Clonal heterogeneity and tumor evolution: past, present and the future

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Abstract

Intratumor heterogeneity, which fosters tumor evolution, is a key challenge in cancer medicine. Here we review data and technologies that have revealed intra-tumor heterogeneity across cancer types, and the dynamics, constraints and contingencies inherent to tumor evolution. We emphasize the importance of macro-evolutionary leaps, often involving large-scale chromosomal alterations, in driving tumor evolution and metastasis, and consider the role of the tumor microenvironment in engendering heterogeneity and drug-resistance. We suggest that bold approaches to drug development, harnessing the adaptive properties of the immune-microenvironment whilst limiting those of the tumor, combined with advances in clinical trial-design, will improve patient outcome.

Introduction

In a famous thought experiment, evolutionary biologist Stephen Jay Gould asked, if the 'tape of life' could be turned back to the very beginning, the same outcome would prevail (Gould, 2000). While re-playing the 'tape of

life' remains a hypothetical experiment, the 'tape of cancer' is being played and re-played with increasing regularity and too frequently with lethal results. Cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 cancer-related deaths occurring in 2012 alone (Siegel et al., 2013). Alarmingly, the number of new cases is predicted to rise by approximately 70% over the next two decades.

A key factor contributing to the lethal outcome of cancer, therapeutic failure and drug resistance is intratumor heterogeneity (ITH) (Greaves, 2015). ITH provides diverse genetic and epigenetic material upon which selection and Darwinian evolution can act. However, this diversity also permits the 'tape' of each cancer's life to be deciphered, revealing the temporal order of genomic events and shedding light on constraints and contingencies to cancer evolutionary trajectories. The advent of next-generation sequencing has enabled more powerful analysis of tumor evolution and has improved our understanding of tumor initiation and development, as well as the interaction between cancer cells and the immune-microenvironment. Despite these advances, knowledge of ITH and clonal evolution and the potential for competitive release of resistant subclones is infrequently considered in the therapeutic setting to inform clinical trial design.

In this review, we explore the extent and clinical implications of ITH and discuss how profiling tumor genetic diversity has been used to trace tumors' life histories and their patterns of evolution. We emphasize the importance of viewing tumor development in the context of an evolutionary framework, which includes large-scale genomic alterations, and consider the dynamic evolution of tumor cells and their interaction with the microenvironment. Finally, we outline approaches to address cancer systemically, taking into account ITH and tumor evolution.

Intra-tumor heterogeneity: patterns and prevalence

How much heterogeneity is there?

Genomic diversity within single tumors has long been recognized. Indeed, as early 1958, evolutionary biologist Julian Huxley commented on 'genetic inhomogeneity' in cancer, noting, "*it will be of great interest to discover the extent of such new variance and the rate at which it occurs*" (Huxley, 1958). However, it is only with the advent of next-generation sequencing studies that the full extent of genomic ITH is becoming apparent. Sequencing of spatially and/or temporally distinct tumor regions has begun to uncover the bewildering extent of diversity within tumors (Figure 1).

These studies have revealed that the degree of ITH can be highly variable, with between 1 to over 8000 thousand coding mutations found to be heterogeneous within primary tumors or between primary and metastatic/recurrence sites (Johnson et al., 2014). Despite caveats regarding differences in sampling procedure, tumor stage, and sequencing depth, it is evident that certain tumor types, such as melanoma and lung cancer, harbor a significantly larger homogeneous coding mutational burden than other types. The involvement of powerful exogenous mutagens, such as ultraviolet light and tobacco carcinogens, which a stem cell niche may be exposed to for years prior to the first invasive step, likely explain the elevated clonal mutational burden in these cancers (Alexandrov et al., 2013). Accordingly, the prevalence of different types of base-substitutions (within a trinucleotide context) observed in these tumors reflect their exogenous exposures (Alexandrov et al., 2013).

It is also evident that a large clonal burden does not equate to a large subclonal burden, or vice versa. Predominantly this likely reflects the fact that distinct mutational processes may operate at different times in tumor evolution (de Bruin et al., 2014; McGranahan et al., 2015; Nik-Zainal et al., 2012). Indeed, the most notable outliers with regard to high subclonal mutational burden, but low clonal burden, are low-grade gliomas that

recur as glioblastomas after treatment with the alkylating agent temozolomide (Johnson et al., 2014; Kim et al., 2015). In this case, the abundance of subclonal mutations can be directly linked to therapy-induced mutations that are compounded by loss of mismatch repair machinery. As more data accumulates, the impact of therapy on mutational load and ITH will likely become clearer.

In other cancer types, such as non-small cell lung cancer (NSCLC) and bladder cancer, the presence of a large subclonal mutation burden can be attributed to the action of the APOBEC family of cytidine deaminases (de Bruin et al., 2014; McGranahan et al., 2015; Zhang et al., 2014). In colorectal and prostate cancers, alterations to mismatch repair or proofreading machinery can occasionally play a key role in generating both clonal and subclonal mutations (Kumar et al., 2016; Uchi et al., 2016).

Consistent with results from mathematical modeling (Tomasetti et al., 2013), these studies also suggest that the clonal mutation burden in certain cancer types, including lung cancer and melanoma, predominantly reflects mutations accumulated prior to carcinogenesis, while in others, mutation burden may be more reflective of somatic events occurring after tumourigenesis. As such, the mutation rate of a tumor cannot necessarily be inferred from a single biopsy and simply considering the proportion of heterogeneous mutations represents a poor surrogate for diversity (Figure 1).

Importantly, heterogeneity does not simply affect coding mutations. A multitude of epigenetic mechanisms, including DNA methylation, chromatin remodeling and post-translational modification of histones, can contribute to diversity within tumors (for a review see (Mazor et al., 2016)). Analysis of ITH in gliomas (Mazor et al., 2015) as well as prostate cancers (Aryee et al., 2013) and esophageal squamous cell carcinomas (Hao et al., 2016) has suggested that the extent of ITH calculated from DNA methylation mirrors ITH measures captured at the genomic level.

Genomic copy number heterogeneity can also be extensive within tumors, to the extent that copy number ITH in clear cell renal cell carcinoma (ccRCC) mirrors the extent of copy number diversity between tumors (Martinez et al., 2013). Large-scale chromosomal alterations may have profound impact upon the genome, disrupting hundreds of genes and can be considered macro-evolutionary events (see below), which may be required for tumor transformation (Notta et al., 2016). Loss of genomic material through chromosomal instability may also contribute to mutational ITH (McPherson et al., 2016; Murugaesu et al., 2015), highlighting the importance of considering both copy number and mutation data when inferring the evolutionary history of tumors.

Heterogeneity can reveal a tumor's life history

A tumor's mutational catalogue represents a historical record of alterations that have accumulated during its life history. The heterogeneity present between cancer cells can be used to illuminate the temporal order of these events. Alterations identified in every sequenced cancer cell can be considered to form the trunk of a cancer's somatic evolutionary tree, while subclonal mutations, present in only a subset of cancer cells, make up the branches (Figure 2). Further, the prevalence of subclonal mutations in different cancer cells can be used to infer the subclonal hierarchy of the tumor's phylogeny.

