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Death-ision: the link between cellular resilience and cancer resistance to treatments

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Abstract

One of the key challenges in defeating advanced tumors is the ability of cancer cells to evade the selective pressure imposed by chemotherapy, targeted therapies, immunotherapy and cellular therapies. Both genetic and epigenetic alterations contribute to the development of resistance, allowing cancer cells to survive initially effective treatments. In this narration, we explore how genetic and epigenetic regulatory mechanisms influence the state of tumor cells and their responsiveness to different therapeutic strategies. We further propose that an altered balance between cell growth and cell death is a fundamental driver of drug resistance. Cell death programs exist in various forms, shaped by cell type, triggering factors, and microenvironmental conditions. These processes are governed by temporal and spatial constraints and appear to be more heterogeneous than previously understood. To capture the intricate interplay between death-inducing signals and survival mechanisms, we introduce the concept of *Death-ision*. This framework highlights the dynamic nature of cell death regulation, determining whether specific cancer cell clones evade or succumb to therapy. Building on this understanding offers promising strategies to counteract resistant clones and enhance therapeutic efficacy. For instance, combining DNMT inhibitors with immune checkpoint blockade may counteract YAP1-driven resistance or the use of transcriptional CDK inhibitors could prevent or overcome chemotherapy resistance. *Death-ision* aims to provide a deeper understanding of the diversity and evolution of cell death programs, not only at diagnosis but also throughout disease progression and treatment adaptation.

Introduction

Cancer resistance to therapies along with its progression (including metastasis) are the causes of most failure in curing this disease. The "classical" theory is that cancer is initiated by sequential mutation(s) in normal cellular populations then undergoes a series of transformations which allow mutated cells to progress through rounds of selective processes, as suggested by Fearon and Vogelstein in the colorectal cancer context [1]. Some cancers are linked to genetic mutations but not all individuals with germinal cancerous mutations develop cancer. Heavy somatic mutation burdens found in cancer are also present in normal cellular ageing populations but not all these mutated cells develop into cancer [2].

Cancer is an inherently ineffective disease (see Sect. 1) due to the existence of protective barriers (i.e. cell death or senescence) the failures of which initiate the onset and progression of cancer [3]. However,

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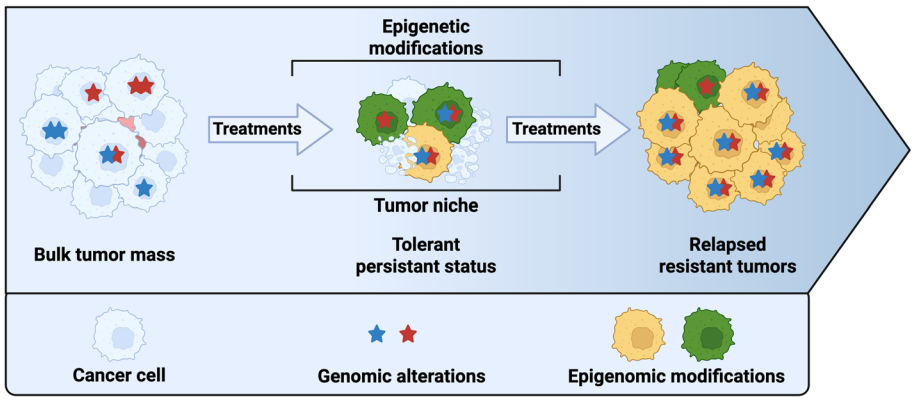

Fig. 1 Genomic and epigenomic modifications concurrently contribute to the response to anticancer treatments. Bulk tumor masses are composed of cancer cells with diverse genomic alterations and epigenetic landscapes. Under treatment pressure, tolerant or persistent cells are selected as those in which specific genomic and epigenomic changes confer a survival advantage. These selected clones can expand, leading to drug-resistant tumor relapse. The same epigenetic state may either support or hinder treatment efficacy, depending on the underlying genomic context and the microenvironmental niche in which the cancer cells reside

several mechanisms used by cancer cells to resist the initial surveillance/selection processes, also participate in the resistance to treatments. The resistance to treatments exploits multiple pathways that can be divided into specific routes (i.e. resistance to a class of drugs through a dedicated mechanism) or more general mechanisms (multidrug pumps or cell death inhibition for example). Additional escaping strategies using "duck" (drug tolerance/persistent, slow cycling, senescence, dormancy…) or "cover" (hide in a niche, hypoxia, metabolism changes, modifications of the immune environment…) tactics could allow cancer cells to survive aggressive therapies.

Once mutated, pre-cancerous cells that evade constraining mechanisms—such as immune surveillance, cell death induction, or senescence/cell cycle arrest—can further acquire genetic and epigenetic alterations, enabling rapid tumor growth. These alterations can then act as survival/escape mechanisms, activated by cancer cells as adaptation/selection processes to survive the pressure of anticancer therapies. Of note, clonal selection follows a Darwinian rule with a touch of Lamarckism as adaptation can be partially controlled by dynamic metabolic/epigenetic rewiring [4]. Genetic driver alterations can significantly influence the epigenetic response to therapy, ultimately contributing to the development of resistance under treatment pressure. In the context of specific driver mutations, only a subset of epigenetic modifications may promote the emergence of drug-resistant relapses (Fig. 1). Elucidating the interplay between genetic and epigenetic alterations in shaping therapeutic responses is of critical translational and clinical relevance.

For example, inhibition of the EZH2 methyltransferase enhances therapy efficacy in PIK3 CA-driven lung cancer models, but not in tumors driven by KRAS mutations [5]. Similarly, leukemogenesis involves extensive, dynamic methylation reprogramming that becomes a substrate for evolutionary selection during treatment [6]. In such malignancies, founding driver mutations—such as the BCR-ABL translocation in Chronic Myeloid Leukemia or FLT3 mutations in Acute Myeloid Leukemia—collaborate with specific epigenetic regulators to orchestrate a reprogramming of the methylome, further promoting therapy resistance [6].

In this review, we describe some (but not all) cellular and genetic/epigenetic mechanisms linked to cancer development and resistance to treatment. We discuss what we know and what we need to know to consolidate our knowledge on cancer evolution and to elaborate more efficient strategies to dodge the resistance to therapies, the main obstacle to achieving cures for most cancers.

Cell death is a predominant mechanism for evading internal surveillance of cancer initiation and for resisting external pressures during treatments, ultimately influencing subsequent cancer progression [7]. In recent years, cell death has been observed to coexist within cancer cells through distinct programs that can be either antagonistic or interconnected, depending on cellular physiology. This dynamic interplay may ultimately dictate cell survival or death [7]. We propose to call this step "Death-ision". Because, tumors undergo drastic changes during the course of treatment, we suggest that a better knowledge of "Death-ision" during tumor evolution, taking into account the heterogeneity of cancers, could prove to be actionable for more efficient treatments. Through this review we will provide evidence that the "Death-ision" for cancer cells to live or die and

then clonally evolve under the pressure of the different types of therapies including, chemo- radio- targeted- and immune-therapies are the results of balanced influences driven by the driver genomic alterations of each single disease and the epigenetic changes induced by the micro-environmental pressure (Fig. 1). Identifying and targeting the molecular mechanisms underlying the "Death-ision" to induce cell death overall cell survival in each specific context will eventually improve cancer treatments and possibly prevent the appearance of resistant disease. For instance, targeting Bcl-2 overexpression or EZH2 mutation resulted in objective clinical response in patients with follicular lymphomas, likely through induction of cell death in cancer cells [8]. The future challenge will be to precisely identify the patients that could better respond to "Death-ision" modifiers, thereby defining the best personalized therapies.

