Non-genetic mechanisms of therapeutic resistance in cancer

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Abstract | Therapeutic resistance continues to be an indominable foe in our ambition for curative cancer treatment. Recent insights into the molecular determinants of acquired treatment resistance in the clinical and experimental setting have challenged the widely held view of sequential genetic evolution as the primary cause of resistance and brought into sharp focus a range of non-genetic adaptive mechanisms. Notably, the genetic landscape of the tumour and the non-genetic mechanisms used to escape therapy are frequently linked. Remarkably, whereas some oncogenic mutations allow the cancer cells to rapidly adapt their transcriptional and/or metabolic programme to meet and survive the therapeutic pressure, other oncogenic drivers convey an inherent cellular plasticity to the cancer cell enabling lineage switching and/or the evasion of anticancer immunosurveillance. The prevalence and diverse array of non-genetic resistance mechanisms pose a new challenge to the field that requires innovative strategies to monitor and counteract these adaptive processes. In this Perspective we discuss the key principles of non-genetic therapy resistance in cancer. We provide a perspective on the emerging data from clinical studies and sophisticated cancer models that have studied various non-genetic resistance pathways and highlight promising therapeutic avenues that may be used to negate and/or counteract the non-genetic adaptive pathways.

As our understanding of the molecular pathogenesis of cancer continues to expand, we are drawn further towards the promise of personalized cancer care. This form of molecular medicine seeks to eradicate cancer cells by interfering with specific targeted molecules needed for tumour growth and/or maintenance. The past two decades have witnessed the rapid development of a range of targeted therapies, including small-molecule inhibitors and monoclonal antibodies that aim to block immune checkpoints or interfere with key cancer-promoting pathways[1–4]. These agents, many of which have now become the standard of care either in isolation or as part of combination strategies, have revolutionized patient outcomes. Unfortunately, however, most patients with cancer, particularly those with advanced or metastatic disease at the time of treatment, continue to experience therapeutic resistance manifested most commonly as local or distant disease recurrence. Cancer relapse occurs because all available therapeutic modalities frequently leave behind residual cancer cells, traditionally called 'minimal residual disease' (MRD), providing a reservoir from which relapse inexorably emerges. The primary cause often invoked for therapeutic resistance is genetic evolution, whereby one or a group of malignant cells either carry or acquire a specific genetic alteration (that is, mutation, gene amplification, gene deletion or chromosomal translocation) that provides the cancer cells with a clonal advantage to escape the therapeutic pressure. While genetic clonal evolution unquestionably plays a role in mediating resistance to some targeted therapies and conventional chemotherapies, therapeutic resistance in the absence of a clear genetic cause is increasingly being recognized in several cancers[5–10]. The true prevalence of genetic and non-genetic mechanisms of resistance in cancer is largely unknown, and the molecular mechanisms underlying non-genetic resistance remain elusive. Here we provide a perspective on this critical issue and discuss how addressing these questions may help develop new innovative, effective and rational combination therapies. Importantly, we also provide recent evidence that points to a convergence of the molecular mechanisms underlying drug adaptation and non-genetic resistance across distinct tumour types, raising the possibility that common combination treatments may be applicable to overcome this resistance across a wide range of cancers.

Therapeutic resistance in cancer

Resistance to cancer therapies can be broadly classified as primary (intrinsic) or secondary (acquired) resistance. Primary resistance is manifested by the lack of an objective clinical response following therapy. By contrast, secondary resistance represents local or distant recurrence of the malignancy after a clinical response. While these terms are part of our everyday vernacular, it is important to realize this distinction is entirely dependent on the mode of response assessment. Response assessment in cancer is a complex and rapidly evolving area that involves multiple modalities, including clinical and pathological assessment, various imaging methods and an ever-increasing suite of molecular diagnostics. While a detailed discussion of response assessment in cancer is beyond the scope of this Perspective, it is important to emphasize that each modality differs in sensitivity and specificity, and while one monitoring method may suggest a clinical response, another may raise the prospect of primary resistance. We believe that common principles underpin the inability to completely eradicate the malignant population in both primary and secondary resistance; however, this Perspective will focus on malignant relapse following an initial response to therapy, so-called acquired resistance.

Acquired resistance is often conceptualized and portrayed as genetic evolution of cancer in response to therapeutic challenge (BOX 1; FIG. 1). However, there is a growing appreciation that genetic evolution is unlikely to represent the

Box 1 | **The genetic lens through which cancer evolution is often seen**

While it is well recognized that all cancer cells are heterogeneous in terms of their genetic, epigenetic and transcriptional states, tumour evolution resulting in therapy resistance is most commonly viewed through a genetic lens. The 'Darwinian' selection of mutant cells carrying a relevant mutation acquired by chance before (or during) treatment[163] leads to a shift in clonal composition whereby certain mutant cells are passively selected by the therapy over time. Intratumour genomic heterogeneity has been extensively studied through multiregional tumour tissue sampling[164–166], paired primary tumour–metastases studies[167] and post-mortem analyses[168,169], which have provided key insights into the complex clonal architecture of cancers and the way in which subclonal structure evolves over time. Patterns of genomic evolution have been broadly described as branching (to describe the emergence of divergent subclones) or linear (where additional mutations are acquired sequentially). Analyses of serial biopsy samples are often depicted as showing the sequential accumulation of genomic changes over time but, in part, this imagery often depicted in fish plots has propagated an oversimplification and adoption of linear models of disease progression (FIG. 1). Moreover, sequencing studies have traditionally been performed using bulk DNA pooled from multiple cancer cells, making it impossible to fully resolve whether particular mutations co-occur in the same cell. More recently, single-cell sequencing studies have helped to overcome these limitations and are challenging the view of linear progression by revealing far greater clonal complexity than was previously appreciated. Importantly, emerging evidence has emphasized the fact that tissue-specific cancer stem cells, due to their longevity and duration of exposure to genotoxic insults, harbour the greatest subclonal genetic diversity, resulting in diverse non-linear evolutionary trajectories[170]. If this variation in clonal dominance is analysed at the population level by bulk sequencing, it will likely be misrepresented as a linear gain and/or loss of mutations providing evolutionary fitness (FIG. 1a). However, when analysis is performed at single-cell resolution, the reality reveals that genetic evolution has not played a part in treatment resistance but instead the various stressors experienced throughout the treatment course have simply favoured the dominance of pre-existing subclones (FIG. 1b).

In the context of therapeutic pressure, this important distinction between clonal selection versus acquired de novo mutations is often made more unclear owing to the compounded technical limitations of bulk tumour sequencing assays, sampling a single region alone through tissue biopsy and the difficulty in obtaining serial samples over time. Consequently, novel approaches are required that provide greater resolution. When tissue is available, single-cell genomic approaches should be strongly considered. However, serial tumour tissue samples are often impractical and, in this context, complementary strategies such as the use of circulating tumour DNA to track subclonal mutations are being increasingly explored to help overcome the challenges of obtaining comprehensive spatial and temporal representation[171–174]. An accurate characterization of genomic changes in space and time, in parallel with an improved understanding of the interplay between genetic and non-genetic resistance pathways, will be essential for the development of therapeutic approaches to prevent, circumvent and overcome disease relapse following treatment.

sole or indeed the most common mechanism for therapeutic evasion. Non-genetic priming or adaptation in cancer cells contributes substantially to the intratumour heterogeneity that underpins resistance to cancer therapies (FIG. 2). The changes in chromatin structure and function that facilitate the transcriptional priming or dynamic transcriptional adaptation seen as part of non-genetic resistance are starting to be uncovered and highlight the fact that a unilateral approach to only monitor genetic evolution during cancer therapy to guide future therapeutic decisions will fail to deliver on the promise of precision medicine. Instead, it is important for our field to recognize the pervasive contribution of non-genetic heterogeneity and to develop strategies to effectively counteract this, in addition to the genomics-guided approach frequently used with targeted therapies.

While the models of genetic and non-genetic therapeutic resistance are outlined here as separate entities to illustrate the concepts associated with them (FIG. 2), it is vital to emphasize that we strongly believe most, if not all, cancers leverage multiple available processes for therapeutic evasion concurrently, and these are not mutually exclusive evolutionary trajectories. As discussed herein, it is very likely that different genetic mutations within cancer cells favour and enable particular modes of non-genetic therapeutic evasion. Moreover, the repertoire of mechanisms involved in therapeutic evasion include a diverse range of cell-autonomous[11] and cell-non-autonomous[12] processes, consequently posing a major challenge to understanding resistance to all anticancer therapies[13,14]. Devising therapeutic strategies that target only resistance-conferring genetic events becomes a 'whack-a-mole' game, which is unwinnable, especially considering that the vast majority of oncogenic mutations are not druggable[15]. Therefore, an alternative and attractive approach would be to focus our attention on identifying the common and cell type-specific mechanisms of resistance and use this knowledge to inform upfront therapeutic strategies that effectively target both genetic and non-genetic mechanisms of resistance.