A plethora of bioinformatics tools have been developed to help decipher the temporal order of mutations and determine which are clonal or subclonal. The majority of tools focus on somatic point mutations, and either restrict the analysis to copy neutral regions of the genome or make the assumption that a single mutation will be present at the same copy number state in every cancer cell (Carter et al., 2012; Miller et al., 2014; Roth et al., 2014). While tools to infer copy number heterogeneity have also been developed (Ha et al., 2014; Shen and Seshan, 2016), more recent tools seek to combine copy number and mutational data (Fischer et al.,

2014; Jiang et al., 2016) and furthermore, attempt to infer evolutionary relationships between subclonal populations (Deshwar et al., 2015; Jiang et al., 2016). Relatedly, orthogonal tools to dissect heterogeneity by using data from single-cell sequencing have also been developed (Roth et al., 2016).

However, despite considerable advances, it is self-evident that even cutting-edge tools can only dissect the ITH within the sample(s) subject to sequencing. As such, our ability to distinguish truly clonal from pseudo-clonal mutations thereby determine the true clonal and subclonal burder of alterations is largely dependent on the number of tumor regions sequenced (de Bruin et al., 2014; Yates et al., 2015), the depth and purity of what is sequenced, and, further, whether single-cell sequencing is also implemented (Roth et al., 2016).

Driver alterations and heterogeneity

Studies exploring ITH within solid tumors have demonstrated a tendency for established cancer genes to harbor clonal mutations (McGranahan et al., 2015). However, despite this tendency, even mutations in cancer genes can be present in only a subset of cancer cells within a tumor.

Subclonal driver mutations can give an illusion of clonality due to sampling bias. Whole-genome sequencing of 33 pairs of medulloblastomas pre and post-therapy found the majority of putative drug targets identified pre-treatment appeared clonal, but were revealed to be subclonal or absent at recurrence (Morrissy et al., 2016). Equally, ITH may lead to significant underestimates of the number of driver alterations present in a tumor. Analysis of 86 cases of diverse primary tumors and brain metastases revealed that in 53% of cases, putative drug targets, including *PTEN* and *PIK3CA,* were exclusively identified in the brain lesions and not in the primary tumor (Brastianos et al., 2015).

Accumulating evidence suggests certain driver alterations may be more likely to be subclonal than others. Subclonal mutations in *PIK3CA* have

been found in, among other cancer types, lung (de Bruin et al., 2014), breast (Yates et al., 2015), colorectal (Uchi et al., 2016), melanoma (Harbst et al., 2016), esophageal squamous cell carcinoma (Hao et al., 2016), ccRCC (Gerlinger et al., 2014a) and ovarian cancers (Bashashati et al., 2013). In keeping with these results, across 9 cancer types, mutations in the PI3K-AKT-mTOR pathway were found to harbor a higher proportion of subclonal mutations compared to genes associated with RAS-MAPK pathway (McGranahan et al., 2015). However, other driver mutations exhibit a tendency to be clonal in certain cancer types but not others. Mutations in *TP53* appear almost exclusively clonal in NSCLC (de Bruin et al., 2014; Zhang et al., 2014), esophageal adenocarcinomas (Murugaesu et al., 2015) and ovarian cancers (Bashashati et al., 2013), yet are often subclonal in ccRCC (Gerlinger et al., 2014a) and chronic lymphocytic leukemia (CLL) (Landau et al., 2013). Such differences may reflect the importance of epistasis in cancer evolution and are in agreement with findings that co-occurrence and mutual exclusivity relationships between cancer driver alterations can vary extensively in different cancer types (Park and Lehner, 2015).

The subclonal nature of genomic driver alterations can have important clinical implications. A recent report demonstrated that two gastric cancers with high clonal amplification of *FGFR2* responded to the FGFR inhibitor AZD4547. Conversely, the six tumors with low or subclonal amplification of *FGFR2* did not respond (Pearson et al., 2016). In CLL, the presence of subclonal driver alterations is associated with decreased relapse free survival (Landau et al., 2013).

Processes of cancer genome evolution, and evolutionary debates:

Selection and neutral evolution in cancer

"the fittest will survive, and a race will be eventually produced adapted to the conditions in which it lives", (Wallace, 1867)

Although originally framed in relation to the evolution of individual organisms within a population, the fundamental principles of Darwinian evolution, involving variation with differential fitness that is heritable, can be applied in the context of tumor evolution (Nowell, 1976). In this setting, the population of cancer cells are subject to selection and the genetic variation between these cells, influenced by endogenous and exogenous mutational processes, provides the fuel for selection to act (Figure 3).

However, although heterogeneity is required for Darwinian evolution, positive selection does not necessarily lead to heterogeneity (Waclaw et al., 2015). As such, the extent to which positive selection can account for the degree of ITH in tumors has been called into question (Ling et al., 2015; Williams et al., 2016). Specifically, while evidence for selection of driver events in cancer development, as well as the selection pressures imposed by therapy, are undisputed, following a 'big-bang' of diversity early in tumor evolution, ITH development can follow the laws of neutral growth (Sottoriva et al., 2015). In support of this, Williams and colleagues noted that, for a subset of tumors, the relationship between the number of subclonal mutations and their relative abundance was consistent with a neutral growth pattern rather than subclonal expansions (Williams et al., 2016). Likewise, in an extensively sampled colorectal cancer the degree of heterogeneity appeared more consistent with neutral growth (Ling et al., 2015), and lineage-tracing studies in mice have suggested ITH may emerge from a stem-cell hierarchy of cancer cells evolving under neutral evolution (Driessens et al., 2012).

Further work is warranted to explore the temporal and spatial dynamics of clones in human tumors. Longitudinal sequencing data from CLL has demonstrated clonal dynamics and shifts in selection pressures even in the absence of therapy (Nadeu et al., 2016). Conceivably, during Darwinian evolution, both selection and neutral growth may operate simultaneously within the same tumor and this may alter dynamically over time. The observation that multiple different diversity measures in Barrett's

esophagus predict progression to esophageal adenocarcinoma (Maley et al., 2006; Merlo et al., 2010) is indicative that diversity may lead to selection of aggressive subclones, even without therapeutic selection pressures. Relatedly, the fact that cancer genes can harbor a statistically significant enrichment of subclonal mutations suggests a signal of selection can be present throughout tumor evolution (McGranahan et al., 2015). It remains an open question whether distinct clinical behaviors can be observed depending on the mode of tumor evolution, or whether the occurrence of neutral evolution and drift may limit the ability to predict a tumor's next step (Lipinski et al., 2016).