Clonal evolution and drug resistance

The current view of tumor evolution is that tumors evolve as complex Darwinian systems in which the most fitted clones are selected over time and under the pressure of therapies eventually leading to chemoresistance and to recurrent/metastatic diseases [9]. This concept was originally proposed in a seminal work that consolidated the then emerging evidences indicating that for a cancer to become clinically evident, would necessitate the accumulation of subsequent genetic (and we now know also epigenetic) alterations that render the originally transformed cells (cell of origin) fitted to grow and eventually metastasize [10]. Despite the urgency to dedicate more research efforts "toward understanding and controlling the evolutionary process in tumors" [10], this line of research remained mostly undeveloped for decades. Then, the massive introduction of Next Generation Sequences (NGS) revealed extensive intratumor heterogeneity as a driver of resistance (reviewed in [11]). Intratumor heterogeneity is considered the principal cause of acquired drug resistance in cancer and several models have been proposed to explain how intratumor heterogeneity can have an impact on drug resistance. Although very convincing clinical and preclinical data support the hypothesis that tumors evolve as complex Darwinian systems [12, 13], when the process is observed from a molecular point of view it seems that cancer evolution follows a non-Darwinian path in some contexts [14]. The fact that 1) tumors consist of extremely large populations, with hundreds of billions of cells ensuring an extraordinary diversity; 2) are characterized by chromosomal instability that may confer adaptive advantages during evolution; 3) exhibit phenotypic changes independent of genetic variation, support their non-Darwinian evolution distinct from typical evolutionary systems [14]. It is therefore convincingly clear that while a primary tumor typically starts from a single cell or a small, homogeneous group, as it grows, mutations and epigenetic changes lead to a diverse mix of distinct clones or lineages. These clones form novel spatial arrangements and engage in competitive or cooperative interactions, resulting in shifts in the tumors' phenotypic composition [15]. These differences support the possibility that early-stage tumors follow Darwinian processes to grow locally and invade the surrounding tissues. These first steps in tumorigenesis and Darwinian evolution are likely linked to genetic variation that ensure the acquisition of phenotypic configurations necessary to override local controls (see Sect. 1.2 and Fig. 2). Once diverse groups of phenotypic configurations are acquired, it is conceivable that tumors evolve following the concept of selection for function [15]. This model extends beyond classical Darwinian evolution, incorporating the complexity of tumor dynamics from early precancerous to aggressive metastatic stages [15]. Recent evidence elegantly confirmed in an in vivo model of pancreatic cancer evolution that the acquisition of mesenchymal plasticity, driven by Epithelial to Mesenchymal Transition (EMT), is necessary for malignant progression and tumor growth, promoting the emergence of high-fitness populations characterized by complex patterns of genomic instability [16]. In view of this, it is likely that genomic and epigenomic modifications can together explain the selection-for-function model of cancer evolution which then poses critical questions regarding the possible targeting of advanced and drug-resistant diseases. Understanding whether tumors evolve through a Darwinian path or a more complex selection-for- function model is not only important in advancing our knowledge on tumor progression and resistance but also will have direct implications in the selection of the most appropriate treatment of cancer patients.

Therefore, to understand how tumors evolve under the pressure of chemo or targeted therapies we must consider at least two major variables. On one hand, we must clearly identify the tumor cell of origin, defined as the cell that acquires the first genetic hit(s) that eventually leads to the initiation of cancer. The identification of these cells should improve early detection, prediction of tumor behavior, and potentially lead to development of novel preventive therapies [17]. On the other hand, we need to face the higher complexity of advanced metastatic cancers that continuously evolve at genomic, transcriptomic, proteomic and metabolic levels, allowing them to survive in hostile environments. This pushes the tumor into a second dynamic characterized by selection for function, proposed as the driving mechanism of all evolving systems, which poses higher complexities in the definition

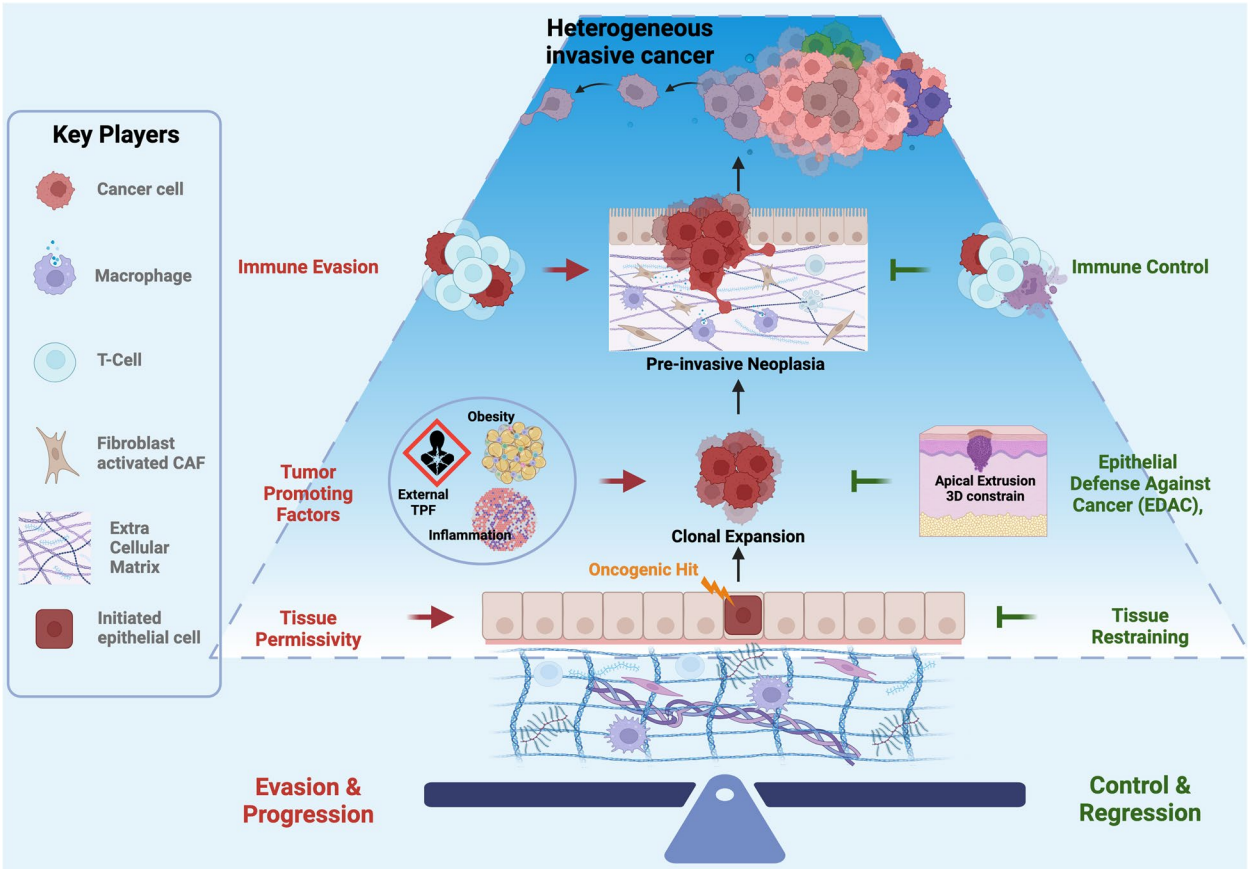

Fig. 2 Key steps from initiated cell of origin to invasive cancer development. Carcinogenesis is an inefficient process where the evolution of initiated cells into the appearance of a locally invasive cancer is the balance between tumor promoting (red) and opposing (green) factors. In the image some of the key players involved are depicted. It is noteworthy that in this process the crosstalk between the initiated/transformed cells and the host local environment plays a central role; with the host controlling tumor evolution (e.g. tissue restraining factors or immune control) and cancer cells directly influencing the surrounding environment (e.g. transforming fibroblast to CAF etc.)

of both mechanisms of resistance and possible therapeutic opportunities [18].

Under the pressure of treatments, the Darwinian evolution could likely explain the recurrences due to the appearance of specific driver gene mutations like those emerging after targeted therapies as for instance, in non-small cell lung cancer treated with EGFR inhibitors (e.g. resistance mutations in EGFR or KRAS genes) or in luminal breast cancer treated with hormonal therapies (e.g. resistance mutations in ESR1 gene, see also Sect. 1.2).

