue in future studies.

While the causal relationship between CHIP and myeloid malignancies has been recognized, interestingly, it has been reported that at least 0.5-1% of all individuals with CHIP progress further to develop hematological malignancies each year despite its high prevalence [96,106]. Thus, the progression of CHIP to malignancies appears to be a relatively slow, stepwise process likely punctuated by iterative rounds of positive selection utilizing several cooperating parameters [107–111]. These parameters include contributions from both cell-intrinsic determinants (genetic and non-genetic) and cell-extrinsic factors (e.g., persistent infections driving chronic inflammation and immune cell regulation of developmental niches) which simultaneously steer the clonal selection process [106,112–117]. The exact identity of such parameters and the combinatorial effect of the selective forces that shape the ultimate clonal architecture underlying an overt malignancy remains to be fully understood. Moreover, CHIP is not the only bonafide pre-malignant state associated with hematological malignancies; other well studied examples include pre-malignant conditions like monoclonal B cell lymphocytosis (MBL) [118] and monoclonal gammopathy of undetermined significance (MGUS) [119] with pre-dispositions to chronic lymphocytic leukemia (CLL) and multiple myeloma, respectively. It would be interesting to study how these pre-malignant hematological states are conceptually related in their origin and eventual progression to frank neoplasms.

Advancement in single-cell multi-omic profiling approaches have introduced new paradigms wherein certain molecular states of cells are preferentially selected in pre-malignant conditions such as CHIP. Importantly, such cellular states likely arise from integration of multiple parameters and represent a more robust and stable readout than features influenced by individual parameters. Therefore, longitudinal identification and tracking of cellular states–similar to lineage tracing approaches recently used in the hematopoietic system [120]–could offer complementary approaches in defining the evolutionary history of pre-malignant conditions and the associated malignancies. Moreover, such clonal states or architectures could be more generalizable in addition to characterizing clonality by mutational spectrums alone [121]. In summary, the hematopoietic compartment offers an attractive and tractable system to perform longitudinal studies to monitor the progression of CHIP to hematological neoplasms for revealing quantitative and systems-level principles that underlie clonal evolution in cancers.

Beyond hematological malignancies, another well-studied example of pre-malignant clonal evolution is the pre-cancerous state called Barrett's esophagus. In Barrett's esophagus, squamous cell lining in the esophagus acquires stochastic genomic alterations and clonal expansion which could transform into esophageal adenocarcinomas [30,33,122,123]. Similar to CHIP, multiple clones in Barrett's esophagus can coexist over long periods of time and could accumulate additional mutations and copy number variations, eventually leading to development of esophageal adenocarcinomas [124,125]. However, in contrast to CHIP, the progression of Barrett's esophagus to esophageal adenocarcinomas appear to be much less frequent and intriguingly associated with genetic architecture that is distinct between pre-malignant and malignant states [126]. Therefore, unique modes of clonal evolution associated with distinct pre-malignant states may be operational in different tissue types. Future studies could test the broad

relevance of the conceptual frameworks developed in particular systems to unrelated tissue and cancer types.

Next frontiers

The continued progress in developing links between clonality and clinical ramifications may pave the way for systematic and periodic monitoring of clonality in individuals with clinically-defined pre-malignant states. In the near future, high-risk individuals such as those with chronic systemic inflammation, persistent infections, or familial history of cancers could be monitored for clonality changes, at least in the hematopoietic compartment, as routine care. In general, recent progress in cell-free DNA technologies could also allow identification of robust biomarkers that can faithfully track origins and progression of cancers across multiple tissue types [127]. Among the various challenges that remain, simultaneous measurement of multiple modalities to identify traceable early biomarkers—beyond curated gene sets—will be important towards unbiased and robust monitoring. Furthermore, universal or perhaps system specific interventions must be defined that consider the functional relevance of clones regardless of their profusion [56].