Contingency and convergence

Using the 'tape of life' metaphor, Gould emphasized the importance of chance and unpredictability in the evolution of life on earth, suggesting the end result is causally dependent on antecedent steps, or 'historical contingency' (Gould, 2000).

Examples of genetic contingency impacting upon the clinical course of the disease can be found in cancer evolution. The order in which the two driver events in *JAK2* and *TET2* are acquired in myeloproliferative disorders affects the clinical course of the disease (Ortmann et al., 2015). If a *TET2* mutation is acquired first, expansion of hematopoietic stem and progenitor cells occurs, blocking expansion of erythroid progenitors until cells acquire a *JAK2* mutation. Conversely, if a *JAK2* mutation is acquired first, megakaryocyte number increases, with no expansion of the hematopoietic stem and progenitor pool until a *TET2* mutation is acquired. Patients acquiring a *JAK2* mutation first are younger at disease onset and are more likely to present with polycythemia rubra vera and develop thrombosis than they are to develop essential thrombocythemia. The cell of origin may also have important consequences on the impact of identical somatic events. Despite the fact that it is possible to induce pancreatic adenocarcinoma and non-small cell cancers from the same initiating events (*TP53* inactivation, coupled with *KRAS* activation), these tumors have been

found to exhibit distinct metabolic requirements, making use of branched-chain amino acids in different ways (Mayers et al., 2016).

An alternative (and likely complementary) view of evolutionary convergence, advocated by Conway Morris and others, is encapsulated by Darwin discussing analogical variation, noting "*the common rule throughout nature is infinite diversity of structure for gaining the same end*" (Darwin, 1859) and echoed nearly 150 years later by Conway Morris the "*recurrent tendency of biological organization to arrive at the same solution*" (Conway Morris, 2003).

Evidence supporting both historical contingency and convergence towards the same solution is apparent from cancer evolutionary studies. Indeed, in germline *VHL* mutant carriers with synchronous renal cell carcinomas developing in the same patient, evidence for both contingency and convergence can be found; despite distinct secondary 3p loss of heterozygosity events and driver mutations in different cancers from the same patient, there was evidence for convergent PI3K signal transduction pathway activation (Fisher et al., 2014).

There is also extensive evidence for convergence of both genotype and phenotype in cancer evolution. Indeed, the notion of cancer hallmarks, supports the occurrence of convergence in cancer evolution. Further, Conway Morris's assertion that "*it matters little what our starting point may have been: the different routes will not prevent a convergence to similar ends*" (Conway Morris, 2003), could be used to describe the tendency for a cancer stem cell transcriptome to derive on multiple occasions across multiple distinct tumor types (Chen and He, 2016).

Moreover, convergence is frequently seen within individual tumors, termed parallel evolution, which, in the context of cancer, refers to the independent evolution of similar traits starting from a single ancestral clone. Campbell and colleagues reported two overlapping out of frame

deletions in exon 6 of *PARK2* in distinct pancreatic cancer metastases from the same patient (Campbell et al., 2010). Similarly, recurrent and independent acquisition of copy number events such as deletions in *PAX5*, *ETV6* and *CDKN2A* have been described in acute lymphoblastic leukemia (ALL) within distinct subclones (Anderson et al., 2011). Recurrent disruption of the SWI/SNF complex or activation of the PI3K pathway through distinct mutations in *mTOR*, *TSC1*, *PTEN* and *PIK3CA* are frequently observed in different subclones from the same renal cancer (Gerlinger et al., 2014a; Gerlinger et al., 2012; Voss et al., 2014).

As the resolution of cancer evolutionary analyses improves, the number of examples of parallel evolution increases, including but not limited to events involving *EGFR* in glioblastoma (Francis et al., 2014; Kim et al., 2015), *TP53* and *ATRX* in glioma (Johnson et al., 2014), activation of the MAPK pathway in multiple myeloma (Bolli et al., 2014; Melchor et al., 2014), *NOTCH1* and *GNPTAB* recurrent mutations in esophageal adenocarcinoma (Murugaesu et al., 2015), *SMO* mutations in medulloblastoma (Morrissy et al., 2016), distinct AR amplification events in prostate cancer (Gundem et al., 2015), *KMTD2D* and *CREBBP* mutations in follicular lymphoma (Okosun et al., 2014) and *PTEN* and *TP53* mutations, *FGFR2* amplifications and *RUNX1* deletions in primary breast cancer(Yates et al., 2015), as well as distinct *CCNE1* amplifications in ovarian cancers (McPherson et al., 2016). In clear cell renal carcinoma, an early clonal event is 3p loss of heterozygosity, which appears to prime the tumor for second hits in *SETD2*, *PBRM1* and *BAP1* (all of which are encoded on chromosome 3p) later in tumor evolution (Gerlinger et al., 2014a). Similarly, in breast cancer, three of the four parallel evolutionary events (*TP53*, *PTEN* and *RUNX1*) documented by Yates and colleagues occurred as the second hit, following a clonal event (Yates et al., 2015).

During the selection pressures of targeted therapies, parallel evolution driving polyclonal acquired drug resistance has been frequently documented. For example, in 13/16 patients with *BRAF*-mutant melanomas

with resistance to RAF inhibition, multiple parallel mechanisms of resistance were observed (Shi et al., 2014). Likewise, following *EGFR* monoclonal antibody therapy, multiple KRAS mutations have been observed in circulating free DNA (Bettegowda et al., 2014; Misale et al., 2012). One patient acquired a codon 12 *KRAS*, codon 61 *KRAS* and a codon 61 *NRAS* mutation together with a *BRAF* codon 600 mutation following acquired resistance to *EGFR* monoclonal antibody therapy that were not detectable prior to therapy (Bettegowda et al., 2014). Following acquired resistance to a PI3K alpha inhibitor, Juric and colleagues found parallel evolution of 6 distinct *PTEN* aberrations across 10 metastatic sites on the background of a clonal single copy *PTEN* deletion, reminiscent of second hit tumor suppressor gene loss *following* an early clonal event witnessed in breast and renal cancers (Juric et al., 2015).

These observations suggest that despite the stochastic nature of genomic change, microenvironmental, epistatic and lineage constraints operate that might allow the prediction of a limited set of subsequent evolutionary moves.

Gradualism versus punctuated evolution

Another longstanding evolutionary debate that has reemerged in the context of tumor development centers on whether tumor evolution occurs gradually, through the sequential accumulation of mutations and clonal expansions, or, whether it is characterized by punctuated bursts (Figure 3). Such a dichotomy has been framed in the context of micro- versus macro- evolution, with gradual accumulation of point mutations (micro-evolution) presented in opposition to a saltationist view, which emphasizes the importance of large-scale chromosomal alterations and bursts of mutations (macro-evolution) (Gerlinger et al., 2014b).