On the other hand, selection-for-function evolution likely drives transcriptional reprogramming, leading to diverse phenotypic cellular states (e.g. the appearance of senescent or drug tolerant persister cells). This process can profoundly impact treatment strategies, with a need for not only selecting the right therapy for a specific tumor but also optimizing its timing and administration for maximum effectiveness [19, 20]. In this context, it is likely that transcriptional reprogramming associated with non-Darwinian evolution might be particularly responsive to epigenetic inhibitors, offering a potential strategy to target reversible non-genomic resistance (e.g. ITGA6 overexpression in ovarian cancer, see Sect. 1.2). Alternatively, optimizing treatment schedules for administering the most appropriate therapies could also be considered to target tumors that have undergone selection for function under the pressure of previous treatments. For instance, it has been proposed that in the case of non-eradicable tumors, adaptive therapy could be more effective in prolonging survival than the classical treatment-for-cure strategy. This strategy was empirically defined and then proved in ovarian cancer xenografts [21]. Of note, this mathematical and preclinically demonstrated hypothesis, is now largely accepted in the management of ovarian cancer, now considered as a chronic disease in which each relapse might be defined and managed differently with available therapeutic options [22].

Toward the understanding of how cancer cells evolve in unperturbed environments or under the pressure of different therapies in complex environments, major advances are coming from the application of single cell omics to genetic approaches able to track the evolving cells over time. Single-cell sequencing is a powerful technology used to study the transcriptomes of individual cells, providing deep insights into cellular heterogeneity and complex biological systems. This genomic approach has facilitated the identification of molecular pathways that enable us to predict survival, therapeutic response, resistance probability and suitability for alternative intervention [23]. One of these approaches successfully used to track the evolution of pancreatic cancer is a lentiviral barcoding platform which can track clonally identical tumors in large cohorts of animals. This approach demonstrated that clone fitness is the most relevant aspect in determining metastatic dissemination in an unperturbed model; and that in the primary tumors, functionally heterogeneous subpopulations of cells co-exist with differential degrees of drug sensitivity and expressing specific transcriptional signatures, which could be used to predict patient survival [24, 25]. Recently, coupling single cell genomic and transcriptomic analyses to in vivo tracing systems has enabled the characterization of the evolution of metastatic disease in pancreatic cancer models [16].

On this basis, we will briefly discuss the most recent research findings on the cell of origin and clonal evolution in solid tumors. These studies represent the basis of imagining novel provocative ways to ameliorate early diagnosis and treatments for cancer patients.

Cell of origin and tumor promotion

Cancer is mostly a genetic disease, a concept now widely recognized and strongly supported by advancements in NGS technologies, which revealed that genetic alterations in bona fide cancer driver genes exhibit striking tissue specificity [26]. Some driver gene alterations are exclusive to cancers arising in specific tissues, whereas a few, (e.g., TERT, TP53, MYC) are observed across multiple tissue types [27]. To understand this tissue specificity, it is useful to to keep in mind the concept of the tumor-initiating cell, or cell of origin, defined as a normal cell acquiring the first cancer-promoting mutation(s) [17]. The transition from this mutated cell to clinically evident tumors is inherently inefficient, requiring the interplay of tumor-promoting factors and the weakening of host defense mechanisms [3] (Fig. 2). For instance, it has been estimated that less than 1% of precursor lesions detected during autopsy in breast tissue could progress to undiagnosed invasive cancer, suggesting that the majority of these lesions will never develop into clinically evident tumors [28]. Recent single-cell DNA sequencing studies have provided deeper insights into this phenomenon. Approximately 3% of luminal epithelial cells in histologically normal breast ducts and lobules show expanded copy number alterations (CNAs), suggesting that clonal expansions of rare aneuploid epithelial cells exist even in healthy women [29]. Notably, the prevalence of these aneuploid cells correlates with age, and many of their CNAs overlap with those found in invasive breast cancers [29]. However, while CNAs in normal breast tissue are typically polyclonal, those in invasive breast cancers tend to be clonal in origin [29, 30]. Although it remains uncertain whether aneuploid cells in normal breast tissue eventually give rise to tumors, these findings offer valuable clues for identifying the tumor cell of origin in human tissues. In breast cancer, for instance, aneuploid luminal epithelial cells may represent the cell of origin for both estrogen receptor (ER)-positive and ER-negative subtypes [29]. Supporting this hypothesis, over 25% of luminal progenitor cells in BRCA2 mutation carriers exhibit sub-chromosomal copy number variations, a phenomenon rarely observed in non-carriers. This suggests that aneuploid luminal progenitors may be a critical point of origin for certain breast cancer subtypes [31]. Despite these findings, driver mutations in oncogenes and tumor suppressor genes display significant tissue specificity, indicating that aneuploidy may act as a cofactor in tumor development rather than the sole driver. Experiments have demonstrated that somatic CNAs affecting proliferation-regulatory networks operate in a highly cell-type-specific manner and may even exhibit antagonistic effects in different cell types depending on the contextual genetic network architecture [32]. This underscores the importance of studying tissue-specific mechanisms, such as cell–cell interactions and cell competition, in limiting clonal expansion of mutant or aneuploid cells [33]. Interestingly, genes commonly mutated across cancer types, such as TERT (via non-canonical effects independent of its catalytic function) and MYC (through regulation of apoptosis), play pivotal roles in regulating cell competition in both cancer and development [33–35]. Nevertheless, in solid tumors, specific oncogenic signals are usually associated with the development of a specific subset of cancer. For instance, among epithelial ovarian cancers, the development of high-grade serous carcinomas is linked to the combined inactivation of TP53 and genes regulating the homologous recombination DNA repair pathway like BRCA1 or BRCA2 while the development of low-grade serous carcinomas is usually linked to activating mutations in the RAS-MAPK pathway. In both cases, while the cell of origin is the epithelial cell of the ovary or the fallopian tubes, the oncogenic signal is different [22, 36–42]. Similarly, breast carcinomas that are clinically divided into three main groups based on the

presence/absence of Estrogen (ER) and Progesterone (PgR) and HER2 receptors in luminal (ER/PgR +) HER2 + and Triple Negative (TNBC) breast cancer, might have the same or distinct cell of origin but are commonly associated with specific genetic alterations linked to the activation/inactivation of specific signaling pathways [43, 44]. Modern cancer treatments increasingly target these molecular distinctions and in breast cancer patients are now tailored depending on tumor type and molecular profile [45], confirming the relevance of studying the molecular mechanisms driving tumor onset and progression. It is worth noting that these early alterations could also bias clonal evolution toward resistance, as seen in BRCA1/2 gene reversion mutations under PARP inhibitor pressure [46]. Moreover, a tumor carrying loss of tumor suppressor genes like BRCA1/2 could activate transcriptomic programs leading to the reciprocal upregulation of synthetic lethal genes which in turn could alter the response to targeted therapies and favor the appearance of drug resistant recurrences [47]. Recent studies in small cell lung cancer (SCLC) reinforce this hypothesis demonstrating that ancestral clones already present in the primary tumor emerge from the common precursor and give rise to subclones shaping clinical relapse, linking the genomic asset of the cell of origin to the appearance of drug resistance [48]. Accordingly, recent data demonstrated that the transcriptomic make-up of human cancers is partially shaped by patterns of TSG loss and reciprocal upregulation of synthetic lethal genes.

Among the possible therapeutic choices, it is worth mentioning that early stages of breast cancers, such as in situ ductal carcinomas, have a very good prognosis and the appropriate treatments might have no effect on survival outcomes. A major challenge for future research is to precisely identify patients who may benefit from treatment de-escalation, avoiding unnecessary interventions [49]. This likely requires integrating transcriptional and proteomic biomarkers with genetic data, as genetic alterations alone may not reliably predict which cancer will have the higher chances to progress if left untreated.