Non-genetic mechanisms of evolution

There is accumulating evidence that the inevitable path to drug resistance cannot be reduced to a simple genetic cause. The notion that a single cancer genome has the ability to produce multiple phenotypic states and that cancer cells can switch between these states without genomic alterations is gaining greater recognition[16]. Importantly, such non-genetic reprogramming events are observed on therapy exposure, and these adaptive responses are associated with increased resistance to the treatment. An early report clearly showed that while the vast majority of cells in a cultured non-small-cell lung cancer (NSCLC) cell population were rapidly killed on exposure to therapy, a rapid and transient accumulation of viable or residual 'drug-tolerant' cells was observed with kinetics and frequency that could not be explained by mutational mechanisms[17]. Notably, as opposed to drug resistance, drug tolerance manifested itself as a state in which tumour cells could transiently survive but not proliferate on treatment. Similar protective responses to therapeutic pressure were subsequently reported in cultures originating from other types of cancer in response to a variety of (therapeutic) challenges[18]. For instance, chemotherapy induces a phenotype-switching event in various epithelial tumours, such as colon[19], gastric[20], lung[21] and breast[22] cancers, known as epithelial-to-mesenchymal transition (EMT). This reprogramming event has long been associated with chemoresistance[23,24]. Similarly, it is well accepted that melanoma cells can switch back and forth between a 'proliferative' and an 'invasive' (mesenchymal-like) cell state[25], with the invasive (also referred to as 'undifferentiated') phenotype being intrinsically resistant to MAPK inhibitors[26,27]. Consistently, exposure of melanoma to targeted therapy causes a shift in the entire cell population towards the undifferentiated cell state, an event that contributes to drug tolerance and/or resistance[27–29]. Notably, this undifferentiated programme also appears to be a hallmark of resistance to programmed cell death protein 1 (PD1) inhibitors[30]. Similarly, in NSCLC treated with PD1 checkpoint inhibitors, upregulation of alternative immune checkpoints leads to resistance[31], indicating that non-genetic adaptive responses occur

in response to most, if not all, therapeutic modalities.

Transient and stable non-genetic resistance. Drug-tolerant persister (DTP) cells display the hallmarks of being slow-cycling cells with altered cellular metabolism. Following drug withdrawal, these persister cells can reinitiate cell cycle progression, and remarkably their progeny are often sensitive to rechallenge with the initial therapy[27,32] (FIG. 3a). These findings may represent the reawakening of an ancestral stress response first described after therapeutic challenge in prokaryotes. In landmark experiments performed in the early 1940s, Joseph Bigger[33] demonstrated that when drug-naive staphylococci were exposed to penicillin, a small proportion of bacteria were able to withstand this lethal insult. When these DTP cells were recovered and rechallenged with penicillin, most of the daughter cells were sensitive, and remarkably the numbers of persister cells noted were similar to those seen with drug-naive bacteria. These results mirror the findings described recently with DTP cancer cells, and suggest that analogous to the situation in bacteria, the DTP cancer cell state is transient and not stably inheritable. In bacteria, there is evidence that DTP cells exist within the drug-naive population and the persister phenotype is underpinned by gene expression programmes that arrest bacterial cell growth and reduce cellular metabolism[34]. However, these critical insights from microbiology need further evaluation in the context of cancer. For example, although DTP cancer cells are seen to rapidly emerge on therapeutic challenge, it remains unclear whether these DTP cells represent an enrichment of a specific 'primed' drug-tolerant state or whether it occurs through active, therapeutically induced, non-genetic reprogramming (FIG. 2). In this regard, it was recently reported that emergence of drug-tolerant neural crest stem cells (NCSCs) in melanoma occurs transiently through active cell state transition rather than passive selection[28]. Such a directed somatic evolution towards an advantageous phenotype may be referred to as 'Lamarckian' induction: a better 'adapted' inheritable state induced by an environmental input. This observation that melanoma cells exploit such a mechanism has been corroborated by two additional studies[35,36]. Arguably, such an adaptive cell state transition is more likely to contribute to drug tolerance or resistance in tumours with a low mutation burden. However, the fact that this phenomenon was also observed in cutaneous melanoma, the tumour type with the highest mutation load, strongly suggests that Lamarckian induction is not uncommon and is likely to constitute a common or default reaction of cancer cells to therapy-mediated killing.

Can non-genetic resistance be stable and heritable? When patients relapse after achieving a remission, clinical experience over many decades has clearly demonstrated that rechallenging the patient with the same therapy is invariably futile. This clinical scenario is often seen even though the patient may not have been exposed to the anticancer therapy for months or years, and it is for this reason that a different anticancer drug schedule is invariably used as salvage therapy. Remarkably, recent efforts to characterize the genomic landscape of recurrent and resistant tumours have failed to identify a clear genetic cause for this 'stable' drug resistance in up to 40% of tumours. This is not only seen in tumours with a low mutation burden such as acute myeloid leukaemia (AML)[37–40] but has also been noted in various solid malignancies, including breast cancer[6] and melanoma[9,41,42]. These findings highlight the fact that non-genetic resistance can clearly be stable and heritable, and understanding the principles that underpin this evolution is the next critical phase.

The process of stable non-genetic adaptation is likely to have many avenues that ultimately result in a new equipoise that is unaffected by the therapeutic pressure (FIG. 3b). This frequently manifests itself as a different transcriptional programme and cellular plasticity that is associated with a phenotypic switch including EMT, transdifferentiation to a different lineage within the cancer tissue and reversion to an immature stem and/or progenitor phenotype[43]. In haematopoietic tumours such as AML, recent evidence in both mouse models of AML[5] and from human patient samples[8] has shown that cells that show stable non-genetic therapeutic resistance have transcriptional programmes,

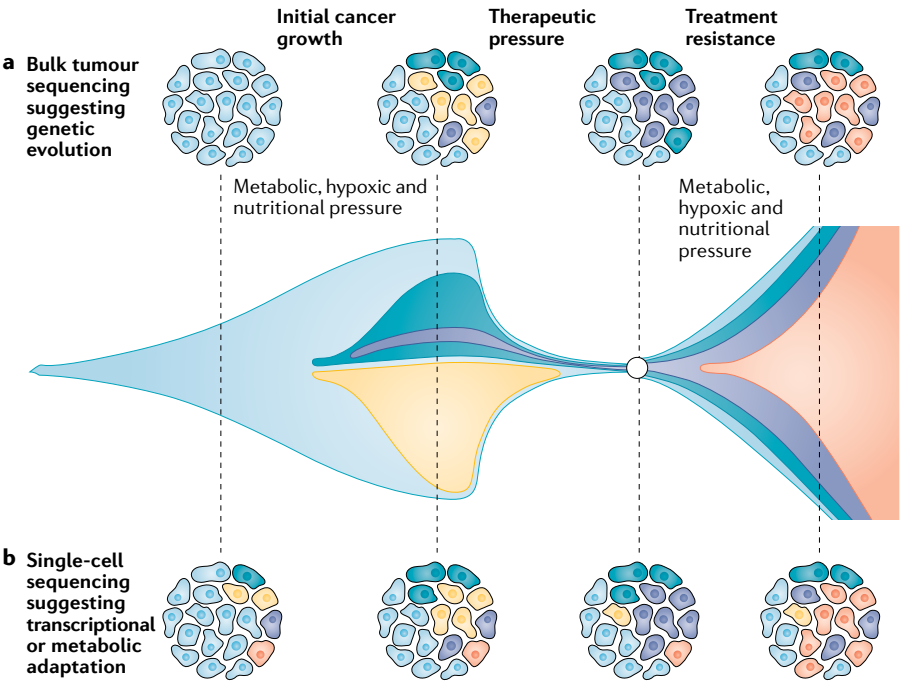

Fig. 1 | **Interpretation of mechanisms of resistance are influenced by the resolution of the technology.** Illustrated here is an often-used schematic, known as a fish plot, which is used to describe tumour evolution and the consequences of therapeutic pressure. As a tumour grows, it is subjected to various pressures, including hypoxia and metabolic and nutritional limitations; consequently, some clones containing certain mutations may be more adept at coping with this pressure and grow in clonal size. Similarly, when challenged with therapy, some clones are eradicated, whereas others have mutations that confer a clonal advantage in the context of therapeutic pressure. **a** | When analysis is done by bulk tumour sequencing, the depth of sequencing primarily allows detection of mutations within the dominant clones present within a population. Therefore, this natural evolutionary trajectory in the life of a cancer cell population can be interpreted as sequential genetic evolution. **b** | By contrast, high-resolution single-cell analyses indicate that all of the clones present throughout the natural history of the cancer may be present at the beginning, but in different clonal sizes. In this scenario, the entire evolutionary path can be explained by non-genetic mechanisms of adaptation to the various pressures, and no new genetic mutations arise.

immunophenotypic features and functional properties of leukaemia stem cells. Here, cancer cells that were fated to relapse existed before therapy, and these cells did not require any further coding mutations to become stably refractory to therapy. Using molecular barcoding in a mouse model of AML to try to understand whether these cells were transcriptionally primed before drug exposure or whether the resistance was an active adaptive process, Bell et al.[44] found that a proportion of cells present in the drug-naive tumour population not only survive the initial therapeutic challenge but are also able to adopt a transcriptional state that is not just tolerant of the drug but can confer them with the ability to actively proliferate in the presence of therapeutic pressure. Unlike the case with DTP cells, if these cells are withdrawn from drug pressure for prolonged periods and then rechallenged, they continue to maintain their resistant phenotype despite there being no new coding mutations[5,44] (FIG. 3b). The transcriptional plasticity, which enabled the resistant phenotype, was because these malignant stem and/or progenitor cells were able to leverage a developmental process called 'enhancer switching'[44]. Enhancer switching is essential in normal development, whereby stem and/or progenitor cells use different enhancers compared with differentiated lineage-specific cells to maintain the expression of important broadly expressed genes such as *MYC* that are ubiquitously required for cellular homeostasis[45,46]. In an analogous manner, inherently plastic cancer stem cells (CSCs) are able to exploit the available pioneer factors or cofactors to nucleate different enhancers to sustain expression of key genes essential for cell survival and proliferation[44]. The hijacking of developmental programmes and the associated enhancer remodelling to enable non-genetic adaptive resistance to a broad range of anticancer therapeutics is a rapidly emerging theme in cancer biology[47–50]. While there is clear evidence for extensive reactivation of regulatory elements during oncogenesis, which are usually silenced during the process of differentiation, it is also likely that this plasticity is not limitless[51]; hence, as discussed below, this may offer therapeutic opportunities to either constrain or target this mechanism of adaptation.