An incremental, gradual, accumulation of mutations during the life history of a tumor is evidenced by the presence of clock-like mutational signatures that correlate with the chronological age of the patient (Alexandrov et al., 2015). However, not all mutations accumulate in a clock-like manner. In several cancer types (Alexandrov et al., 2013), a phenomenon termed kataegis has been observed; describing a small localized mutational process that results in hyper-mutation (a few to several hundred C>T and/or C>G substitutions, enriched at TpC sites) on the same DNA strand. A punctuated mode of tumor evolution is also supported from lineage tracing studies. Graham and colleagues used lineage-tracing techniques based on nuclear and

mitochondrial DNA lesions in human colon adenomas to identify stem cell populations within adenoma crypts with multipotent potential and map their evolution over time. A punctuated model of rare clonal expansions interspersed with prolonged periods of stasis was suggested (Humphries et al., 2013).

Cancer evolution is conceptually similar to evolution in asexually reproducing organisms, and in yeast it was recently demonstrated that tetraploid strains showed faster adaptation to a poor carbon source and accumulated more genomic diversity compared to diploid counterparts (Selmecki et al., 2015). In cancers, genome doublings have been estimated to occur at high frequencies (Zack et al., 2013), and are also associated with elevated rates of chromosomal aberrations (Dewhurst et al., 2014; Fujiwara et al., 2005; Zack et al., 2013). Genome doublings may serve to reduce the impact of Muller's ratchet, a process by which asexual genomes accumulate deleterious mutation in an irreversible manner. Specifically, although the impact of a deleterious mutation cannot be removed through sexual reproduction, it can be mitigated by the presence of additional, doubled, wild-type alleles.

Chromothripsis, characterized as a single catastrophic event resulting in tens of hundreds of locally clustered rearrangements affecting one or a few chromosomes, has also been documents to be widespread in cancers, occurring in over 30% of bladder cancers (Morrison et al., 2014), lung adenocarcinomas (Malhotra et al., 2013), oesophageal adenocarcinomas (Nones et al., 2014), glioblastomas (Malhotra et al., 2013), uterine leiomymoas (Mehine et al., 2013) and pancreatic cancers (Notta et al., 2016). These events occurred both clonally and subclonally during tumor evolution. Single-nucleus sequencing of 1000 cancer cells from 12 triple-negative breast cancers found evidence for copy number alterations that had accumulated in short punctuated bursts early in tumor evolution, but not late (Gao et al., 2016). The progression of esophageal adenocarcinomas from Barrett's esophagus is thought to involve a punctuated path whereby a *TP53*-mutant cell undergoes a whole genome doubling event, followed by the acquisition of oncogenic amplifications (Stachler et al., 2015), conceivably through chromothripsis (Nones et al., 2014).

Chromothripsis and large-scale genomic rearrangements have parallels with evolutionary biologist Richard Goldschmidt's notion of '*hopeful monsters*' and 'macromutations' (Goldschmidt, 1982). Such mutations were described as '*of the most extraordinary rarity to provide the world with the important material for evolution*" and appear analogous to the simultaneous disruption of multiple pre-neoplastic driver events (*CDKN2A*, *TP53* and *SMAD4*) in single chromothriptic event in prostate cancer (Notta et al., 2016).

Perhaps unsurprisingly, while large-scale genomic rearrangements and chromothriptic events are often associated with aggressive cancers, its common occurrence in uterine leiomymoas highlight that it can also be involved in the development of benign tumors. In fact, consistent with "hopeful" and "hopeless monster" evolutionary thought, chromothripsis can even occasionally have a positive impact on patient outcome. McDermott and colleagues reported a case-study in which a chromothriptic event resulted in a cure for a patient with an inherited immunodeficiency disease caused by over-activity of a mutated chemokine receptor CXCR4 (McDermott et al., 2015). Specifically, the chromothriptic event led to deletion of the aberrant allele in a single hematopoietic stem cell, which subsequently repopulated the bone marrow and restored normal immune function.

Taken together, these data highlight the frequent occurrence of macro-evolutionary events in cancers. However, temporal and multi-regional tumor analyses will be required to reveal the true extent to which chromosomal alterations occur dynamically throughout tumor evolution. Nevertheless, notably even static measures of aneuploidy are associated with poor prognosis across cancer types (McGranahan et al., 2012). Moreover, the observation that tumors with an extreme level of chromosomal instability appear associated with improved prognosis compared to intermediate levels (Andor et al., 2016; Birkbak et al., 2011), further supports the hypothesis there may be a delicate balance between too much and too little instability and that there may be potent selection pressures in cancer evolution for a "just-right" level of cell-to-cell variarion.

Speciation and the metastatic process in cancer

Tumor metastasis is frequently cited to be responsible for approximately 90% of all cancer-related deaths. The process has been likened to a speciation event with macro-evolutionary leaps required to endow a tumor cell with metastatic potential (Gerlinger et al., 2014a; Turajlic and Swanton, 2016).

In certain tumors metastatic spread has been found to be monophyletic, with a single subclone in the primary tumor appearing to seed multiple metastases at different sites, resulting in low inter-metastatic ITH (McPherson et al., 2016; Schwarz et al., 2015). However, in other tumors, subclones at distinct metastatic sites are more closely related to subclones within the primary tumor than they are to each other, indicative of a polyphyletic metastatic process. Importantly, polyphyletic metastatic spread suggests multiple distinct evolutionary trajectories within a single tumor can result in metastatic dissemination. A study of seven patients with

ovarian cancer found five patients exhibited monoclonal and uni-directional seeding from the ovary to intraperitoneal sites, while the remaining two patients exhibited polyphyletic spread and reseeding (McPherson et al., 2016). However, convergent selection pressures in the metastatic setting, even in the context of polyphyletic spread, is evidenced by the occurrence of parallel evolution at distinct metastatic sites (Campbell et al., 2010). Finally, multiple rounds of metastasis, involving re-seeding, may also occur, highlighting the diverse patterns of metastatic spread that can occur, even within single tumors (Turajlic and Swanton, 2016).

Lineage tracing studies have informed our understanding of the patterns of tumor metastatic seeding. In an autochthonous model of mouse pancreatic cancer Maddipati and colleagues used multi-color lineage tracing strategies to track early development of KRAS/p53 mutant pancreatic pre-invasive lesions through to metastatic disease (Maddipati and Stanger, 2015). Each pancreatic mass contained an average of four single color lesions, indicating the presence of distinct tumors originating from independent genetic events in the pancreas. A quarter of pre-malignant precursor pancreatic lesions, acinar-to-ductal metaplasias (ADMs), displayed heterogeneous colors, indicative of their evolution from multiple acinar cells. However, pancreatic intra-epithelial neoplasia (PanIN) lesions displayed single colors, indicative of a bottlenecking event in the evolution of the pre-malignant disease from ADMs to PanIN lesions. Analysis of metastatic lesions in the lung, liver and peritoneum revealed a high frequency of polyclonal metastasis suggesting potential cooperativity between cancer subclones facilitating metastatic colonization. Evidence for polyclonal seeding of metastases was also observed in a common mouse model of breast cancer (Cheung et al., 2016). Supporting a clonal cooperativity model of tumor metastases, the authors found evidence for collective invasion and migration of polyclonal clusters of cells within the circulation seeding polyclonal disease at metastatic sites. These data reflect reports of circulating tumor cell clusters associated with poor prognosis in

breast and prostate cancer (Aceto et al., 2014), and highlight the need to view cancer as an ecosystem of subclones that may act cooperatively or antagonistically.