Toward the understating of how solid tumors progress, we have to take into account that all epithelial tissues have the ability to remove or suppress neoplastic cells. An essential concept in understanding cancer progression is the role of Epithelial Defense Against Cancer (EDAC), a process by which epithelial tissues actively suppress or eliminate transformed cells through mechanisms such as apical extrusion [33, 50]. EDAC is a potent barrier protecting epithelial integrity, but tumors can evade these mechanisms through various strategies. Studies in drosophila concurrent with observations in cancer patients suggest that the primary mechanism evading EDAC and other forms of tumor control are due to the presence and activity of tumor promoting factors such as high sugar diet and obesity [3, 33, 51]. Similarly, activation of inflammation and oxidative stress response can lead to the elimination of cancer cells through the Jun N-terminal kinase (JNK) pathway, that depending on the cellular context can have both pro-apoptotic and pro-proliferative functions [52]. Elegant information coming from the study of the tumor promoting activity of air pollution in lung cancer have implicated the activation of the interleukin-1β (IL-1β) pathway activation in driving the expansion of mutated EGFR alveolar type II cells, the cell of origin of lung adenocarcinoma [53], highlighting how inflammatory pathways may facilitate tumorigenesis. Based on the accepted concept that reducing inflammation without affecting lipid levels may reduce the risk of cardiovascular disease, a monoclonal antibody targeting IL-1β was used to treat patients with previous myocardial infarction. The trial, which enrolled more than 10.000 patients, demonstrated that targeting the IL-1β innate immunity pathway significantly lowered the rate of recurrent cardiovascular events compared to controls [54]. More related to cancer was the observation that, in the same cohort of patients, the anti-IL-1β treatment reduced the incidence of lung cancer and lung cancer mortality in a dose dependent manner [54]. Yet, the risk of serious infections and cancer appearance seems to be increased in patients with rheumatologic diseases treated with IL inhibitors [55], emphasizing the need to accurately select the population at high risk to develop cancers prior to designing trials aimed at intercepting the activity of tumor-promoting factors. Precisely identifying the population at high risk for developing solid tumors represents one of the most important challenges of the next years in cancer research. Yet, targeting tumor promotion and risk factors theoretically represents one of the most convincing ways to lower the incidence of invasive cancer and profoundly impact patient survival.

It is worth noting that microenvironmental mechanisms like EDAC or inflammation can select for fit clones early on, driving the emergence of specific resistant clones under the pressure of treatments. Recent studies using colorectal cancer as a model highlighted how phenotypic plasticity in the cells of origin fosters a "*resistance continuum*" with stromal interactions amplifying resistant traits [56]. This will be discussed in the next section.

Once transformed cells overcome competition within the epithelium, they must navigate additional barriers, including the tissue microenvironment and immune surveillance. Tissue architecture, extracellular matrix (ECM) composition, and interactions with stromal, immune and endothelial cells further constrain tumor progression, rendering the progression to invasive cancers a very inefficient process, with transformed cell

clones remaining indolent for years or even regressing [3]. The role of immune surveillance in cancer clone expansion will not be further discussed here but it has been recently reviewed elsewhere [57–60]. Here, we will briefly reiterate the concept that tissues are composed of epithelial cells that dynamically interact with structural components, like ECM components, and cells from hematopoietic, mesenchymal, and endothelial lineages. This complex interaction between transformed epithelial cells and the local microenvironment can both restrict and promote early tumorigenesis. For instance, ECM composition and remodeling is a prerequisite for cancer cells to proliferate within the tissue and eventually invade and metastasize [61, 62]. ECM may restrict tumor cell dissemination by both creating physical barriers and disrupting signaling pathways that are crucial for tumor cell growth and survival [63]. These antitumor activities can be transformed by the remodeling of the interstitial matrix to support tumor progression, by altering growth factors and cytokines production and availability, and by reducing tissue stiffness and remodeling of the basement membrane, necessary for cancer cells to invade stromal tissue and become a malignant tumor [61, 62, 64]. For instance, cancer cells can directly remodel the ECM by secreting metalloproteases and/or altering their adhesion receptors repertoire. Cancer cells can also act through the conditioning of other cells, including hematopoietic, mesenchymal and endothelial lineages, among which includes the reprogramming of fibroblasts into cancer-associated fibroblasts (CAF). This represents a pivotal passage to overcome the limits imposed by 3D tissue organization and leads to resistance toward anticancer therapies [65]. As an example, pancreatic ductal adenocarcinoma (PDAC) relies on its extraordinary ability to thrive and progress in a challenging tumor microenvironment characterized by an intense desmoplastic reaction that compresses blood vessels and limits nutrient supplies. Yet, PDACs sense nutrient deprivation and activate an epigenetic adaptive mechanism (e.g. miR-15 deregulation) to overcome the limitation in oxygen and nutrient availability in the tumor microenvironment and proliferate in the hostile microenvironment [66].

Overall, tumorigenesis is an inherently inefficient process (Fig. 2), with only a minority of transformed cell clones overcoming the host's barriers by acquiring sufficient functional fitness to become invasive cancers. For instance, TP53 mutations can be found in normal esophageal epithelial cells but, although mutated TP53 cells have proliferative advantage and are capable of clonal expansion, these cells do not disrupt normal epithelial structures [67, 68]. On the other hand, accumulating evidence connects the cell of origin to the emergence of drug-resistant clones in which mutational processes, including CNAs, might restrict the epigenetic landscapes and accessibility of cell states, thus modifying the trajectories of adaptation [48, 56]. Future research should focus on identifying populations at high risk for cancer progression and developing interventions targeting tumor-promoting factors and the surrounding microenvironment. A focus should also be on exploring novel therapeutic approaches aimed at preventing emergence by targeting the cancer cell phenotypes (e.g. persistent, drug tolerant, stem cell-like or mesenchymal cellular states) associated with drug resistance. These efforts hold promises for reducing cancer incidence and improving patient outcomes.

Clonal evolution and therapies resistance

Once a tumor becomes clinically evident, increasingly effective therapeutic options have contributed to a remarkable 33% decline in cancer mortality since 1991, averting approximately 3.8 million cancer deaths [69]. However, advanced metastatic diseases persist as a major challenge, with consistently low five-year relative survival rates across nearly all cancer types [69]. These outcomes underscore the critical influence of tumor heterogeneity and underlying clonal evolution in shaping therapeutic efficacy and limiting survival in such challenging disease contexts. Cancer genomics have revealed significant intra-tumor and -patient heterogeneity, demonstrating that genetically distinct clones co-exist within solid tumors [70]. Yet, understanding how this genomic diversity contributes to intrinsic resistance to anti-cancer treatments remains complex. For instance, an analysis of 76 untreated metastases from 20 patients across various cancer types revealed that the majority of driver gene mutations within an individual patient are shared among all metastases [71]. Furthermore, driver mutations unique to specific metastases generally lack significant functional consequences, suggesting that single biopsies can adequately capture most functionally relevant mutations in metastatic tumors. This insight is crucial for guiding therapeutic decision-making [71].

Further evidence from whole-genome sequencing (WGS) studies supports this notion. Data from 250 biopsy pairs collected longitudinally from 231 adult patients with diverse metastatic solid malignancies showed remarkable concordance. Specifically, 99% of biopsy pairs displayed full genomic agreement, and in 91% of cases, a second WGS analysis did not identify additional actionable biomarkers qualifying patients for clinical trial enrollment. Secondary mutations in driver genes emerged primarily in response to small-molecule inhibitors or hormonal therapies [72]. In our view, these findings emphasize two key concepts in cancer evolution and drug resistance: (1) trunk genomic alterations are

pivotal for tumor progression and therapy resistance, and (2) epigenetic modifications contribute to clonal selection and resistance, especially in response to non-targeted as opposed to targeted therapies (Fig. 1). A glaring example comes from breast cancer studies where targeted hormone therapies and chemotherapy have both been used for many years, providing important information about disease progression under the pressure of different types of treatment. Resistance to chemotherapy in breast cancer appears more closely linked to transcriptional regulation changes, which may be counteracted with RNA transcription inhibitors [73]. Conversely, resistance to hormone therapy has been associated with de novo ESR1 (estrogen receptor 1) mutations, detectable via liquid biopsies in metastatic hormone-therapy-resistant patients. These mutations are now actionable using FDA- and EMA-approved ESR1 degrader molecules [74]. Such examples highlight both the power of genomic biomarkers in personalizing anti-cancer treatments and the need for further research into the mechanisms driving resistance, particularly under chemotherapy, which remains the standard of care for most cancer patients.