On the technological front, advances across disciplines provide promising new toolkits for addressing the open questions in tumor clonality (**Table 1**). For example, advanced spatiotemporal lineage tracing coupled with computational modeling may resolve whether clonal states eventually selected for during tumor evolution are pre-existing or newly acquired during adaptation. Moving forward, defining clonality through surrogate means which go beyond the introduction of synthetic lineage tracers would be ideal to infer evolutionary trajectories emerging via genetic and non-genetic mechanisms. For instance, natural barcodes arising from the mitochondrial genome enable lineage inference in both health and disease [128,129]. Rapid advances in automated image analysis with machine learning also offer complementary tools to leverage phenotypic differences–such as cellular and subcellular morphology and localization–to infer clonality [130–133]. Similarly, computational methodologies relying on transcriptional memory between progenies may also enable barcode-free lineage tracing with single cell RNA-sequencing datasets alone [134,135]. However, while clonality in some cancers can be described well by single cell RNA-sequencing, others may require multi-parameter approaches [136]. Furthermore, it will be critical to perform simultaneous evolutionary measurements of cell-intrinsic factors and extrinsic environments, and identify the chronological order of molecular and cellular events to unravel unifying and cancer-specific systems-level control principles (**Figure 1**). This will entail development of not just experimental multimodal biochemical and imaging techniques, but also computational and analytical frameworks to complement such rich and composite datasets.

Author Contributions

V.S. and Y.G. conceived the project and E.I.G., A.A., V.S., and Y.G. wrote the manuscript.

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Declaration of Interests

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Table 1

Publication	Type	Specifics	System
Rodriques et al. 2019 [137]	sequencing	Spatial	Brain
Payne et al. 2021 [138]			Development
Zhao et al. 2022 [139]			Various
Young et al. 2015 [140]		Error-corrected	Leukemia
Martelotto et al. 2017 [141]		Single-cell genome	Fixed
Dong et al. 2017 [142]			N/A
Chu et al. 2017 [143]			N/A
Biezuner et al. 2016 [144]		Scalable	Various
Tao et al. 2021 [145]			Melanoma
Nam et al. 2022, 2019 [146,147]		Multiomics	MPN, CHIP
Wagner et al. 2018 [148]	barcoding	Transposase	Development
Pei et al. 2017 [149]		Cre Recombination	Hematopoiesis
Biddy et al. 2018 [150]		Retrovirus	Stem Cell Reprogramming
Goyal et al. 2021 [9]			Drug resistance
Fennell et al. 2022 [10]			AML
Weinreb et al. 2020 [81]			Hematopoiesis
Gutierrez et al. 2021 [151]			CLL
Oren et al. 2021 [11]			Drug resistance
Umkehrer et al. 2021 [12]			Drug resistance
Ludwig et al. 2019 [128]		Mitochondrial	Various
Miller et al. 2022 [129]			Hematopoiesis
Klahor et al. 2018 [152]		CRISPR/Cas9	Development
Spanjaard et al. 2018 [153]			Development
Zhang et al. 2021 [154]			Metastasis
Simeonov et al. 2021 [155]			Metastasis
D. Yang et al. 2022 [66]			NSCLC

Chan et al. 2019 [156]			Development
Alemany et al. 2018 [157]			Development
McKenna et al. 2016 [158]			Development
Frieda et al. 2017 [159]			N/A
Raj et al. 2018 [160]			Brain
Clark et al. 2021 [161]		Rabies virus	Brain
Loveless et al. 2021 [162]		CRISPR/Cas9 and TdT	Various
Quinn et al. 2021 [59]		Hybrid (Retrovirus and CRISPR)	Metastasis
Satas et al. 2020 [163]	computational	Phylogeny	Various
Eisele et al. 2022 [164]			Various
Gao et al. 2021 [165]			Various
C. Yang et al. 2021 [45]	Imaging	Timelapse	Drug resistance
Han, Yang, and Spencer 2020 [166]			Various
Chakrabarti et al. 2018 [167]			Drug resistance