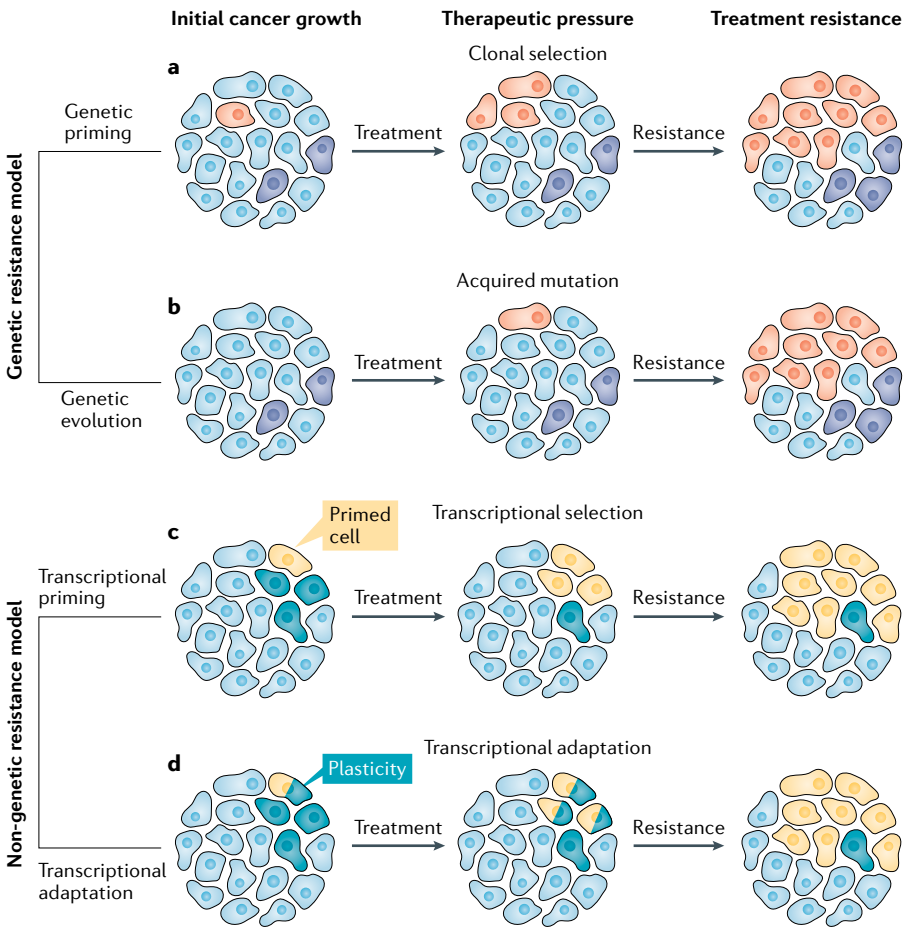

Fig. 2 | **Models of genetic and non-genetic therapy resistance. a** | Genetic mechanisms of drug resistance include mechanisms by which a pre-existing genetic mutation primes the cancer, conferring intrinsic resistance to a particular therapy. **b** | Alternatively, cancer cells may acquire a mutation during the course of therapy that affords a proliferative or survival advantage by negating the effects of the treatment. **c** | Non-genetic mechanisms may result in the expression of a particular transcriptional programme that provides intrinsic resistance to a particular drug. **d** | Cancer cells can rapidly adapt to therapeutic pressure by rewiring their gene expression to acquire a programme that offers a selective advantage in the context of therapy, enabling the cells to escape the therapeutic pressure. It is likely that all cancers to a greater or lesser degree display all of these mechanisms of resistance.

Therapy-induced phenotype switching often refers to a bidirectional phenomenon in which cells switch between a drug-sensitive and a stem-like drug-resilient state. However, this model is likely to be oversimplistic. Recent evidence has shown that EMT is more than a binary on–off switch; indeed, several intermediate states harbouring distinct phenotypic features can be identified between the extreme epithelial and mesenchymal states[52]. In keeping with this, it was recently shown that multiple drug-tolerant states coexist in melanoma[28]. Single-cell RNA sequencing was used to study adaptation to MAPK inhibition during the establishment of MRD with patient-derived tumour xenografts (PDXs) as an in vivo model system. Distinct populations of drug-tolerant cells were identified in a single MRD lesion, including the invasive or undifferentiated mesenchymal-like melanoma cells, a melanoma NCSC population, a highly pigmented or differentiated state and a 'starved-like' melanoma cell (SMC) state. When the time course of transcriptional dynamics and lineage relationships were reconstructed, it was found that these cells were distributed along pseudotemporal ordering paths from proliferative to pigmented cells (differentiation lineage) or to cells that adopted either the invasive state or the NCSC state (dedifferentiation lineage). The SMCs were present at the branching point, preceding both end states, indicating that drug exposure promotes a rapid and transient switch from a proliferative state to a starved-like state from which cells then make the decision to move along either a differentiation or a dedifferentiation trajectory. The NCSC state was the predominant drug-tolerant state in the on-treatment patient biopsy samples analysed[28]. In a follow-up study, evidence was obtained that the cellular composition

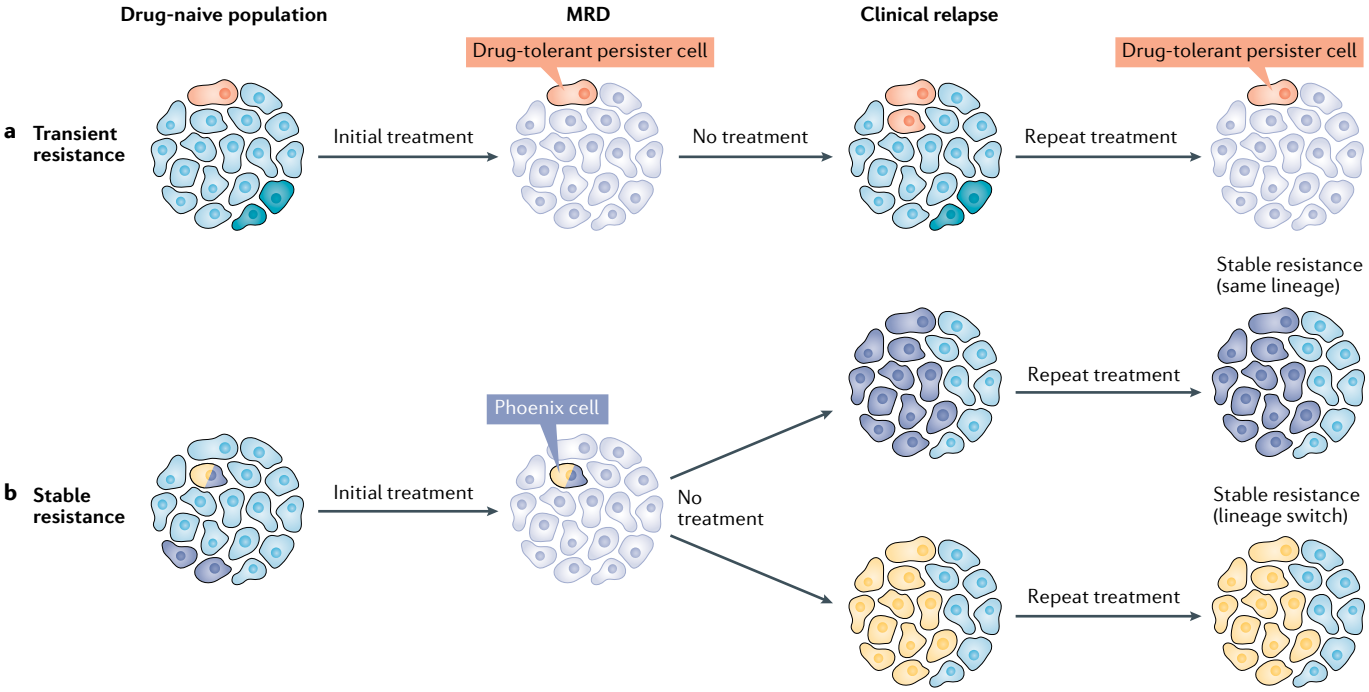

Fig. 3 | **Mechanisms of transient and stable non-genetic resistance.** Underpinning cancer relapse is a rare population of cells that are able to survive the initial therapeutic challenge, giving rise to minimal residual disease (MRD). **a** | The MRD cells may manifest themselves as a transient drug-tolerant persister cell state. Drug-tolerant persister cells are able to regenerate the tumour cell population, but most of the population remains sensitive to the initial therapy. **b** | By contrast, the MRD cells may adopt the phoenix cell state. This population of cells is able to undergo rapid transcriptional and metabolic reprogramming and may regenerate a tumour population that is morphologically and phenotypically identical to the drug-naive tumour or displays phenotypic features that are distinct from those of the drug-naive tumour. In both cases, the regenerated tumour has adapted to the initial therapy and is no longer sensitive to rechallenge. It is also theoretically possible that the phoenix state can emerge transiently on therapy and may be lost during tumour regrowth.