In recurrent chemo-resistant ovarian cancer, WGS rarely identifies recurrent actionable point mutations, suggesting that, at best, only low frequency events are likely to be uncovered using personalized genomic evaluation of patients with recurrent ovarian cancer [75]. Resistance to chemotherapy appears to leave an imprint on the tumor genome, characterized by non-silent coding changes. However, these changes seldom involve known mutations in driver or actionable genes. Conversely, resistance to PARP inhibitors—a maintenance therapy targeting PARP1 in platinum-sensitive, BRCA1- and BRCA2-mutated ovarian cancers—is frequently linked to reversion mutations in BRCA1/2. These mutations, identifiable in cell-free DNA, correlate with shorter patient survival, reinforcing the association between targeted therapies and de novo mutations in actionable genes [46]. These observations reinforce the concept that targeted therapies are more often associated with the appearance of de novo mutations in actionable genes.

The advent of single cell sequencing technologies has raised hopes in identifying genomic alterations linked to chemotherapy resistance. A notable clinical application is in triple-negative breast cancer (TNBC) patients treated with neoadjuvant chemotherapy (NAC), such as taxol and doxorubicin, the standard of care for most TNBC patients [76]. Resistance to NAC is observed in about 30–50% of the cases and is associated with worse prognosis [76]. Single-cell DNA and RNA sequencing revealed that resistant genotypes pre-existed and were adaptively selected, while resistant expression profiles emerged through transcriptional reprogramming in response to chemotherapy. These findings support a model in which adaptive and acquired evolution jointly establish chemoresistance [77]. This work also demonstrated that, although the chemo-resistant transcriptional programs were not pre-existing and were acquired via transcriptional reprogramming after treatment, small subsets of primed tumor cells expressing resistant genes may pre-exist, gaining a survival advantage under therapeutic pressure [77]. Similarly, studies in ovarian cancer have shown that platinum treatment can activate a transcriptional program linked to Integrin α6 (ITGA6) expression via epigenetic regulation. In resistant clones, this program becomes constitutively active, contributing to chemoresistance [78]. Subclones expressing higher ITGA6 levels within platinum-sensitive tumors may also gain a survival advantage under chemotherapy, highlighting the role of clonal selection [78]. Of course, this hypothesis of course needs to be further validated and it is likely that only studies using the combination of single-cell DNA and RNA sequencing could further elucidate whether adaptive or acquired evolution—or both—drive resistance in specific tumor types under chemotherapeutic pressure [77]. One transcriptional program linked to chemotherapy resistance in TNBC is the epithelial-mesenchymal transition (EMT) program. Interestingly, recent data obtained using scRNA-seq, multiplex immunohistochemistry and RNA fluorescence in situ hybridization in melanoma patients treated with immunotherapy demonstrated that a substantial fraction of melanoma cells manifest "hybrid" phenotypes, indicative of an ongoing cell state transitions due to cell plasticity. This could be due to the presence of mesenchymal (MES) melanoma cells in the early stages during immunotherapy treatment that are associated with resistance to immune checkpoint inhibitors. Although the proportion of MES cells is relatively low, they may contribute to resistance through non-cell- autonomous mechanisms, influenced by the local tumor microenvironment [79]. Co-culture studies have further demonstrated that extracellular vesicles can prime chemo-sensitive cells and the microenvironment toward a resistant phenotype [78]. Moreover, this multi-omics as well as spatial approach also suggested that the local tumor microenvironment directly contributes to the spatial organization of melanoma cells expressing overlapping transcriptional programs [79].

Collectively, emerging evidence suggests that resistance to targeted therapies often involves specific genomic alterations affecting drug-response genes such as ESR1 mutations in luminal breast cancer or BRCA1 reversion in PARPi-treated ovarian cancer [46, 74]. Resistance to non-targeted therapies, like chemotherapy and immunotherapy, is more closely tied to transcriptional changes

and clonal evolution influenced by the tumor microenvironment due to alteration in transcriptional programs under the pressure of chemotherapy [73, 78] or the activation of specific pathways involved in tumor-microenvironment interaction such as YAP1/TAZ1-regulated cellular mechano-transduction in driving immunotherapy resistance [80, 81]. This non-cell-autonomous crosstalk underscores a functional resistance phenotype that persists through interactions between resistant and sensitive cancer cells.

If resistance is a functional state, a central challenge lies in identifying and targeting persistent cancer cells that survive and adapt under therapeutic pressure. These cells exhibit high plasticity and transient phenotypes, complicating their targeting. However, recent advances in single-cell and spatial *omic* technologies offer promising avenues in the understanding of persistent cell phenotypes and their vulnerabilities. As these tools mature, their integration into clinical practice may unlock new strategies to counteract or prevent resistance, paving the way for improved patient outcomes [82, 83].

Epigenetic mechanisms

Epigenetic modifications—heritable changes in gene expression that occur without alterations to the DNA sequence—play crucial roles in the development of drug resistance in cancer [84]. These modifications include DNA methylation/hydroxymethylation, histone covalent modifications, ATP-dependent chromatin remodeling, and noncoding RNAs (ncRNAs). Epigenetic modifications occurring in cancer cells, immune cells, and cells of the tumor microenvironment, alter gene expression and contribute to both intrinsic and acquired resistance to therapeutics. Central to driving these epigenetic changes is the dysregulation of essential epigenetic regulators, which elicit genomic alterations, allowing cancer cells to escape immune surveillance and resist therapeutics [85].

DNA methylation is frequently altered in cancer and plays a significant role in driving drug resistance. Methylation occurs primarily at the 5′ position on the pyrimidine ring of cytosine residues in the context of CpG dinucleotides. The addition of the methyl group is catalyzed by DNA methyltransferases, including DNMT1, DNMT3A, and DNMT3B using s-adenosylmethionine (SAM) as a methyl donor. DNMT3A and DNMT3B establish and DNMT1 maintains the epigenetic landscape during organismal development and across the lifespan, particularly during aging, a process closely tied to tumorigenesis [86]. A family of methyl-DNA binding proteins specifically recognize and bind to methylated DNA, converting the DNA methylation signal into functional outcomes, including mediating crosstalk with chromatin regulators [87]. DNA methylation at regions of high CpG density, known as CpG islands and CpG island shores proximal to gene promoters and enhancers, silences gene expression while methylation in gene bodies is associated with active transcription [88, 89]. DNA methylation impacts gene expression by inhibiting transcription factor binding, promoting repressive chromatin structure, and affecting alternative splicing, as well as regulating expression of non-coding RNAs, which then impact expression of protein coding genes [90]. DNA methylation also maintains genome integrity by suppressing transposable elements and other repetitive sequences [91].

Ten-eleven translocation (TET) proteins oxidize 5-methylcytosine to generate 5-hydroxymethylcytosine (5- hmC), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC) using alpha-ketoglutarate (αKG) as a cofactor [92]. The oxidized methylcytosines can be stable epigenetic marks or serve as intermediates for DNA demethylation through the base excision repair pathway. TET proteins have dual roles in cancer and can act as oncogenes and tumor suppressors depending on cellular context and tumor microenvironment. One explanation for this dual role is that changes in the tumor microenvironment can alter the function of TET proteins. For example, in the hypoxic microenvironment of many tumors, TET proteins may cooperate with hypoxia inducible transcription factor (HIF1) or other oncogenic transcription factors, to promote drug resistance and tumor progression [51–55]. As a result, both the DNA methylation and hydroxymethylation landscapes are significantly altered in cancer, disrupting normal gene regulation. This occurs in cooperation with changes in the chromatin landscape as well as altered expression of non-coding RNAs [85].