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Figure Legends

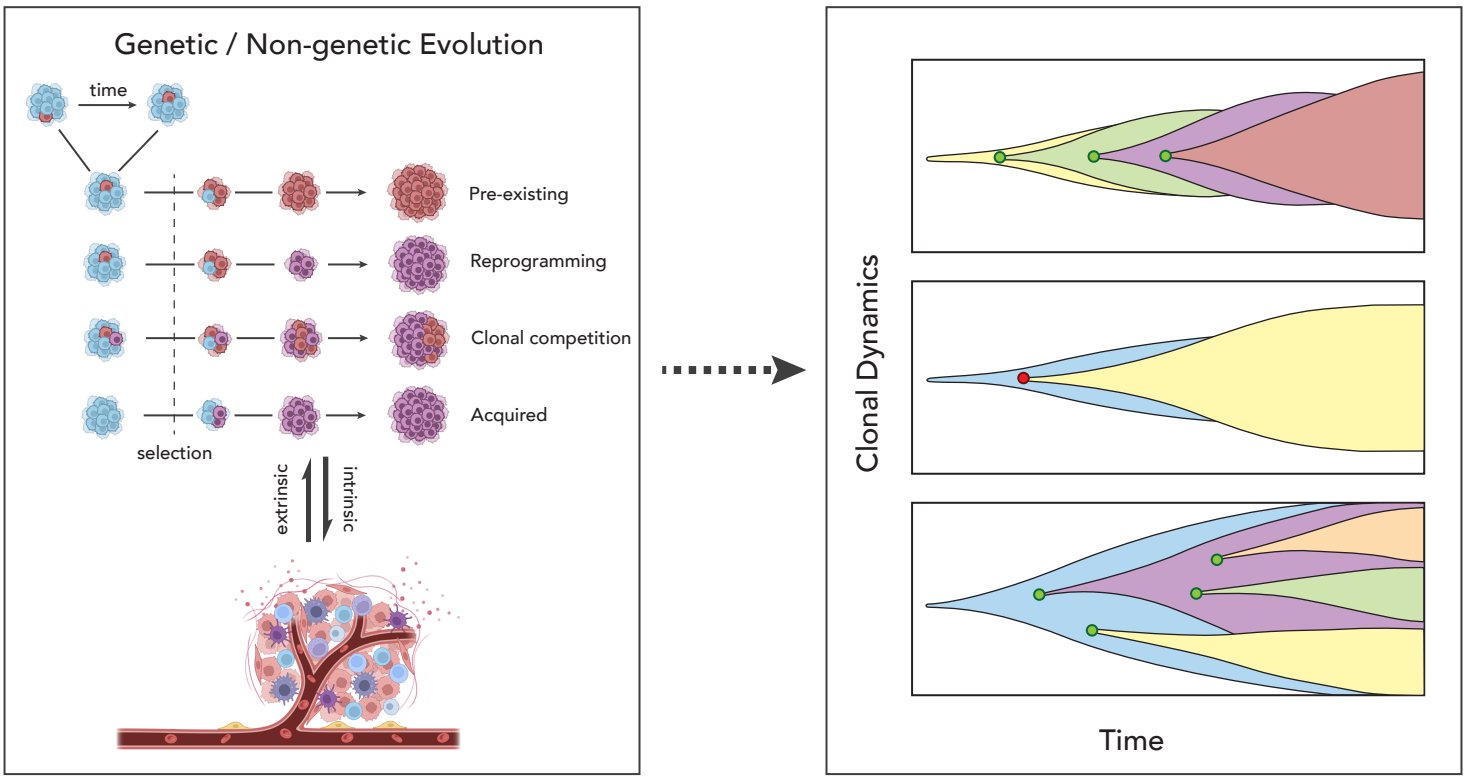
Figure 1: Tumor evolution is shaped by intrinsic and extrinsic factors. (left) Intrinsic genetic and non-genetic variations together with extrinsic tumor microenvironmental factors dynamically interact to shape fate responses and tumor evolution to selective pressures. Diversity within tumors can result from a number of factors, including but not limited to pre-existing genetic/non-genetic diversity; may be acquired as a response to selective pressures; as a result of reprogramming or phenotypic switching from one state to another; or perhaps as a consequence of clonal competition. Tumor composition has a bidirectional interaction with the microenvironment and response to selection pressures. **(right)** These various modes of tumor evolution are responsible for the changing clonal trajectories and structure in tumors. Models depicted are representative of detectable clonal formation and collapse over time. In the top pane, several successive waves of dominant clones drive the growth of the tumor, as might be seen in response to different therapeutic interventions. In the middle pane, a single clone outcompetes the founding clone and holds dominance for the majority of the tumor's