The cancer ecosystem

Functional cooperativity

The common occurrence of ITH challenges the view that tumor phenotypes are entirely driven by the dominant tumor clone in a cell-autonomous manner, in which driver mutations only confer a benefit to the cancer cell in which they occur. If cancer cells act in a non-cell autonomous way, whereby driver mutations confer benefits to neighboring cells it will result in ITH and cooperative or social networks governing tumor behavior.

Anton Berns and colleagues studied metastatic potential in a mouse model of small cell lung carcinoma (Calbo et al., 2011). Mesenchymal and neuroendocrine cells derived from a common progenitor, when engrafted into mice as a heterogeneous population triggered metastatic behavior of the neuro-endocrine cells. In adult GBM, Inda and colleagues noted that EGFRvIII deletion mutants account for a minority of the total population in some tumors. The authors found a paracrine mechanism sustaining growth of the dominant EGFR wild type clones through IL-6 and/or LIF from the EGFRvIII deletion mutants leading to wtEGFR activation in neighboring clones, sustaining tumor heterogeneity (Inda et al., 2010).

To investigate subclonal cooperativity further, Polyak and colleagues studied subclonal interactions in mouse xenograft models. Sub-populations of tumor cells could sustain the survival and growth of all tumor cells through IL-11 mediated microenvironment change. Notably, if minor subclones, sustaining the growth of the majority, were outcompeted by tumor subclones with greater proliferative capacity, tumor collapse resulted, suggesting non-cell autonomous drivers may be required for tumor development (Marusyk et al., 2014). Similarly, using a mouse model

of breast cancer, Cleary et al (Cleary et al., 2014) demonstrated that clonal cooperation can be essential for tumor maintenance. Bi-clonal mouse tumors containing genetically distinct luminal and basal subclones were separated into their component subclonal populations and subsequently transplanted into wild-type host animals, either separately or as a 1:1 admixture. Whereas the bi-clonal cell mixture was highly tumorigenic, mono-clonal populations failed to elicit tumor formation.

Such cooperativity also extends to the field of drug resistance. Hobor and Bardelli noted that only a fraction of some cetuximab resistant colorectal cancer samples harbored *KRAS* mutations, commonly described to result in acquired resistance to *EGFR* directed therapies. The authors found evidence that TGF alpha and amphiregulin secretion from *EGFR* inhibitor resistant cells was capable of sustaining the growth of *KRAS* wild type drug sensitive cells in a paracrine manner (Hobor et al., 2014).

The tumor microenvironment

The tumor microenvironment likely imposes profound constraints upon cancer evolution both at primary and distant sites. Such constraints arise through resource limitations, immune predation and adverse growth conditions in the form of tissue hypoxia, acidosis and cancer therapeutics, amongst others. Increasing evidence supports the ability of tumor cells to shape their own advantageous growth environment and the ability of the microenvironment to protect tumor cells from the deleterious impact of exogenous sources of microenvironment change derived from systemic therapy. Therefore, cancer evolution cannot be fully understood without a detailed understanding of the source and impact of micro-environmental selection pressures.

Computational, pathological as well as tumor imaging approaches are increasingly being used to describe the complex tumor microenvironment in a relatively unbiased manner. Aerts and colleagues applied radiomics, which refers to the quantification of tumor phenotype using multiple

imaging features, to head and neck and lung cancers (Aerts et al., 2014). The authors found that multiple radiomic features associated with heterogeneity were linked to poorer survival outcome in both tumor types. Through the integration of gene expression and somatic copy number data with automated microenvironment analysis from standard hematoxylin and eosin slides, Yuan and colleagues demonstrated that survival predictions in ER negative breast cancer can be optimized (Yuan et al., 2012). The authors found that the spatial distribution of stromal cells was an independent prognostic factor for survival outcome. Similarly, in ovarian cancers the percentage of stromal cells (assessed from hematoxylin and eosin slides) was significantly associated with poor overall and progression free-survival, even after controlling for clinical parameters including surgical debulking status and age (Natrajan et al., 2016). In prostate cancers, a measure of genomic instability coupled with intratumoral hypoxia was found to be able to significantly improve prognostic accuracy, beyond conventional clinical parameters (Lalonde et al., 2014). A landmark study in melanoma, which sequenced over 4,500 single cells from 19 patients (including malignant, immune, stromal and epithelial cells), identified therapy resistance tumor subpopulations present prior to treatment, which may have been missed with bulk-sequencing, as well as a relationship between cancer-associated fibroblasts and preferential expression of an AXL-high/MITF-low transcriptional program (Tirosh et al., 2016).

Recently, approaches to analyze the tumor microenvironment in 3D have been developed, allowing a quantitative measure of micro-environmental heterogeneity to be assessed (ecosystem diversity index) (Natrajan et al., 2016). In grade 3 breast cancers, high micro-environmental diversity was associated with poor prognosis, independent of tumor size or genomic features. The authors suggest these data are indicative of cooperation between tumor cells and the microenvironment. Further, the spatial diversity of resources inherent in a heterogeneous tumor microenvironment may select for a metastatic phenotype. A complementary explanation is that a heterogeneous tumor

microenvironment may also contribute to unequal drug penetration brought about by disordered blood vessel development that might contribute to resistant cell populations emerging through therapy (Fu et al., 2015; Junttila and de Sauvage, 2013) or to the development of diverse niches including hypoxic or perivascular regions that might support cancer stem cell phenotypes and chemo-resistance (Mao et al., 2013).

Immune-mediated editing

Seminal work from the Schreiber laboratory in mouse models demonstrated the capacity of the immune system to maintain tumors in a state of equilibrium, where clonal expansions are attenuated by adaptive immunity (Koebel et al., 2007). These observations begin to shed light on tumor dormancy and how patients with early stage breast cancer may have disseminated tumor cells in bone marrow, which never give rise to metastatic disease (Hartkopf et al., 2014).

One substrate for immune-mediated disease control of tumor growth can be patient-specific neo-antigens that arise as a consequence of tumor-somatic mutations. A number of studies have revealed an association of tumor mutational burden with response to immune checkpoint blockade. In both melanoma and non-small cell lung cancer, evidence is building that the mutational load and or neo-antigen burden correlates with benefit to anti-CTLA4 therapy in melanoma ((Snyder et al., 2014; Van Allen et al., 2015)) and anti-PD1 therapy in NSCLC (Rizvi et al., 2015). Likewise, hyper-mutated mismatch repair deficient tumors are significantly more responsive to immune-checkpoint blockade than their mismatch repair proficient counterparts (Le et al., 2015).