Alterations in chromatin structure disrupt gene regulation, driving tumorigenesis. Chromatin is regulated by two classes of enzymes: those that add or remove covalent modifications on histone proteins and those that use the energy from ATP hydrolysis to disrupt nucleosome structure [93]. Covalent modifications, including acetylation, phosphorylation, methylation, SUMOylation and ubiquitination, influence chromatin structure and gene expression [94]. Proteins with reader domains, such as bromodomains, chromodomains, plant homeodomain (PHD) fingers, Tudor domains, PWWP domains, and YEATS domains, bind to specific histone modifications and play a critical role in regulating downstream processes [95]. ATP-dependent chromatin remodelers play crucial roles in development, enable the cell to respond to environmental cues, maintain genome integrity, and control gene expression in a highly orchestrated manner. These remodelers often function in large multi-subunit complexes and work in coordination with histone-modifying enzymes and other epigenetic regulators to ensure

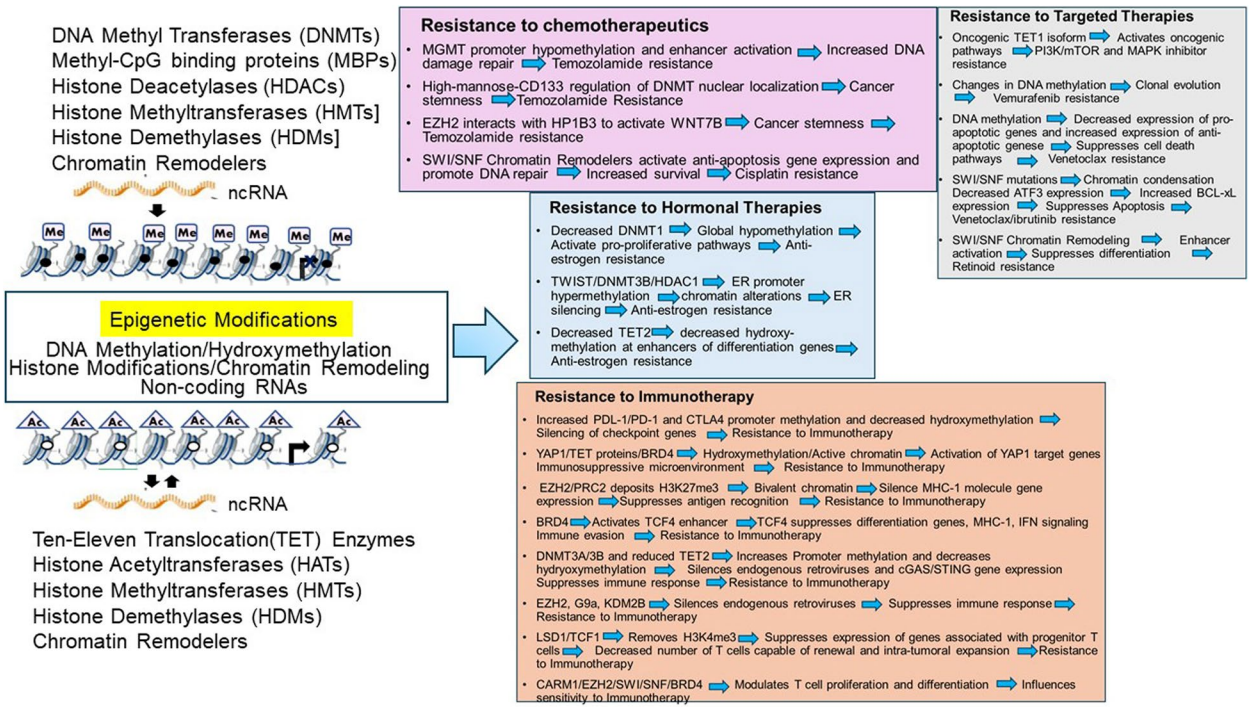

Fig. 3 Epigenetics in drug resistance. Epigenetic modifications contribute to drug resistance in cancer cells by altering gene expression profiles. The mechanisms include DNA methylation, hydroxymethylation, nucleosome repositioning, histone modifications, and the regulation of non-coding RNAs. These changes enable cancer cells to develop diverse strategies to evade the effects of cancer therapeutics. In the figure several examples on how epigenetic modifications impact the response to different anti-cancer treatments is shown (see text for more details)

proper cellular function. The activities of histone-modifying enzymes, chromatin readers, and chromatin remodelers are often dysregulated in cancer cells, leading to profound alterations in chromatin structure and gene expression. These disruptions contribute to tumorigenesis and resistance of cancer cells to therapeutics [96]. A diverse range of epigenetic modifications play a crucial role in the evolution of cancer cells and their ability to develop resistance to therapeutics (Fig. 3). The following sections explore key examples of how epigenetic mechanisms drive resistance to chemotherapeutics, hormonal therapies, and targeted treatments, concluding with a discussion of related therapeutic opportunities.

Epigenetics in resistance to cancer therapeutics

Epigenetics in resistance to chemotherapeutics

Deregulation of the normal DNA methylation landscape contributes to resistance against chemotherapeutics like Temozolomide (TMZ), an oral alkylating agent frequently used as an adjuvant to radiotherapy in the treatment of glioblastoma multiforme (GBM) [97–99]. The response to TMZ is heavily influenced by the methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) promoter. Silencing of MGMT by promoter hypermethylation is associated with a favorable response to TMZ, while hypomethylation and high expression of MGMT lead to a poor response [100]. This is because elevated MGMT expression enables glioma cells to repair DNA adducts, thereby reducing the apoptosis-inducing efficacy of TMZ [100, 101]. However, despite the observation that MGMT promoter methylation is seen in about 30–60% of GBM patients, the status of MGMT promoter methylation is not a sufficiently useful biomarker for predicting drug response. One reason is that a subset of patients with MGMT promoter hypermethylation still express MGMT and show poor response to TMZ [102]. In some GBM cells that harbor a methylated MGMT promoter, an alternative epigenetic mechanism activates a distal enhancer by promoting histone 3 lysine 27 acetylation (H3 K27ac) and overrides promoter methylation, driving MGMT expression and promoting TMZ resistance [103]. Furthermore, as discussed below, other epigenetic mechanisms beyond MGMT can play a significant role in determining sensitivity to TMZ. A better understanding of these diverse epigenetic mechanisms is needed in order to understand transcriptional reprograming that occurs under the selective pressures of TMZ administration and to identify useful biomarkers for predicting drug response.