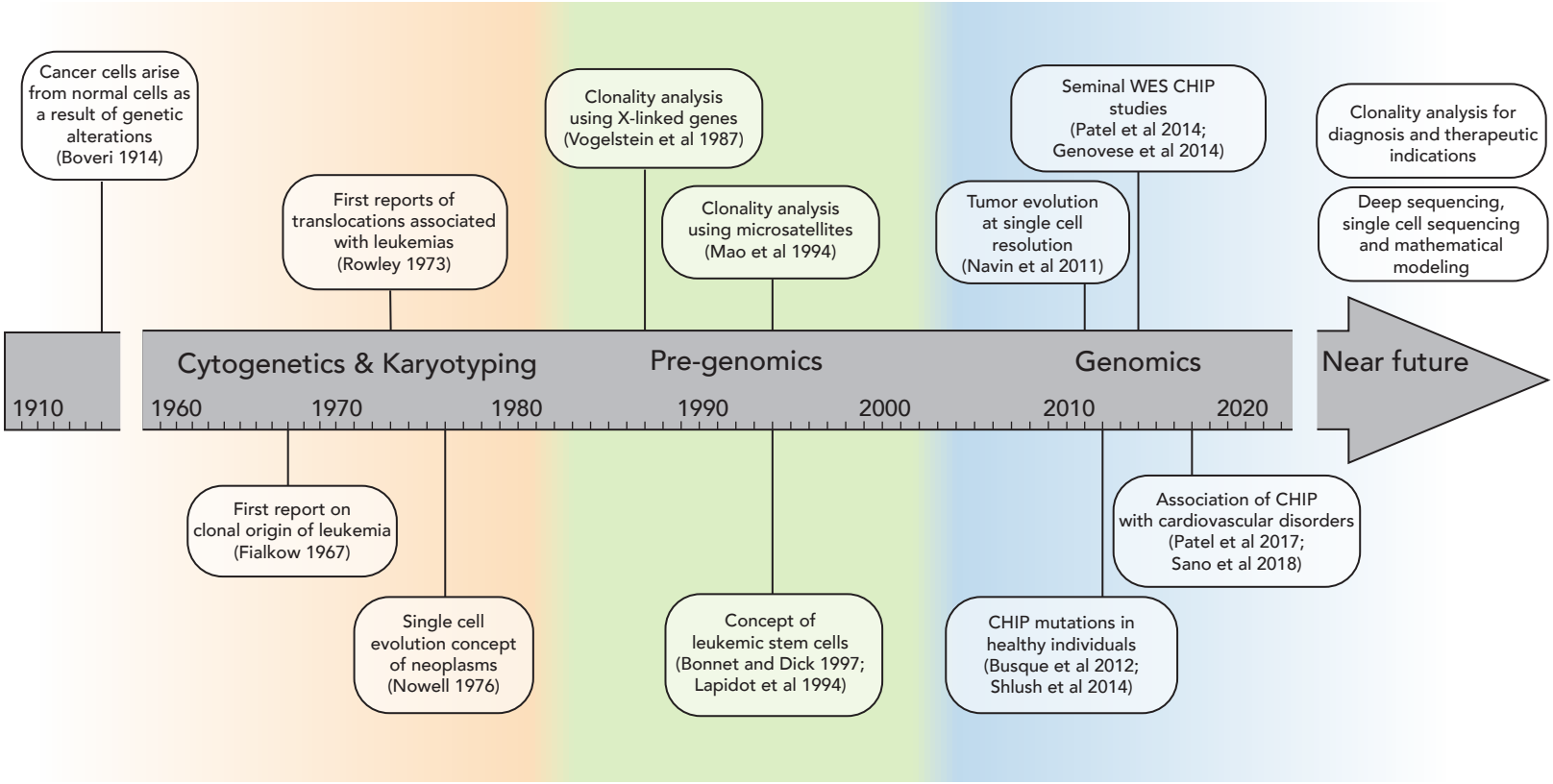
development, as might be seen in a tumor with low heterogeneity. In the bottom pane, several clones coexist and expand over time without completely outcompeting each other.