of MRD may deterministically impose distinct drug resistance evolutionary paths. The presence of the NCSC subpopulation in MRD was shown to concur with the rapid development of resistance through non-genetic mechanisms. Complete ablation of this subpopulation delayed the onset of resistance in the PDX setting, and, strikingly, all tumours that eventually escaped the NCSC-targeting regimen exhibited resistance-conferring genetic alterations (J-C.M, unpublished observations). These data also raise the possibility that this NCSC population exhibits increased 'epigenetic' plasticity that permits random activation of alternative gene regulatory networks and thus allows acquisition of specific phenotypic properties through non-genetic reprogramming. Some of these properties may be maintained through cell division and may eventually lead to the selection of drug-resistant 'epiclones'. These observations are consistent with other reports highlighting the increased plasticity or pliancy of cancer stem-like cells and their important contribution to therapy resistance[49,53–55].

In the future, it will be important to find out whether the cellular composition of MRD is indeed predictive of the subsequent resistance mechanism in the context of other cancer types and/or drugs and whether the presence of specific DTP cells favour the acquisition of resistance-conferring genetic alterations. Solving these outstanding questions in the field may offer a new set of principles for future exploration of therapeutic options. Importantly, the available findings so far show that to be effective, novel therapeutic strategies will have to take into account both the emergence of transient drug tolerance and the constant reprogramming effect of the therapy that leads to stable non-genetic resistance.

Cell of origin for non-genetic therapeutic resistance. Cells that exhibit therapy resistance have been shown in multiple cancers to have features of CSCs with increased tumour-initiating capacity[5,8,28,56]. By contrast, more-differentiated tumour cells are generally more sensitive to cancer therapies. Cellular plasticity and the ability to respond rapidly to extrinsic cues and/or pressure is an inherent capacity of tissue-specific stem cells[25,57]. Moreover, because of their longevity in tissues, normal stem cells and by extension CSCs are endowed with increased xenobiotic resistance. Therefore, it is tempting to surmise that therapy resistance is simply driven by passive Darwinian selection. However, an alternative possibility is that tumour recurrence may be caused by surviving cells that do not a priori exhibit increased tumour-initiating capacity but that through the process of adaptive reprogramming acquire the phenotypic features of CSCs and result in the continuous replenishment of the CSC pool throughout the treatment course. In the latter scenario, the cancer therapy itself actively induces intrinsic and extrinsic non-genetic signals that directly contribute to an adaptive non-genetic switching to a stem-like state with increased tumour (re) initiating capacity. We hypothesize that Lamarckian induction is at play in many diverse (if not all) types of cancer and that the dedifferentiated or stem-like state is a major contributor to tumour recurrence. We hereafter refer to this particular state as the 'phoenix state', paying homage to the long-lived mythological bird that symbolizes rebirth from the ashes, defeating hard times and immortality (FIG. 3b). We use this term as a generic term to refer to the

drug-tolerant population (likely to harbour stem-like cell features) at the origin of relapse, acknowledging that multiple such states may exist (and even coexist within the same tumour or MRD lesion). For instance, in the context of melanoma, there is evidence that relapse may arise from both the NCSC population and the invasive or undifferentiated population[28,29], indicating that both dedifferentiated melanoma states may be assimilated by phoenix cells.

Thus far, conclusive experimental proof to distinguish between the passive selection and active reprogramming models is lacking. In part this is driven by technical limitations in our methods of analysis. By their nature, the serial genetic, epigenetic and transcriptional analyses of samples used to study resistance mechanisms in animal models or patient samples provide at best cross-sectional data. Whether these assays are performed at a population level or at single-cell resolution, the snapshot in time does not enable unequivocal serial clonal tracking to truly understand whether the surviving cells are selected for from pre-existing genetic, epigenetic and transcriptional signatures or whether the emergence of malignant stem cell properties is a dynamic adaptive process. To develop these insights, we will require the development and application of new technologies that simultaneously facilitate serial clonal tracking and the assessment of the genome, epigenome and transcriptome of cancer cells at single-cell resolution. Several new technologies involving CRISPR–Cas9 (REF.[58]), self-reporting transposons[59] and lentiviral genomic barcodes[60] have started to emerge in this area that have enabled the coupling of lineage tracing with functional phenotypes, transcription factor dynamics and transcriptional programmes that drive cell fate decisions at single-cell resolution. These promising new platforms are likely to substantially increase our understanding of cancer biology and as discussed later guide therapeutic decision-making.

The genetic and non-genetic interplay

Analogous to the evolutionary theories of Darwin and Lamarck is the interplay between genomic and non-genomic evolution in cancer. Darwin believed in "survival of the fittest" whereby organisms all display inherent differences and that these differences contribute to certain organisms being more likely to survive. In the context of cancer, all tumours show intratumour heterogeneity, with significant cell-to-cell and spatial differences. Embodied in this is the notion that some cancer cells, usually by virtue of specific genomic changes, have a distinct survival advantage and can expand over time. By contrast, Lamarck hypothesized that organisms are able to adapt to their environment during their lifetime in order to survive and pass these changes on to their offspring, similar to a scenario whereby cancer cells harness an epigenetic and transcriptional adaptive process to evade cancer therapies. The complexities of drug resistance in cancer are such that Darwinian and Lamarckian principles are both at play. Invoking a model of either exclusive genetic or non-genetic evolution is fraught with dangerous assumptions, and there is now an accumulating body of evidence to show that these processes are not mutually exclusive. Interplay between genetic mutations and the evolving non-genetic landscape is widespread and shows the importance of understanding these processes collectively, rather than in isolation. For example, it is possible that the emergence of drug-tolerant cells in MRD may also jump-start classic (mutation-based) somatic evolution. Similarly to genotoxic agents, precision medicines may, at least in some specific contexts, induce resistance-conferring mutations more directly by increasing the mutation rate. It was recently suggested that some cancer cells harbouring certain genetic alterations are able to awaken the ancestral programme of enhanced mutagenicity in the context of therapeutic pressure[61]. Specifically, colorectal cancer cells that contain amplifications of the epidermal growth factor receptor (*EGFR*) gene or gain-of-function mutations in *BRAF* when challenged with targeted therapies against these oncogenes showed a transient repression of key genes required for the functional integrity of the mismatch repair pathway and homologous recombination, and instead transiently increased the expression of error-prone DNA polymerases. Similarly, another study reported that human cancers under non-genotoxic drug selection paradoxically enhance adaptation at a competing intrinsic fitness cost. A genome-wide approach was used to identify mTOR as a stress-sensing rheostat that increases mutagenesis across multiple cancer types and conditions[62]. These observations are consistent with a two-phase model for drug resistance, in which an initially rapid expansion of genetic diversity is counterbalanced by an intrinsic fitness penalty, subsequently normalizing to complete adaptation under the new conditions. To what extent this form of transient enhanced genetic evolution contributes to acquired resistance in the clinical context remains to be established.

Similarly, there is also accumulating evidence that certain genetic mutations in cancer cells can facilitate non-genetic mechanisms of acquired resistance. For instance, mutations in members of the SWI/SNF chromatin remodelling complex are prevalent in up to 20% of all cancers, and emerging evidence is beginning to shed light on the evolutionary advantage conferred by these acquired oncogenic mutations[63]. In mantle cell lymphoma, mutations in the SWI/SNF complex result in marked transcriptional heterogeneity within the cancer cell population[64]. This form of transcriptional noise provides an ideal adaptive milieu as it means there is always a population of cells transcriptionally primed with a gene expression programme to survive an imposed stress, be it in the form of nutritional deprivation, hypoxia or targeted therapies. Likewise, in oestrogen receptor (ER)-positive breast cancer, mutations in the SWI/SNF component AT-rich interactive domain 1A (ARID1A) have been shown to potentiate a switch from ER-dependent luminal cells to ER-independent basal cells[65,66]. This enhanced cellular plasticity and consequent change in the transcriptional programme ultimately results in resistance to anti-oestrogen therapies. Lastly, although to our knowledge clear experimental evidence for this is still lacking, one cannot exclude the possibility that both genetic and non-genetic mechanisms may independently contribute to tumour regrowth by giving rise to distinct subclones that may co-emerge through parallel evolution.