Epigenetic mechanisms that promote a cancer stem cell (CSC) phenotype play a key role in mediating TMZ resistance [104]. CSCs possess a highly plastic phenotype, enabling them to adapt to dynamic microenvironments, drive tumor progression, and evade the effects of therapeutic drugs. High expression of stem cell markers is accomplished by selective promoter hypomethylation and permissive chromatin structure in response to cues from the tumor microenvironment, such as hypoxia, nutrition, and cytotoxic stress [105]. For example, promoter hypomethylation drives over-expression of CD133, a transmembrane glycoprotein that marks a subset of tumor-initiating cancer cells capable of driving aggressive tumor growth and resistance to chemotherapeutics in a diverse group of cancers [106–112]. To promote the stem cell phenotype, the CD133 protein must also be differentially glycosylated. CD133 is glycosylated by Mannosidase Alpha Class 1 A Member 1 (MAN1A1) in differentiated cells but not in CSCs where MAN1- A1 is epigenetically silenced [113, 114]. This enzyme converts high-mannose glycans into hybrid and complex N-glycans [115], which influences protein–protein interactions and suppresses the ability of CD133 to promote tumor progression. In contrast, CSCs, lacking MAN1A1 expression, accumulate the high-mannose form of CD133. High mannose-CD133 inhibits nuclear localization of DNMT1, leading to promoter hypomethylation of cell cycle inhibitory genes, CDKN1 A and CDKN1B, thereby activating their expression and promoting the slow cycling state and self-renewal capacity that makes glioma stem cells resistant to TMZ [113]. Since CD133 is downregulated by promoter methylation, the loss of DNMT1 may create a positive feedback loop: increasing CD133 leads to progressive reduction of DNMT1 activity, thereby further increasing CD133 expression. This leads to robust activation of cell cycle regulators, potentiating the self-renewal and the slow-cycling phenotype, ultimately enhancing resistance to TMZ [116]. Although high levels of CD133 promote the CSC phenotype, there have been conflicting reports regarding the usefulness of CD133 expression as a biomarker for predicting response to TMZ. Rather than total CD133 levels, the levels of high mannose-CD133 and MAN1 A1 expression may hold greater clinical significance [114, 117]. Hence, epigenetic rewiring of multiple genes enhances the plasticity of CSCs, allowing them to evade TMZ treatment. As discussed below, chromatin alterations also contribute to emergence of cells which no longer respond to TMZ.

Several chromatin regulators are involved in maintaining GBM stemness, thereby also promoting resistance to TMZ. The high expression of the histone methyl transferase, SUV39H1, which deposits the repressive H3 K9 me3 mark, is associated with poorer GBM prognosis. SUV39H1 drives GBM stem cell resistance to TMZ by promoting G2/M cell cycle progression, stem cell maintenance, and suppressing cell death pathways [118]. The histone methyltranserase, EZH2, the catalytic subunit of Polycomb repressive complex 2 (PRC2) is also highly upregulated in GBM stem cells. In this context, EZH2 works independently of PRC2, interacting with heterochromatin protein 1 binding protein 3 (HP1BP3) to activate expression of WNT7B and promote proliferation, stemness, and TMZ resistance [119]**.** Supporting a PRC2-independent role for EZH2 in glioma, EZH2 was found to be overexpressed in later stages of the disease—unlike a different PRC2 subunit, EED,—and its elevated expression was associated with poorer patient survival [120–123]. These findings suggest that EZH2 may serve not only as a biomarker but also as a potential target for therapeutic intervention in glioma. However, one study reported that although short-term depletion of EZH2 suppresses murine tumor growth and improves survival, prolonged inhibition results in undifferentiated, highly proliferative tumors with upregulated expression of DNA repair genes, rendering them resistant to TMZ [124]. Therefore, effective therapeutic targeting of EZH2 would require a carefully optimized dosing regimen.

In addition to these histone methyl transferases, the BRG1 catalytic subunit of the SWI/SNF chromatin remodeling complex promotes GBM stemness, contributing to resistance to TMZ as well as other chemotherapeutics [125, 126]. Notably, BRG1 depletion or pharmacological inhibition of BRG1 can sensitize gliomas harboring histone H3 mutations to TMZ and other chemotherapeutics, reducing tumor growth and increasing survival in animal models [127–131]. While BRG1 is frequently mutated in some brain cancers, mutations are rare in GBM, where BRG1 is frequently over-expressed [132, 133]. In combination, these studies suggest BRG1 may be a useful therapeutic target as a strategy to overcome GBM resistance to TMZ.

SWI/SNF chromatin remodeling enzymes can modulate sensitivity to various other chemotherapeutics through both transcriptional regulation and DNA repair mechanisms. In melanoma cells, BRG1 prevents cisplatin-induced cell death by promoting the expression of the anti-apoptotic gene BIRC7 (ML-IAP). This process operates through a lineage-specific mechanism that prevents EZH2 from silencing chromatin on the gene's promoter [134]. Additionally, disruptions in chromatin remodeling enzymes impair the repair of DNA damage, often making cancers with loss-of-function mutations in SWI/SNF genes more vulnerable to chemotherapeutic agents [135]. In lung cancer cells, BRG1 and the alternate SWI/SNF ATPase, BRM modulate cisplatin resistance by promoting recruitment of the nucleotide repair protein,

ERCC1 to damaged DNA [136]. In non-small cell lung (NSCLC) patients, tumors with low BRG1 expression exhibit increased sensitivity to platinum-based drugs, suggesting that BRG1 status may serve as a predictive biomarker for treatment response in NSCLC patients [137].

Epigenetics in resistance to hormonal and targeted therapies

Epigenetic dysregulation plays a crucial role in cancer resistance to hormone-based therapeutics. For example, in BRCA1-mutated breast cancer cells, hypermethylation of an E2F1 binding motif in the DNMT1 promoter suppresses DNMT1 expression, leading to global hypomethylation and resistance to anti-estrogens [138]. Although the precise mechanisms driving hypermethylation of the DNMT1 promoter remain unclear, the loss of BRCA1, a known activator of DNMT1 transcription is likely to be a causal factor [139]. Additionally, overexpression of DNMT3A and DNMT3B, driven by hypomethylation of their promoters contributes to region-specific hypermethylation patterns that promote tamoxifen resistance [140, 141]. In breast cancer cell lines, DNMT3B overexpression is associated with a hypermethylator phenotype, characterized by high levels of DNA methylation at tumor suppressor sites and poor prognosis [142]. This leads to methylation driven silencing of Estrogen receptor (ER), driving resistance to tamoxifen and other anti-estrogens. In one study, overexpression of the transcription factor TWIST was shown to recruit DNMT3B and histone deacetylase 1 (HDAC1) to the ER promoter. This recruitment led to promoter hypermethylation and chromatin condensation, resulting in reduced ERα transcript levels [143]. It was recently demonstrated that administration of the DNA hypomethylating drug, decitabine, reduced growth of ER + metastatic breast tumors in mice. The effects on tumor growth were linked to decreased methylation of ER enhancers, leading to the decompaction of higher-order chromatin structure, and the upregulation of ER-mediated transcription pathways. These findings highlight the clinical potential of DNA hypomethylating drugs in resensitizing ER + resistant breast cancer to antiestrogen therapies and also demonstrate the coordinated effects of both DNA methylation and chromatin modifications in the development of drug resistance [144].

Disruptions to the hydroxymethylation landscape also drive resistance to hormonal therapies. In breast cancer cells, TET2 plays a crucial role in regulating ER-dependent gene expression [145]. Loss of TET2 was found to increase the population of breast cancer stem cells, prevent luminal cell differentiation, and reduce sensitivity to tamoxifen. TET2 was also found to be crucial for the demethylation of enhancer regions associated with luminal differentiation and its loss associated with resistance to endocrine therapy in ER positive breast tumors [146, 147]. Similarly, the repression of TET2 in prostate cancer was associated with reduced hydroxymethylation and the development of hormone insensitivity in advanced tumors [148]. In contrast to the tumor-suppressive functions of TET2, an oncogenic TET1 isoform was found to be overexpressed in triple-negative breast cancer (TNBC), where it activates oncogenic signaling pathways and resistance to PI3 Kinase/mTOR and MAPK inhibitors [149]. Studies suggest that TET1 overexpression in triple-negative breast cancer (TNBC) is associated with a worse prognosis and may serve as a biomarker for poor response to various therapeutic strategies [150, 151].