Figure 2: Key discoveries and scientific advances in the area of tumor clonality. The timeline depicts three eras – Cytogenetics & Karyotyping, Pre-genomics, and Genomics – when research was guided by the prevailing philosophies and experimental tools. We postulate that in the near future, clonality analysis by deep sequencing and mathematical modeling may have broad implications in diagnosis and therapeutics. WES (whole exome sequencing); CHIP (Clonal Hematopoiesis of Indeterminate Potential). [15,23,94,95,99,151,166–177].

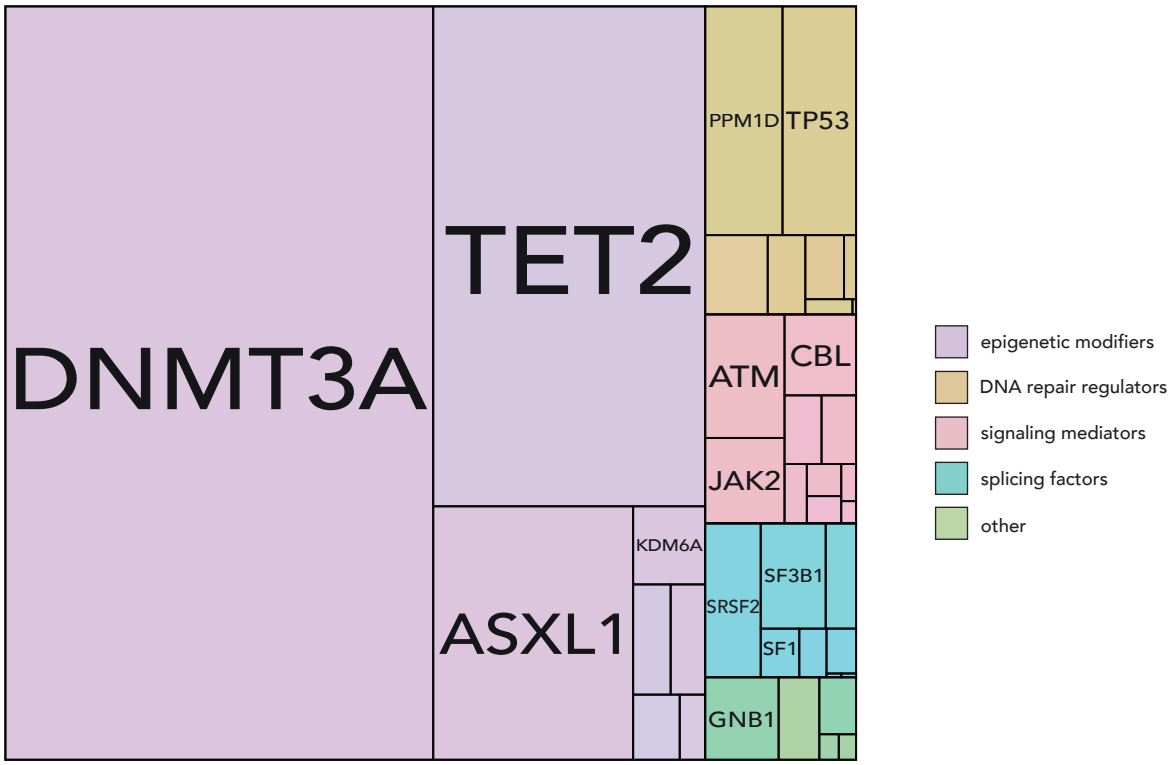
Figure 3: Clonal hematopoiesis development shares similarities with tumor evolution. A) The most commonly mutated genes in clonal hematopoiesis, categorized by the major pathways that they are associated with as reported by Kar et al [104]. Among the common genetic alterations associated with CHIP, epigenetic modifiers such as *DNMT3A*, *TET2,* and *ASXL1* are the top most recurrently mutated genes. Other known cancer associated genes and pathways such as *TP53, ATM*, and *JAK2* also feature prominently. **B)** A model for clonal selection and progression of CHIP. Changes in HSC clonal diversity in CHIP is influenced by cell-intrinsic and -extrinsic factors that wax or wane as an individual ages. Certain genetic mutations serve as initiating events and confer selective advantage to a clone that may skew HSC diversity and function. The initiating genetic events may cause further selection through genetic and non-genetic mechanisms leading to enhanced clonal diversity. The genetic alterations and skewed clonal architectures would in turn promote immune dysfunction leading to increased risk of infections and chronic inflammatory states that could further drive the clonal selection and eventual transformation.