Lineage infidelity as a mechanism of therapeutic evasion. Lineage infidelity, or transdifferentiation, is a process by which cells within a tissue transition from one cell identity to another, a process referred to as 'lineage plasticity'[67]. This remarkable phenomenon is best described in the context of neuroendocrine transformation from epithelial tumours in the context of lung cancer[68] and prostate cancer[69]. It has also recently been described in basal cell carcinoma of the skin[70]. In all these cases, lineage switching appears to be more commonly induced in the context of targeted therapy against a molecular pathway that provides cellular identity. Consequently, lineage switching has brought into focus the cell of origin of the cancers prone to this mechanism of therapeutic evasion.

Elegant studies in mouse models of lung cancer have demonstrated that type 2 alveolar cells can serve as the cell of origin for small-cell lung cancer (SCLC)[71] and NSCLC such as adenocarcinoma[72,73] (FIG. 4). The genetic hallmarks of SCLC include nearly ubiquitous biallelic loss of *TP53* and *RB1* (REF.[74]). While mutations in these genes are also frequently seen in NSCLC, these cancers, particularly adenocarcinoma, also have frequent mutations in *KRAS* and *EGFR*[75,76]. Consistent with the fact that EGFR signalling promotes epithelial differentiation and is required for the normal development of alveoli[77], it is plausible that activating mutations in *EGFR* provide the cue for cellular identity and direct differentiation of the cancer cell of origin down an epithelial path. However, when the cancer cell is challenged with potent inhibition of EGFR, the major stimulus for epithelial differentiation is negated, and there is a strong negative selection placed on the cell of origin to continue to follow an epithelial differentiation pathway. In this context, the multipotent cell of origin, such as type 2 alveolar cells, can undergo neuroendocrine differentiation instead and continue to expand and proliferate with a new cell identity that is unimpeded by EGFR inhibition. Clinical evidence to support this possibility includes the fact that SCLC transformation is invariably associated with the retention of the original *EGFR*-activating mutations[78]. Moreover, up to 14% of patients with *EGFR*-mutated adenocarcinoma who were rebiopsied after clinical evidence of resistance to EGFR inhibitors showed histological evidence of SCLC[78]. It is important to note that small cell transformation is not confined to *EGFR*-mutated adenocarcinomas, and similar events have also been described in *ALK*-rearranged lung cancers treated with potent ALK inhibitors[79], suggesting that these tumours may also share a cell of origin with inherent multipotent potential. The cell of origin of these tumours has important therapeutic implications as current clinical classifications remain binary and patients are labelled and treated as having either SCLC or NSCLC even though up to 10% of diagnostic tissue biopsies show the concurrent existence of both histological subtypes before any therapeutic challenge[80]. Therefore, a molecular pathology approach to help inform therapeutic choice and clinical behaviour is potentially more informative.

Lending weight to a molecular classification and stratification is the fact that lineage infidelity is greatly facilitated by the genotype of the cancer cell. In both lung cancer and prostate cancer, neuroendocrine transformation is highly correlated with mutations in *TP53* and *RB1* (REFS[81–83]). While multiple lines of evidence point to the fact that loss of *RB1* and loss of *TP53* are insufficient by themselves to result in transdifferentiation[82,83], recent evidence has clearly emphasized the important role that *RB1* plays in maintaining the differentiated state and lineage fidelity[84]. To investigate the specific role of *RB1* in lung cancer driven by *KRAS* activation and *TP53* deletion, this recent study used an elegant genetic system that enables the inactivation and subsequent reactivation of *RB1*. The results highlighted the fact that loss of *RB1* enables a more primitive cellular state with higher metastatic potential that features the concurrent expression of lineage-defining transcription factors such as NKX2-1 and forkhead box protein A2 (FOXA2) alongside chromatin factors such as high mobility group AT-hook protein 2 (HMGA2) that enable transcriptional plasticity and whose expression is usually confined to embryonic tissues[84]. Similar findings have also been experimentally demonstrated in prostate cancer[85]. Here again there was clear experimental proof that although *RB1* loss and *TP53* loss were indispensable for neuroendocrine transformation, they were not sufficient and required the cooperation of other factors to drive a transcriptional programme that enabled this transdifferentiation[85]. While we have gained substantial insights into the key determinants that mediate neuroendocrine transformation in both lung cancer and prostate cancer, it remains unclear whether this phenomenon is driven by the dedifferentiation of epithelial cells in the context of potent therapeutic challenge or whether this simply represents the activation of an alternative lineage programme in the multipotent cancer-initiating cells.

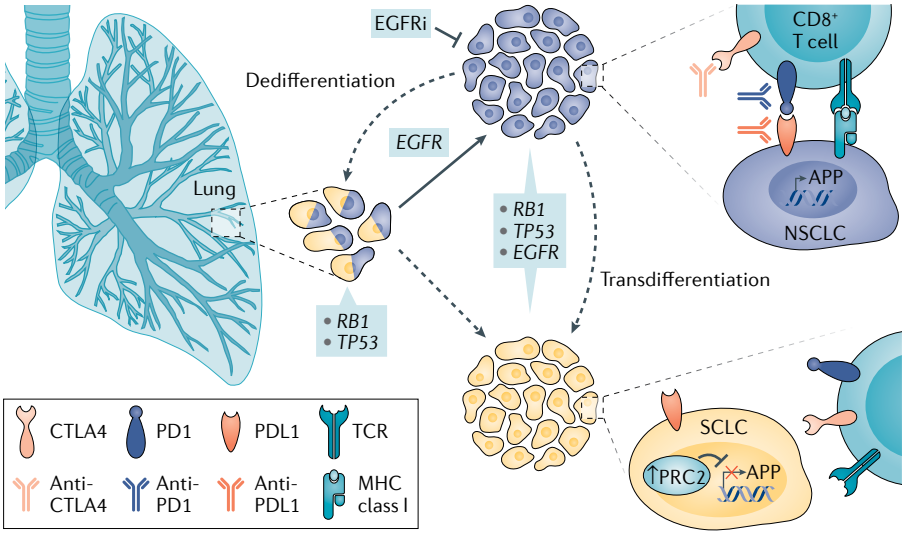

Fig. 4 | **Cell of origin and genetic composition influence mechanisms of resistance.** Multipotent cells within a particular tissue, such as type 2 alveolar cells, can serve as the cell of origin for epithelial (non-small-cell lung cancer (NSCLC)) and neuroendocrine (small-cell lung cancer (SCLC)) tumours. Certain early genetic mutations such as those in *RB1* and *TP53* provide the cancer cell of origin with greater plasticity. Mutations acquired later (for example, those in the epidermal growth factor receptor (*EGFR*) gene) provide a differentiation drive towards a particular lineage. However, potent therapeutic pressure particularly against the oncogenic driver (using EGFR inhibitors (EGFRi)) can provide an evolutionary bottleneck, and these tumours with inherent plasticity can switch lineages either by direct transdifferentiation or through an intermediate step of dedifferentiation. The new lineage is no longer subjected to the initial therapeutic pressure. Lineage switching can also provide a mechanism for evading cancer immunotherapies (such as anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA4), anti-programmed cell death protein 1 (PD1) and anti-PD1 ligand 1 (PDL1)), which have been proven to be more efficacious in NSCLC. In SCLC, transcriptional repression of the antigen processing and presentation (APP) genes by Polycomb repressive complex 2 (PRC2) potentiates immune evasion and is likely one of the reasons why SCLC is less responsive to immunotherapies despite having a similar mutation burden to NSCLC. MHC, major histocompatibility complex; TCR, T cell receptor.

Cellular plasticity enables immune evasion in cancer. Although the precise mechanisms that govern lineage plasticity remain to be established, an emerging theme from multiple studies emphasizes the central role of pioneer transcription factors such as SRY-box 2 (SOX2) and chromatin

regulators, particularly the highly conserved components of Polycomb repressive complex 2 (PRC2)[82,83,86]. Remarkably, inhibition of enhancer of zeste homologue 2 (EZH2), the catalytic component of PRC2, was able to reverse the neuroendocrine transformation, suggesting that the processes mediated by this chromatin complex are essential to the maintenance of the transdifferentiated state. The enhanced activity of PRC2 in facilitating and maintaining the cellular plasticity required for neuroendocrine transformation in lung and prostate cancer may also have additional benefits for the cancer cell, including the evasion of immunosurveillance (FIG. 4).

It was recently demonstrated that PRC2 is the dominant chromatin complex required for the repression of the antigen processing and presentation pathway[87]. Transcriptional repression of major histocompatibility complex class I (MHC-I) was previously described as a prominent feature in several neuroendocrine tumours, including neuroblastoma, SCLC and Merkel cell carcinoma[88,89]. Notably, this repression of MHC-I in neuroendocrine cancers was recently identified as a mechanism of resistance to immunotherapy[87,90], and several patients with adenocarcinoma of the lung who underwent small cell transformation in the context of EGFR inhibition were noted to have loss of MHC-I in the transformed tumour and were consequently refractory to immunotherapies[87]. These findings are also consistent with the fact that MHC-I-deficient neuroendocrine tumours are associated with highly aggressive behaviour, including early metastasis, which likely reflects an inability to be constrained by an effective antitumour immune response. Here too, the cell of origin appears to be critical as PRC2-mediated repression of MHC-I in embryonic and tissue-specific stem cell subsets likely evolved to protect these cells from inflammatory insult[91]. However, cancers that have a cell of origin where this pathway is functional can exploit the activity of PRC2 to silence MHC-I antigen presentation and gain immune privilege. Remarkably this ability of PRC2 to regulate antigen presentation and enable immune evasion is evolutionarily conserved and accounts, in part, for the extreme form of immune evasion seen in transmissible tumours such as Tasmanian devil facial tumour disease[87].