How tumor cells evade such hostile immune predation is becoming an active research area. Hacohen and colleagues devised an RNA-seq based signature of cytolytic activity, incorporating Granzyme A and Perforin, genes which are up-regulated following CD8+ T cell activation (Rooney et al., 2015). Application of the signature to the TCGA dataset, revealed that

certain tumors such as renal clear cell carcinoma exhibit high cytolytic activity while others such as glioma and prostate cancer tend to display low cytolytic activity. Somatic mutations in specific genes, such as inactivating mutations in Caspase 8, were associated with higher cytolytic activity. These data are consistent with previous work revealing that Caspase 8 blockade results in tumor T cell escape in two murine tumor models (Medema et al., 1999). CTL activity was associated with both the rate of mutations and the rate of mutations resulting in predicted neo-antigens across multiple tumor types (Rooney et al., 2015). Intriguingly, colorectal and kidney cancer harbored significantly fewer putative neo-antigens per non-silent than expected, consistent with immune-editing, where immune activity likely results in the depletion of emerging tumor clones with productive neo-epitopes.

The evolving somatic mutational landscape may also influence immune surveillance and response to checkpoint blockade. Evidence is emerging that the clonal status of a neo-antigen might influence immune checkpoint benefit. Tumors with a high clonal neo-antigen burden and low subclonal neo-antigenic heterogeneity appeared to be enriched in patients benefiting from anti-PD1 therapy in NSCLC or anti-CTLA4 therapy in melanoma (McGranahan et al., 2016). Whether subclonal neoantigens developing in a rapidly evolving tumor actively distract the immune response from the effective targeting of clonal neo-antigens is unclear. We have recently shown that APOBEC-induced mutagenesis contributes to branched evolution and the acquisition of subclonal mutations in adenocarcinoma of the lung, ER negative breast cancer, head and neck squamous carcinoma and esophageal adenocarcinomas (de Bruin et al., 2014; McGranahan et al., 2015; Rosenthal et al., 2016). In this regard the parallels with HIV-based evolution of diversity mediated by APOBEC activity are intriguing. Evidence suggests that APOBEC 3G/3F induced mutations in HIV are less immunogenic and reduce CD8+ T cell responses against common HIV epitopes *ex vivo* (Monajemi et al., 2014). Whether

APOBEC induced mutagenesis provides the tumor with a similar immune evasion escape warrants further investigation.

The dynamic nature of the "predator-prey" relationship between the immune microenvironment and the tumor has recently been highlighted by work demonstrating the ability of tumors to lose the expression of neo-antigens (Anagnostou et al., 2016; Verdegaal et al., 2016). Thus, therapeutic efforts may have to be oriented towards the targeting of multiple clonal neo-epitopes to optimize disease control and minimize the potential for immune escape.

Safe havens

Recent studies have suggested mechanisms of tumor resistance need not always be mediated by the selection for resistant populations of tumor cells (Hirata et al., 2015).

Using detailed intravital imaging in a mouse model of cancer, Sahai and colleagues have demonstrated that resistance to a BRAF inhibitor PLX4720 is mediated through melanoma associated fibroblast induced matrix remodeling. This promotes integrin Beta 1-FAK-Src signaling within melanoma cells and reactivation of ERK signal transduction and BRAF inhibitor resistance that can be circumvented by combined BRAF/FAK inhibition. Consistent with these data, fibronectin matrices (Hirata et al., 2015) or fibronectin induction (Fedorenko et al., 2016) are sufficient to circumvent the impact of BRAF inhibition on tumor cells. Co-cultures of fibroblasts, stromal cells and tumor cells revealed stromal derived HGF as a mediator of RAF inhibitor resistance in BRAF mutant melanoma cells via activation of its cognate receptor cMET (Straussman et al., 2012). Moreover, BRAF inhibition results in TGF beta release from melanoma cells, which promotes the differentiation of fibroblasts, fibronectin expression and HGF secretion which together triggers PI3K/AKT pathway activity (Fedorenko et al., 2016). Similar evidence in the field of anti-angiogenic therapies implicates the stroma in resistance to therapy through release of PDGF-C by cancer-associated fibroblasts (CAFs) (Crawford et al., 2009).

Just as genotoxic damage can foster the emergence of drug resistant cells and histological transformation of the tumor into a high-grade recurrence (Johnson et al., 2014; Kim et al., 2015), the stroma can also be adversely affected by genotoxic agents to support the survival of cancer cells in the face of such selection pressures. Genotoxic therapy can promote the microenvironmental secretion of WNT16B that reduces the impact of cytotoxic therapy upon prostate cancer cells *in vivo* (Sun et al., 2012). Genotoxic chemotherapy can also result in secretion of IL-6 and TIMP-1 from thymic endothelium in a mouse model of Burkitt's lymphoma, which promotes the survival of minimal residual disease in the thymus (Gilbert and Hemann, 2010). Exploring this chemoresistant perivascular niche phenomenon further, Hodivala-Dilke and colleagues demonstrated that following DNA damage, FAK loss from endothelial cells sensitizes cancer cells to doxorubicin through the suppression of NF-kB induced cytokine production from endothelial cells and concomitant reduced phospho-STAT3 in tumor cells following doxorubicin exposure, without having any measurable impact on endothelial function (Tavora et al., 2014).

Finally, increasing evidence supports the ability of the immune microenvironment to support the survival of tumor cells. MAPK pathway inhibition increases macrophage infiltration into the tumor microenvironment, promoting resistance to BRAF and MEK inhibition through TNF alpha release from myeloid cells, mediated the induction of the melanocytic-specific transcription and survival factor, MITF (Smith et al., 2014).

Taken together, these data are in keeping with the concept that the microenvironment can provide a "safe haven" for the evolution of drug tolerant cells, providing an explanation as to how cells survive in the period between initial tumor response and disease progression. Conceivably, through this permissive microenvironment, tumor cells may proceed to acquire genetically encoded drug resistant mechanisms that

might dominate specific lesions at progression. Targeting the microenvironment itself may therefore present an effective strategy to manage cancer clonal evolution. Using a humanized mouse model Bcl2/Myc driven lymphoma, treated with alemtuzumab (anti-CD52 antibody), Hemann and colleagues demonstrated that infiltration of resistant leukemia cells into the bone marrow rewires the tumor microenvironment to inhibit engulfment of antibody-targeted tumor cells. Combination therapy of cyclophosphamide with the antibody eliminated residual disease by inducing a secretion phenotype that increased infiltration of macrophages into the bone marrow and enhanced phagocytic activity (Pallasch et al., 2014).

Managing clonal evolution

Despite extensive clinical data documenting ITH and its clinical relevance, drug development and novel clinical trial designs to account for the dynamic evolution of tumors have lagged behind.