Table 1: **Recent advances in the study of clonality.** We have provided recent studies of technological advances, including new hardware, computational frameworks, and experimental designs. The publications are primarily grouped by type of technology developed. Sequencing refers to technological advances in sample preparation and sequencing data acquisition that increase resolution for studying carcinogenesis and clonality. Barcoding refers to the use of synthetically introduced or endogenous inheritable DNA sequences for identification of cellular lineage. Computational refers to analytical methods and frameworks for interpreting clonal evolution over time. Imaging refers to microscopy-based tracking of tumor evolution using either reporter or label-free systems. The specifics of the technology are further described and similar technologies are presented together. Lastly, the model system in which the technology was applied in the cited publication is listed wherever relevant. Publications cited are not comprehensive, rather are representative examples; an omission from this list does not reflect the potential importance nor impact of that publication, but merely an oversight.

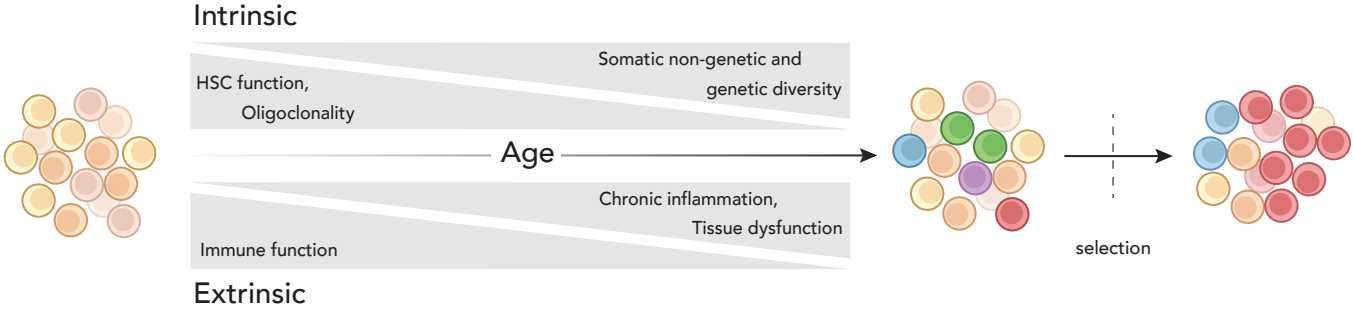

Genetic / Non-genetic Evolution
time
Pre-existing
Reprogramming
Clonal competition
Acquired
selection
extrinsic
intrinsic
Clonal Dynamics
Time


Cancer cells arise from normal cells as a result of genetic alterations (Boveri 1914)
First reports of translocations associated with leukemias (Rowley 1973)
Clonality analysis using X-linked genes (Vogelstein et al 1987)
Clonality analysis using microsatellites (Mao et al 1994)
Seminal WES CHIP studies (Patel et al 2014; Genovese et al 2014)
Tumor evolution at single cell resolution (Navin et al 2011)
Clonality analysis for diagnosis and therapeutic indications
Deep sequencing, single cell sequencing and mathematical modeling
Cytogenetics & Karyotyping
Pre-genomics
Genomics
Near future
1910
1960
1970
1980
1990
2000
2010
2020
First report on clonal origin of leukemia (Fialkow 1967)
Single cell evolution concept of neoplasms (Nowell 1976)
Concept of leukemic stem cells (Bonnet and Dick 1997; Lapidot et al 1994)
CHIP mutations in healthy individuals (Busque et al 2012; Shlush et al 2014)
Association of CHIP with cardiovascular disorders (Patel et al 2017; Sano et al 2018)

A


DNMT3A
TET2
ASXL1
KDM6A
PPM1D
TP53
ATM
CBL
JAK2
SRSF2
SF3B1
SF1
GNB1
epigenetic modifiers
DNA repair regulators
signaling mediators
splicing factors
other

B


Intrinsic
HSC function,
Oligoclonality
Somatic non-genetic and
genetic diversity
Age
Chronic inflammation,
Tissue dysfunction
Immune function
Extrinsic
selection