Cellular plasticity as a mechanism to evade antitumour immunity is not confined to epithelial malignancies or immune checkpoint therapies. It was reported that mouse melanoma can resist T cell therapy through inflammation-induced reprogramming into a dedifferentiated state, reminiscent of the drug-tolerant NCSC state described earlier in this Perspective[92]. The clinical application of this phenomenon was also recently reported in a patient whose metastatic melanoma underwent dedifferentiation as a mechanism of resistance to adoptive T cell transfer therapy for the MART1 antigen[93]. A more recent study confirmed that MHC-I downregulation is a hallmark of resistance to PD1 inhibitors in melanoma as well and that this is associated with the dedifferentiated or invasive phenotype. Furthermore, transforming growth factor-β (TGFβ) was identified as a driver of the treatment-resistant phenotype and downregulation of MHC-I (REF.[30]). Thus, there is increased evidence that the DTP cells, which contribute to resistance to targeted therapy, may also drive, at least partly, intrinsic resistance to immune checkpoint inhibitors. However, more studies will be needed to firmly establish this point and to test its generalizability to other cancer types.

Similarly, the remarkable success of chimeric antigen receptor (CAR) T cell therapy in haematological cancers, particularly acute lymphoblastic leukaemia (ALL), has seen the emergence of resistance to this transformative cellular immunotherapy, including through transdifferentiation from lymphoid to myeloid malignancies[94] (FIG. 5). Although CAR T cell therapy can induce complete remission in up to 90% of patients with relapsed ALL[95], acquired resistance is seen in up to half of the patients[96,97]. The mechanisms of relapse can be quite varied, and include lineage switching particularly in patients with ALL harbouring translocation of the mixed-lineage leukaemia (*MLL*; also known as *KMT2A*) gene[98,99]. Leukaemias harbouring translocations in the *MLL* gene have unique clinical and biological properties. They have few, if any, other mutations[100,101], suggesting that the MLL-fusion protein is entirely sufficient for the full malignant phenotype[102]. The inherent plasticity conferred by MLL-fusion proteins is evidenced by the fact that they give rise to both lymphoid and myeloid leukaemias and can reinstate stem cell transcription programmes in committed progenitors[103]. Modelling of lineage plasticity in the context of therapeutic pressure exerted by CAR T cells clearly shows that the underlying genotype of the cancer is important[104]; however, the molecular mechanisms that underpin the transdifferentiation remain unclear. Notably, lineage plasticity to evade immune eradication in leukaemias with *MLL* fusions and other enabling oncogenic drivers is not confined to cellular immunotherapies but is also seen to occur in the context of potent bispecific T cell engager (BiTE) antibodies[105,106] (FIG. 5). From the findings together, there is an accumulating body of evidence demonstrating that in both solid malignancies and haematological cancers potent therapeutic pressure results in cellular plasticity that not only results in lineage switching but also facilitates immune evasion. Understanding the molecular events used by oncogenic drivers that enable lineage switching is critical to develop therapeutic strategies that constrain this plasticity and prevent acquired resistance via this route (FIG. 5).

Monitoring mechanisms of resistance

The examples presented so far of how the pre-existing mutational landscape of the tumour can directly influence the adaptive mechanisms used serve to highlight the significant challenge ahead. Moreover, as most of these resistance mechanisms are used by only a small fraction of cells that are able to withstand the initial therapeutic onslaught, future studies need to focus on understanding the resistance mechanisms used by the MRD cells that continue to persist at the time of clinical remission. Notably, non-genetic resistance often develops in the absence of selection of a single, genetically distinct, clonal population[44,48]. This observation raises the possibility that non-genetic resistance may not be clonal but may occur, instead, through collective reprogramming. By contrast, although rare examples of parallel genetic evolutionary trajectories have been described[107], these are likely to represent exceptions rather than the rule. These issues need to be collectively considered when one is designing strategies to monitor these adaptive mechanisms of resistance.

The importance of tracking temporal changes in the cancer genome throughout therapy is well described[108,109]. Serial monitoring of cancer genomes through repeated tumour biopsies and/or circulating tumour DNA sampling has revealed the expansion of drug-resistant populations that harbour specific genomic alterations which drive resistance to conventional and targeted therapies[110]. Examples where these approaches have been invaluable include the identification of mutations that alter drug binding (for example, $EGFR^{T790M}$ and $EGFR^{C797S}$ mutations that impair

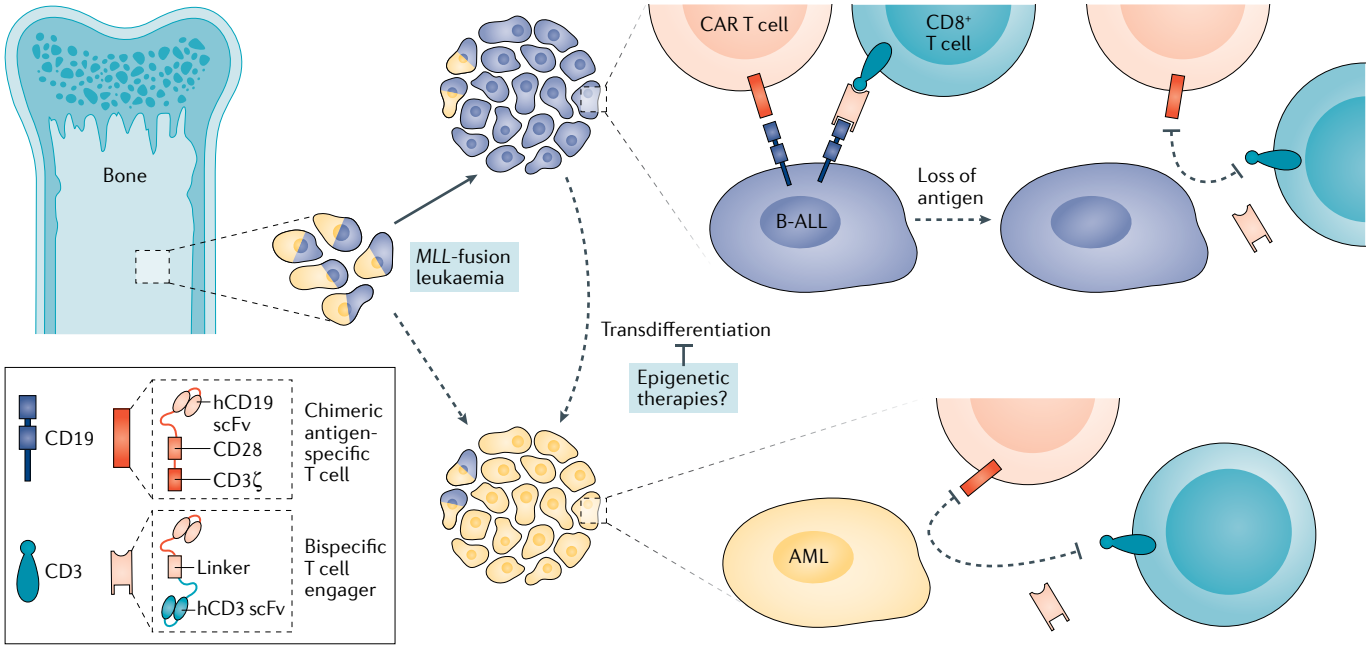

Fig. 5 | **Cellular plasticity as a mechanism to evade anticancer immunosurveillance.** Both acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) can arise from recurrent translocations involving the mixed-lineage leukaemia (*MLL*) gene, so-called *MLL*-fusion leukaemia. Immunotherapies including chimeric antigen receptor (CAR) T cells and bispecific T cell engagers directed against the B cell lymphocyte antigen (CD19) have revolutionized the management of B progenitor ALL (B-ALL). In the context of this potent therapeutic immune pressure, the cancer cells can evade immunosurveillance by losing the expression of the CD19 antigen via transcriptional repression or genetic mutation[94]. Alternatively, the cellular plasticity conferred by the *MLL*-fusion protein can also enable lineage switching, whereby the genetically identical cancer cells switch from a phenotypic B cell malignancy to a myeloid malignancy that no longer expresses CD19. As transdifferentiation requires extensive changes to chromatin architecture and transcriptional programmes, which in turn leverage the activity of epigenetic regulators, it is possible that epigenetic therapies may be used alongside these therapies to constrain this plasticity. h, human; scFv, single-chain variable fragment.

binding of selected EGFR tyrosine kinase inhibitors in lung cancer[111,112]), mutations that result in constitutive activation of the same signalling pathway (for example, RAS gene mutations that mediate resistance to EGFR monoclonal antibodies in colorectal cancer[113,114]), mutations that engage alternative survival pathways (for example, PI3K catalytic subunit-α (*PIK3CA*) gene mutations that can contribute to endocrine resistance in breast cancer through upregulation of PI3K signalling[115]) and somatic BRCA gene reversion mutations leading to poly(ADP-ribose) polymerase (PARP) inhibitor resistance[116]. While these monitoring strategies have been very successful in providing insights into the genomic mechanisms of therapeutic resistance, monitoring of non-genetic mechanisms has been far more challenging and will require significant technical and conceptual advances to be overcome. A leading possibility includes the integration of single-cell transcriptomic platforms into the clinical arena.