Longitudinal sampling strategies

Given the evolutionary capabilities of tumors, monitoring disease evolution to guide therapeutic interventions and to understand evolutionary trajectories of individual tumors has become a vital research area. Serial sampling of tumor genomes from plasma and circulating tumor cells is now increasingly implemented in both clinical research settings to monitor cancer clonal evolution and drug resistance mechanisms over time (Haber and Velculescu, 2014) as well as the evolution of metastatic disease (Carreira et al., 2014). Sequencing of circulating tumor DNA (ctDNA) through therapy can reveal somatic mutations acquired at resistance following cytotoxic and targeted therapies (Murtaza et al., 2013) and the detection of which has higher sensitivity and dynamic range than conventional blood based markers (Dawson et al., 2013). Furthermore, an increase in ctDNA heralds progressive disease in advance of conventional imaging approaches (Dawson et al., 2013).

Problems inherent to tumor sampling bias due to ITH may be mitigated with ctDNA sampling (Jamal-Hanjani et al., 2016; Russo et al., 2016; Siravegna et al., 2015). Moreover, such methods can track mechanisms of resistance to targeted agents, which may emerge during the course of therapy. Multiple mutations in KRAS and NRAS were identified in the same patient through BEAMing analysis of ctDNA, acquired during cetuximab or panitumumab exposure in advanced colorectal cancer, converging upon the reactivation of ERK signaling. These results suggest a rational strategy to limit acquired therapy resistance may be through dual blockade of both EGFR and MEK signaling (Misale et al., 2012). Studies are also revealing the dynamic clonal evolution that occurs subsequent to the acquisition of drug resistance. Bardelli and colleagues (Siravegna et al., 2015) demonstrated the waning of KRAS mutant subclones upon EGFR monoclonal antibody drug withdrawal, suggesting fitness costs following the acquisition of KRAS mutations later in tumor evolution and providing an explanation for further tumor responses following drug re-challenge. However, a drawback of ctDNA sampling strategies is that they cannot necessarily provide an accurate portrayal of the copy number state of the cancer genome nor a detailed phylogeny, and there may be over-representation of DNA from dying cells. In this respect, circulating tumor cells may provide additional information.

Targeting clonal events

Targeting clonal events, present in every tumor cell, may present an attractive model for drug development. Indeed, it is likely that many targeted therapies that have successfully passed through the drug development process, demonstrating robust progression free survival benefits in clinical trials, are targeting early clonal events present at all sites of disease. However, even in the context of a clonal driver, resistance to such therapies is frequent in the advanced disease setting and may be driven by the selection of resistance cancer cells present at low frequencies prior to therapy (Bhang et al., 2015; Su et al., 2012; Turke et al., 2010) or may evolve through de novo mutations that are acquired during therapy (Hata et al., 2016).

Modeling approaches have estimated that most lesions identifiable through radiographic techniques harbor ten resistant subclones (Bozic and Nowak, 2014). Combination therapy approaches that act through distinct pathways may help circumvent this problem (Bozic et al., 2013). However, in practice the feasibility of such approaches may be complicated by the toxicity of combination therapy, as well as the occurrence of mutations that confer resistance to multiple drugs.

Conceivably, vaccine or adoptive T cell therapy approaches targeting multiple clonal neo-antigens may provide the specificity required to minimize normal tissue toxicity and maximize tumor cell kill, whilst minimizing the possibility for acquired drug resistance to occur. We have recently found evidence for CD8+PD1+ T cell populations recognizing clonal neo-antigens present in all cells of a tumor (McGranahan et al., 2016). However, the extent to which tumors could circumvent such strategies through chromosomal instability-driven loss of neo-antigens remains unclear.

Attenuating or exploiting genome instability

Genome instability acts as a fuel for cell-to-cell variation and hence selection and evolution. The clinical relevance of genomic instability is evidenced by the association of chromosomal instability with outcome across multiple cancer types (McGranahan et al., 2012), and accumulating evidence linking chromosomal chaos with metastasis (Turajlic and Swanton, 2016). Targeting specific genome instability mechanisms may provide a means to arrest tumor evolution and limit disease progression, particularly in the early disease.

The success of PARP inhibitors in the treatment of *BRCA*-mutant cancers exemplifies how genomically unstable cancers can be targeted by elevating instability to lethal levels and exploiting synthetic lethality (Lord and Ashworth, 2016). However, even in this context resistance can occur, for example through BRCA reversion either directly through additional

mutations to *BRCA* (Lord and Ashworth, 2016) or indirectly through, for example, inactivation of 53BP1(Lord and Ashworth, 2016).

Maley and colleagues have explored the impact of non-steroidal anti-inflammatory agents (NSAIDs) upon the evolution of pre-invasive Barrett's esophagus to invasive esophageal cancer. In a longitudinal analysis of 13 patients who had been exposed to NSAIDs over a period of several years, the authors found evidence that NSAID use was associated with a reduced rate of somatic genomic abnormalities as defined by SNP array analyses (Kostadinov et al., 2013).

Evolutionary studies are revealing distinct mutagenic processes that occur through the disease course. Evidence is emerging in lung adenocarcinoma, bladder cancer, estrogen receptor negative breast cancer, head and neck squamous carcinoma and esophageal squamous carcinoma that APOBEC induced mutagenesis is enriched later in tumor evolution, suggesting that efforts to target the cytidine deaminase family may demonstrate utility to limit ongoing mutagenesis (de Bruin et al., 2014; McGranahan et al., 2015). A recent study, using clinical data and xenograft experiments, found evidence that APOBEC3B can facilitate tamoxifen resistance in ER-positive breast cancer (Law et al., 2016). Similarly, chemotherapy refractory chemotherapy-resistant urothelial carcinomas exhibited an enrichment of APOBEC3B mutations following therapy (Faltas et al., 2016). Inhibiting APOBEC3B may provide an effective strategy to improve efficacies of cancer therapies, by limiting the evolutionary potential of cancer cells.

Competitive release and Adaptive Therapy

Whilst benefits in progression free survival times are commonly reported in clinical trials, these rarely translate to equivalent clinically relevant overall survival benefits (Fojo et al., 2014). Notwithstanding the complexities of clinical trial design, the mismatch between progression free and overall survival times may reflect clonal competition and competitive release. Conceivably, elimination of a dominant drug sensitive clone in the investigational arm, might allow the competitive release of resistant

subclones to undergo accelerated growth in a resource-rich environment, resulting in more rapid disease progression compared to the control arm after the observed progression free survival benefit (Figure 4).