Single-cell RNA sequencing analyses of matched human tumour samples before and after therapy have been shown to be informative for highlighting potential mechanisms of therapy resistance in several tumour types[6,44,117,118]. Similar methods have also been applied to circulating tumour cells[119,120] and cells within the tumour microenvironment[117,121]. There has also been significant progress in developing methods to couple single-cell genomic and transcriptomic information from the same cell[122,123]. The vital information gleaned from studies such as these has led to major plans to generate a comprehensive atlas to document cancer cell transitions for a variety of tumour types[124]. A potential limitation of these strategies that has been raised is the fact that ex vivo tumour cell processing, including arduous dissociation for solid tumour samples, may introduce spurious variations in transcriptomic profiles[125]. One potential method that reduces the need for dissociation and enables critical spatial information to be obtained involves multiplex transcriptomic analyses performed directly on whole tissue sections[126–130]. However, transcriptome measurements may not necessarily capture adaptive responses that involve post-transcription mechanisms such as translation or metabolic rewiring[131–133]. Studying the involvement of such mechanisms, which have been shown to play important roles in driving tolerance and/or resistance, will require the further development of methods that capture proteomic and metabolic changes at single-cell resolution. While such methods are being developed[134–136], their sensitivity, resolution and range of proteins and metabolites detected remain limited. If the ambition is to use these methods to understand, track and respond to adaptive mechanisms of therapy resistance in real time, it is important to realize that all of these methods rely on the ability to access serial tumour samples. This is not an insignificant drawback, particularly for solid malignancies, where serial tumour biopsies are often not practical because they involve an invasive procedure. This difficulty is further compounded by the fact that at a time of clinical remission, sampling of the MRD cells is not usually technically (and ethically) feasible. Moreover, in the context of multifocal disease, a single tumour biopsy sample is not representative of the global tumour burden. Strategies to overcome these major hurdles will require significant technical

and conceptual advances before these approaches can inform clinical care in real time.

Targeting non-genetic resistance

The promise of precision medicine, in part, relies on the premise that the genomic landscape of cancer will be sufficient to guide therapeutic decisions. As our understanding of the adaptive mechanisms involved in evading anticancer therapies grows, it has become increasingly apparent that this vision is an enormous oversimplification of the task ahead. Therefore, we believe that it is urgent that the contribution of non-genetic mechanisms of therapy resistance gain greater and wider recognition in the field. It is only when their pervasive and potentially much more prevalent role, than in the classical model of sequential genomic evolution, is fully accepted that we will eventually be able to implement newly designed clinical trials to deal with this major source of therapeutic resistance. Understandably, before such novel therapeutic approaches are implemented in the clinical arena, it is imperative that we keep learning much more about the mechanisms involved. However, there are several emerging principles that are already worth considering in shaping the clinical trials of tomorrow, some of which are detailed in the following sections.

What is clear is that there will not be a single universal or uniform effective clinical approach. Some of the strategies described in the following sections may need to be combined and/or adapted on a case-by-case basis. It is also important to recognize that many of these adaptive processes are occurring in the MRD cells present at the time of clinical remission following initial therapeutic measures. Traditionally, this is a time when the patient has just completed a demanding regimen of (chemo)therapy and following the achievement of a complete remission enters a convalescent phase of careful observation. It is potentially at this time that strategies that counter the adaptive mechanisms should be introduced.

Targeting drug-tolerant persister cells. What might these strategies look like? One potential approach involves leveraging cell plasticity for therapeutic benefit by converting a drug-resistant population to one that is drug sensitive, an approach termed 'directed phenotype switching'[137] (FIG. 6). Proof of concept for such an approach was provided in the context of melanoma resistance to targeted therapy[137]. However, the clinical implementation of such an approach will require identification of FDA-approved agents that enable this approach. One may also envisage a strategy that diverts the fate of various DTP cells into a single permanently dormant state. Such a dormancy-directed strategy would limit the heterogeneity of drug tolerance by forcing all drug-tolerant cells to adopt a dormant phenotype (FIG. 6). Thus, the cells may remain dormant for a prolonged period and/or eventually be eradicated by taking advantage of their sensitivity to inhibitors that are yet to be identified or by enabling immune-mediated clearance of the homogeneous dormant cancer cell population.

Targeting DTP cells should also be considered (FIG. 6). Although, there is evidence that drug withdrawal in in vitro models[27] and intermittent drug dosing in animal models may overcome DTP cells[138], this approach is unlikely to gain clinical traction. Not all DTP cells may have the same ability to contribute to relapse; therefore, it will be essential to identify the actual phoenix cells. This is not a trivial task, and experimentally this will require the use of barcoding and lineage tracing approaches to understand the mechanisms that underpin emergence of the phoenix cell state and its interconversion into stably resistant cell states. As highlighted earlier, there is increasing evidence that MRD cells bearing stem cell features (as opposed to differentiated DTP cells) are more likely to contribute to resistance. One can therefore aim therapeutic approaches at these cells as a priority. Targeting stemness is a strategy that has recently attracted attention in a variety of cancers, leading to the development of inhibitors of signalling pathways[139–141], epigenetic regulators[142,143], immune signalling[144] and the tumour microenvironment[145] that all contribute to the properties of CSCs. This is an appealing approach as stemness is governed by a limited number of overlapping pathways (for example, Notch and WNT), and therefore the mechanisms that govern maintenance of the phoenix state are likely to be common to a wide range of cancers and drug treatments. In keeping with this, there is increasing evidence that multiple different drug-tolerant cells that evade other cell death pathways have a greater dependency on the phospholipid glutathione peroxidase 4 (GPX4) pathway[146]. DTP cells that arise after exposure to a range of anticancer compounds were found to have transcriptionally downregulated genes

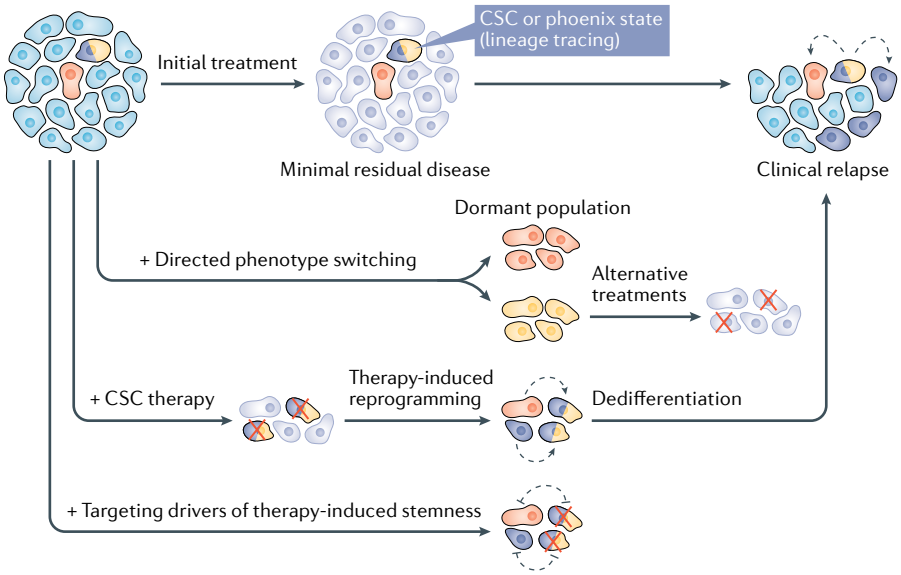

Fig. 6 | **Potential therapeutic strategies to counteract non-genetic resistance mechanisms.** Illustrated here are strategies that may potentially minimize the risk of cancer recurrence owing to non-genetic mechanisms of resistance. In all of the suggested scenarios it is possible that combination therapies using treatments with non-overlapping mechanisms of activity and/or toxic effects are likely to be the cornerstone of management. These approaches include directed phenotype switching towards a drug-sensitive state or a state that does not contribute to relapse (for example, a permanently dormant and/or a fully differentiated state). However, it remains unclear whether such a state exists. Targeting the phoenix cells themselves (by, for instance, using a cancer stem cell (CSC)-based therapy) and/or drivers of therapy-induced reprogramming into the phoenix state could also be used. Future approaches will be guided by further insights into the predominant mechanisms involved with particular cancers and the adaptive pathways associated with their initial treatment.

that contribute to the generation of two major antioxidant cofactors, glutathione and NADPH, and the decreased levels of these cofactors consequently led to a heightened susceptibility to ferroptosis[147]. Therapeutic targeting of GPX4 induced ferroptotic cell death and prevented tumour relapse in animal models[148]. Although the clinical approaches to target this dependency are not mature, this dependency, which appears to be shared among DTP cells across a broad range of cancers, is one attractive novel possibility.

In addition to engaging a robust antioxidant defence, DTP cells that are under constant stress or therapeutic pressure often activate other prosurvival pathways, including metabolic remodelling, autophagy, mitohormesis, unfolded protein response (UPR) and glutaminolysis, some of which may offer novel therapeutic opportunities to target the phoenix cells specifically. While it might be difficult to find a therapeutic window to target these ubiquitous cellular stress response pathways, emerging data suggest that both in animal models and in patients some of these approaches particularly in combination with anticancer targeted therapies may have value. For example, autophagy has been shown to protect various KRAS-, NRAS- or BRAF-driven cancer cells from the cytotoxic effects of MAPK pathway inhibition, and combined inhibition of MEK1 and MEK2 plus autophagy displays synergistic anti-proliferative effects in cultured cell lines in vitro and it also promotes regression of PDX tumours in mice[149]. In line with these findings, the combination of the MEK inhibitor trametinib plus the autophagy inhibitor hydroxychloroquine resulted in a clinically meaningful response in a patient with relapsed pancreatic cancer[149], suggesting that this combinatorial approach may represent a novel strategy to target RAS-driven cancers.

Corralling the adaptive potential of the phoenix cell state. The recent identification of relapse-initiating cells in B progenitor ALL (B-ALL) highlighted distinct metabolic properties (for example, increased oxidative phosphorylation), mitochondrial activities and proteostasis programmes, in addition to stemness features. This analysis may lead to new avenues for eradicating these cells[10] by, for instance, using agents that target mitochondrial metabolism of the UPR pathway before further evolution renders them fully therapy resistant. There are obvious parallels when comparing the biology of drug-tolerant cells across multiple tumour types. For example, as in B-ALL, a slow-cycling population of melanoma cells that emerge in cultures exposed to targeted therapy exhibited elevated oxidative phosphorylation, and targeting mitochondrial respiration blocked their emergence and overcame their non-genetic drug resistance[150]. Elevated levels of gene expression of the UPR (proline-rich receptor-like protein kinase (PERK)–activating transcription factor 4 (ATF4)–C/EBP-homologous protein (CHOP; also known as DDIT3)) and other proteotoxic stress response pathways following drug exposure has been documented in multiple cancers[151]. The role of ATF4 in drug tolerance is increasingly recognized[152]. For example, in melanoma, a link between ATF4 induction, dedifferentiation and acquisition of resistance to both targeted therapies and immunotherapies has recently been established[153]. Taken together, these considerations highlight the potential of rationally designed MRD-directed therapies, taking advantage of specific (and possibly common) vulnerabilities of the phoenix cell states, with the ultimate goal to intervene before mechanisms of resistance are stably established.

Given that therapy itself can directly promote emergence of stem cell-like states, how could we seek to maximize the desired effects of therapy while minimizing the adaptive potential of the surviving cells? An attractive strategy is to directly interfere with therapy-induced reprogramming into the phoenix state (FIG. 6). To reach this goal, there is a need to decipher the governing molecular principles of therapy-induced stemness and to use these to devise pharmacological strategies directed against shared drivers of these events (FIG. 6). Bioinformatic analyses may be used to decipher the gene regulatory network within specific DTP cell states. Each stable transcriptional cell state emerges from the combined action of thousands of active enhancers. These enhancers are bound by sets of transcription factors and cofactors, which assemble enhancer–promoter complexes via chromatin looping and ultimately instruct the expression levels of their target genes[154]. Integrating transcriptome data with genome-wide chromatin accessibility maps, generated, for instance, by assay for transposase-accessible chromatin using sequencing (ATAC-seq)[155], permits the construction of robust gene regulatory networks underlying specific cell types or states and identification of key regulators, including transcription factors, that govern these networks. By studying the 'enhancer logic' controlling therapy-induced phenotype switching, one can gain insights into the molecular mechanisms underlying this process and identify strategies to manipulate it. A proof-of-principle study recently highlighted the feasibility of the approach[28]. The transcription factor retinoid X receptor-γ (RXRγ) was shown to promote emergence of the NCSC drug-tolerant state in melanoma MRD, and targeting RXRγ activity with an antagonist drastically decreased emergence of the NCSCs and delayed the time to progression in a PDX model. However, targeting phoenix state transcription factors using clinically compatible pharmacological strategies is not always possible. In this case scenario, alternative strategies should be envisioned, such as the use of small molecules that affect the conformation of DNA-binding domains or agents that promote proteolysis specifically, among other innovative strategies used to suppress intractable protein targets[156].

Epigenetic therapies to limit cellular plasticity. Restraining cellular plasticity is perhaps the most difficult task. In part, this is difficult because even with genotypes that are prone to transdifferentiation, there are few clear indicators of which tumours will undergo this process of therapeutic evasion. Moreover, there are few detailed molecular insights into the process of transdifferentiation. Although there remains much to be learnt about this process, it is very likely that the major regulators of cell fate determination play a critical role. In line with this notion is the fact that the evolutionarily conserved PRC2, which is required for cell fate decisions in all multicellular eukaryotes, appears to have a central role in enabling and maintaining the transdifferentiated state[82,83,86]. Small-molecule inhibitors of EZH2 have undergone extensive clinical evaluation in a broad range of malignancies, including relapsed and refractory lung and prostate cancer, where they have shown very modest effects[157]. As with other epigenetic therapies, it is very likely that the context-dependent molecular and cellular effects of these compounds have not been fully elucidated[158], and consequently these drugs have not necessarily been tested in the most appropriate clinical context[159]. Potentially a more rewarding approach would be to use PRC2 inhibitors in patients with epithelial malignancies containing *RB1* and *TP53* mutations, particularly following the induction of complete remission with targeted therapies (FIG. 4).

How best to use epigenetic therapies in the clinical arena is a complex and completely unanswered question. Thus far, the vast majority of epigenetic therapies have had

mediocre clinical success[159]. In part, this is because they have been primarily tested in relapsed and refractory cancers that have already undergone significant adaptation and clonal selection. A potentially more promising approach may involve use of these drugs in an alternative clinical setting, at the start of clinical remission to counteract the adaptive chromatin changes occurring in MRD cells. The stable non-genetic adaptation often manifests itself as the expression of an alternative transcriptional programme driven by the collaborative action of transcription factors and epigenetic regulators[44,160,161]. The new enhancers formed as part of the adaptive process are critical to maintaining the resistant phenotype, and consequently targeting co-activators such as bromodomain 4 (BRD4) that are required for the functional integrity of these enhancers uncouples the adaptive transcriptional programme, leading to eradication of these resistant cells[44,160,161]. These findings have raised the possibility that targeting epigenetic regulators in MRD cells will negate the transcriptional plasticity that results in therapeutic resistance. An important area to consider when one is pursuing long-term inhibition of epigenetic regulators as part of maintenance therapies is the tolerability of these agents, particularly as epigenetic regulators are widely expressed and also play a central role in normal cellular homeostasis. However, the recent success of maintenance therapies that inhibit DNA methyltransferases to increase survival in patients with AML[162] provides emerging evidence for the potential merit of this strategy.

Conclusions

The ever-increasing knowledge demonstrating that non-genetic mechanisms make a major contribution to therapy resistance and cancer relapse is a major advance for the field. Yet, the development of efficient curative therapies will require further study at single-cell resolution both spatially and temporally to accurately gauge the challenge ahead. For example, it is important to know what the dominant non-genetic mechanisms that drive resistance and relapse in specific cancers are. Do these mechanisms and/or the phenotype of the resistant population differ in different tissue microenvironments within the same host? Can we prospectively identify different phenotypic subpopulations which exhibit distinct therapeutic vulnerabilities? Importantly, our clinical experience has clearly taught us that our best chance of cure is our first line of therapy in a drug-naive population of cancer cells. Therefore, can we pre-emptively use therapies, in a neoadjuvant-like approach, to homogenize the inherent non-genetic heterogeneity within the tumour cell population, enabling a more uniform sensitivity to first-line therapy. For this we will need to be able to predict the future behaviour of an individual cancer and, in particular, which drug resistance evolutionary path it will take.

The application of technologies aimed at dissecting intratumor heterogeneity at the single-cell level combined with innovative models that better approximate the complexity of human cancer is likely to address many of these key questions. In particular, methods that capture the magnitude and dynamics of both genetic and non-genetic intratumour heterogeneity in the 4D (spatial and temporal) space and at single-cell resolution will be especially useful. Although single-cell profiling techniques have revolutionized our understanding of individual cancer cell behaviours within complex model systems and human tissue samples, there remains much to be learnt beyond a static snapshot in time. If we are truly going to understand and counter the molecular mechanisms that lead to therapeutic resistance and cancer relapse, we will need to develop additional single-cell multi-omics tools, particularly ones which allow the simultaneous profiling of the single-cell genome, (epi)genome and transcriptome in serial samples. Moreover, to better study the dynamic adaptive mechanisms used by cancer cells in vivo, we will need to couple single-cell multi-omics methods and clonal lineage tracing to provide a robust framework for defining cell fate transitions, intermediate states and cell branching lineage trajectories. Together these technological and conceptual advances will facilitate both the development of innovative therapies and more informed therapeutic approaches to improve outcomes for patients with cancer.

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