Thus, new trial concepts accounting for competitive release of resistant subclones may maximize the overall survival benefits with current therapies. Gatenby and colleagues have devised approaches in animal models to exploit the fitness cost of resistant subclones by maintaining a stable population of sensitive subclones, thereby restricting the growth of resistant cells (Enriquez-Navas et al., 2016). In contrast to standard clinical practice where the goals of therapy are to reduce tumor burden, the focus of adaptive therapy is to maximize time to progression by stabilizing tumor size (Enriquez-Navas et al., 2016; Gatenby et al., 2009). Adaptive therapy requires variable drug dosing and schedules in two phases; an induction phase to control tumor progression from exponential growth; and a maintenance phase that might require progressively lower dosing or even omitted schedules resulting in better progression free survival times compared to standard fixed dosing. In keeping with the benefits of an adaptive therapy approach, in the context patient-derived melanoma xenografts. Stuart and colleagues demonstrated how vemurafenib resistant melanomas can exhibit drug dependency, such that an intermittent rather than continuous dosing of the drug can forestall the onset of lethal drug resistance (Das Thakur et al., 2013).

Exploiting evolutionary constraints.

Traditional approaches to cancer management are primarily reactive, focusing on the management of drug resistant disease. Pro-active management of cancers through attempts to predict or attenuate a cancer's next evolutionary move, exploiting evolutionary constraints or synthetic lethality, might be feasible as knowledge of evolution across cancer types increases.

Emerging evidence in renal cell carcinoma suggests that the constraints upon activation of the PI3K/mTOR pathway, manifested as recurrent deleterious or activating mutations in *PTEN*, *PIK3CA*, *TSC1* or *mTOR*, might be exploitable for therapeutic benefit. Voss and colleagues examined renal tumors from 5 patients who had experiences a prolonged benefit from mTOR pathway inhibition with Everolimus or Temsirolimus. Multi-region tumor sampling revealed parallel evolution with distinct somatic mutations predicted to lead to activation of the mTOR pathway in different tumor regions in 3 of the 5 cases (Voss et al., 2014). These data suggest that targeting constraints to tumor evolution might be practical if appropriate biomarker assays could be developed that could detect parallel evolution leading to signal transduction pathway convergence.

A further tractable approach may be derived from exploiting iatrogenic evolutionary selection pressures. An approach termed "collateral sensitivity" leverages the phenomenon where resistance acquired to one drug comes at the expense of sensitivity to another (Hill, 1986; Jensen et al., 1997). Hemann and colleagues have exploited such evolutionary constraints in a murine model of Philadelphia chromosome positive acute lymphoblastic leukemia (ALL) (Zhao et al., 2016). The authors described collateral sensitivity induced by treatment with dasatinib, which resulted in the selection of the acquired resistance BCR-ABL1 V299L mutation at intermediate stages of evolution of Ph+ ALL cells. This rendered the cells sensitive to non-classical BCR-ABL inhibitors such as cabozantinib and vandetanib.

Similar methods have been applied to the targeting of aneuploid populations. Rong Li and colleagues use an "evolutionary trap" by reducing karyotypic heterogeneity to a defined predictable state through initial drug exposure, which can then by targeted by a secondary drug. Specifically, exposure of aneuploid budding yeast to radicol, an HSP90 inhibitor, results in the selection of multiple copes of chromosome XV. Amplification of

chromosome XV results in resistance to radical, however, it also engenders sensitivity to hygromycin (Chen et al., 2015).

Evidence is emerging for the potential of similar strategies in the clinical setting. Engelman and colleagues explored resistance mechanisms in a patient with ALK rearranged non-small cell lung cancer with a subclonal C1156Y mutation in the kinase domain, acquired following progression on crizotinib (Shaw et al., 2016). Although the tumor did not benefit from a second generation ALK inhibitor, it did respond to the 3rd generation ALK inhibitor, lorlatinib. Following progression on lorlatinib, the tumor acquired a L1198F mutation, which, together with the pre-existing C1156Y alteration, prevented drug interaction with the kinase. However the L1198F mutation promoted re-sensitization to crizotinib, thereby resulting in improvement in the patient's symptoms. This case study illustrates how evolutionary constraints and collateral sensitivity can be exploited for patient benefit.

Conclusions

Although our understanding of cancer genome evolution, and the dynamic interplay between tumor cells and the microenvironment, has dramatically increased, the field of cancer evolutionary therapeutics is still in its infancy. As we attempt to forecast evolution and proactively manage a dynamic tumor genome and its microenvironment, it is worth reflecting that Darwin recognized such challenges *"throw up a handful of feathers, and all fall to the ground according to definite laws; but how simple is the problem where each shall fall compared to that of the action and reaction of innumerable plants and animals which have determined, in the course of centuries, the proportional numbers and kinds of trees now growing" (Darwin, 1859).* Predicting the innumerable interactions of cancer subclones with each other and the microenvironment is an equally formidable task. Computational and technological advances, coupled with prospective longitudinal studies exploring the cancer genome and the immune microenvironment, will be needed to gain a deeper understanding of the evolutionary trajectories of tumors and the extent to which a tumor's next

step may be predicted. Such studies may also allow new insights into the processes generating diversity and reveal how constraints to tumor evolution may be exploited, leveraging an adaptive immune response, permitting proactive management of cancers.

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Figure legends

Figure 1 Heterogeneity of non-silent mutations from multiple-sample sequencing across a range of cancer types. For each tumor type, each point represents one tumor, with the proportion of heterogeneous mutations (ITH proportion), as well as the absolute numbers of heterogeneous and homogeneous non-silent mutations shown. Black circles represent treatment naïve tumors, with red triangles indicating tumors that have received treatment. Notably, this data is restricted to non-silent mutations and does not include copy number alterations. The data is extracted from the following primary publications: Diffuse intrinsic pontine glioma (Nikbakht et al., 2016); Neuroblastoma (Eleveld et al., 2015);

low-grade gliomba/glioblastoma multiforme (Johnson et al., 2014; Kim et al., 2015; Wang et al., 2016); breast cancer (Yates et al., 2015); clear cell renal cell carcinoma (Gerlinger et al., 2014a); multiple myeloma (Bolli et al., 2014); lung adenocarcinomas (de Bruin et al., 2014; Zhang et al., 2014); prostate (Gundem et al., 2015; Ju et al., 2014); bladder (Lamy et al., 2016); colorectal adenocarcinomas (Uchi et al., 2016); liver hepatocellular carcinoma (Xue et al., 2016); esophageal squamous cell carcinoma (Hao et al., 2016); ovarian (Bashashati et al., 2013; Eckert et al., 2016); esophageal adenocarcinoma (Murugaesu et al., 2015); melanoma (Harbst et al., 2016).

Figure 1 Evolutionary trees illustrating intratumor heterogeneity across cancer types. For each cancer type, the mean number of clonal and subclonal non-silent mutations are depicted as trunks (blue) and branches (yellow and red) respectively.

Figure 3 Clonal heterogeneity and tumor evolution: modes, mechanisms, ecosystems and evolutionary therapeutics. The first three panels depict different aspects of cancer genome evolution, which all need to be understood to develop improved evolutionary therapeutics (panel four).

Figure 4 Competitive release of resistance subclones. Similar overall survival times, yet divergent progression free survival times, between treated and un-treated patients may reflect competitive release of aggressive subclones.

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