Both DNA methylation and chromatin de-regulation play significant roles in promoting resistance to targeted therapies. Alterations in DNA methylation drive gene expression changes during the clonal evolution of BRAF inhibitor-resistant melanoma cells. In Vemurafenib (VEM)-resistant melanoma, this includes promoter hypermethylation-mediated silencing of resistance suppressors like the transcription factor SOX10, as well as the upregulation of genes such as EPH3, which promote VEM resistance [152]. Similarly, leukemia cells that develop resistance to the BCL2 inhibitor, Venetoclax (VEN), differentially express genes that regulate cell survival and metabolism in response to drug exposure [153]. Resistance to VEN is associated with drug-induced epigenetic inactivation of pro-apoptotic and activation of anti-apoptotic gene signatures [154]. PUMA is one pro-apoptotic gene that was shown to be silenced by promoter hypermethylation after exposure to VEN. Loss of PUMA led to increased oxidative phosphorylation and adenosine triphosphate production, ultimately compromising the response to the drug. Conversely, in mantle cell leukemia (MCL) cells, depletion of the BRG1 component of SWI/SNF led to an increase in anti-apoptotic Bcl-xL expression [155]. The mechanism by which BRG1 loss promotes elevated Bcl-xL levels involves the suppression of ATF3 expression, mediated by reduced chromatin accessibility at the ATF3 locus. Since ATF3 functions as a transcriptional repressor of Bcl-xL, its loss leads to elevated Bcl-xL transcription, contributing to drug resistance. In this study, BRG1 depletion did not affect proliferation in the absence of drug treatment, suggesting that SWI/SNF disruption enables a transcriptional state that promotes survival under selective pressure. This may explain why a significant number of MCL patients who do not respond to VEN/ibrutinib harbor SWI/SNF subunit mutations. Furthermore, it was found that SWI/SNF status in plasma cell free DNA (cfDNA) may serve

as a prognostic biomarker for VEM sensitivity in MCL patients [155].

Although SWI/SNF functions as a tumor suppressor in MCL and its loss is linked to therapeutic resistance, several oncogenic transcriptional programs paradoxically depend on SWI/SNF to sustain open chromatin at promoters and enhancers. Moreover, elevated BRG1 expression has been associated with poorer prognosis in certain cancers. [135, 156]. Therefore, although SWI/SNF functions as a tumor suppressor in certain contexts, it has also garnered significant interest as a potential target to overcome therapeutic resistance, with several clinical trials currently investigating this approach [135].

The dependency on SWI/SNF in certain cancer cells can be therapeutically targeted using small-molecule inhibitors. Notably, BRG1 inhibition was shown to sensitize neuroblastoma to retinoids, which are used in the treatment of high-risk neuroblastoma patients [157, 158]. This study revealed that SWI/SNF activity is essential for survival during the G1 phase of the cell cycle but dispensable in other phases. This is due to its role in chromatin remodeling, which maintains accessibility at enhancers regulated by a core transcriptional circuitry, including MYCN, ISL1, PHOX2B, HAND2 and GATA3. The accessibility of these enhancers oscillates in a cell cycle-dependent manner, with the strongest reliance on SWI/SNF activity occurring at enhancers that peak in accessibility during the G1 phase. Given this role, SWI/SNF inhibition could synergize with retinoids to promote cell-cycle exit. This study revealed the critical role of SWI/SNF in G1/S progression in SWI/SNF-addicted cancers and emphasized how the temporal dynamics of enhancer-related dependencies can guide the optimal context and timing for therapeutic targeting of SWI/SNF activity.

Epigenetics in resistance to immunotherapy

Epigenetic de-regulation plays an essential role in immune evasion and impacts sensitivity to immunotherapy [159, 160]. Cancer cells evade immune detection by epigenetic modulation of immune stimulatory and suppressive genes. The simultaneous activation of immune checkpoint genes, such as PD-L1 and CTLA4, through hypomethylation, and the silencing of co-stimulatory genes (CSGs), such as HLA, via DNA hypermethylation in cancer cells, is inversely associated with the recruitment of functional T cells to the tumor microenvironment [161]. This dual epigenetic modulation enables cancer cells to evade immune surveillance and resist immune-mediated death. To counteract this, immune checkpoint blockade (ICB) targeting PD-1/PD-L1 and CTLA4 have become some of the most widely used cancer therapies, designed to enhance the capacity of the immune system to eliminate cancer cells. However, ICB has also led to a significant number of cases of immune checkpoint resistance. Variations in PD-L1 expression within tumors can impact the effectiveness of ICB [162]. Low PD-L1 expression has been associated with promoter hypermethylation and tri-methylation of histone H3 K27 by EZH2, while high expression is accomplished by promoter acetylation and binding of the bromodomain-containing protein, BRD4 [163–165]. By modulating the epigenetic landscape of immune checkpoint genes, tumors can adapt to immune pressures, influencing their response to immunotherapy. Understanding these epigenetic mechanisms may offer new strategies to improve the effectiveness of immune checkpoint inhibitors and overcome resistance.

YAP1, a key regulator of the Hippo signaling pathway, cooperates with transcription factors and epigenetic regulators to play a critical role in tumor progression and drug resistance [60]. Melanoma cells that developed cross-resistance to both MAPK inhibition and immune checkpoint blockade had transcriptomic and methylomic changes that enriched for a YAP1 signature [166, 167]. DNA demethylation in the 5' untranslated region (5'UTR) of YAP1 drives its high expression in many cancers [168]. YAP1 then interacts with chromatin modifying and remodeling enzymes to promote an immunosuppressive tumor microenvironment and contribute to resistance to immunotherapy and other cancer treatments [169]. In some cancers, YAP1 regulates the hydroxymethylation landscape by hijacking TET proteins to promote immune evasion. Recent research has demonstrated that in liver tumorigenesis, YAP1 activation induces the expression of TET1, which physically interacts with TEAD. This interaction leads to regional DNA demethylation and activation of YAP1 target genes to promote tumorigenesis [170]. In support of this, many tumors with mutations in TET1 are associated with greater immune cell infiltration and respond better to immune checkpoint blockade [171–174].

TET2/3 enhance the response to immune checkpoint inhibition [175, 176]. Supplementation of the TET cofactor, α-ketoglutarate, leads to increased 5 -hmC on the PD-L1 promoter, enhancing STAT1/3 binding to upregulate PD-L1 expression and enhancing the response to anti-PDL1 treatment [177]. A recent study found that NAD +enhances Tet1 activity by modulating levels of its cofactor, α-ketoglutarate (α-KG), leading to increased hydroxymethylation. This, in turn, promotes inducible PD-L1 expression via interferon-γ (IFN-γ)-mediated STAT1/IRF1 signaling, ultimately enhancing sensitivity to ICB [178]. Cancer cells are highly dependent on high NAD +levels to fuel proliferation, however, the decline in NAD +levels during aging and fluctuations that

occur under various nutrient conditions may negatively influence the response to ICB. Collectively, these studies have increased the understanding of the epigenetic mechanisms that promote resistance to ICB by modulating PD-L1. However, resistance to ICB is multifaceted, involving epigenetic silencing of additional genes. For example, in NSCLC, the hypomethylated promoters of Cytohesin 1 Interacting Protein (CYTIP) and TNF superfamily member 8 (TNFSF8) were found to be stronger predictors of ICB response, progression-free survival (PFS), and overall survival (OS) than PD-L1 expression [179].

The epigenetic downregulation of major histocompatibility complex class I (MHC-I) molecules on the cell membrane impairs immune recognition of diverse tumors, reducing the effectiveness of cancer immunotherapies and leading to poorer clinical outcomes. MHC-I molecules are crucial for presenting tumor antigens to cytotoxic T cells, which enables immune-mediated tumor elimination. Cancer cells exploit a normal developmental epigenetic silencing mechanism, using the PRC2 complex to establish bivalent chromatin by depositing H3 K27 me3 marks at MHC-I genes, thereby suppressing their expression and evading immune detection [179]. Treatment with EZH2 inhibitors decreases repressive H3 K27 me3 marks at MHC promoters and restores expression of MHC-1 genes in diffuse large B-cell lymphoma cells that harbor activating mutations in EZH2. This enhances antigen presentation and antitumor immunity while overcoming resistance to immune checkpoint blockade in head and neck cancer. Notably, high expression of EZH2 is associated with immune-cold tumors and poor response to immunotherapy [180–182]. In combination, these studies suggest that EZH2 is a potential biomarker for immunotherapy response in some cancers and that combining EZH2 inhibitors with ICB offers a promising new therapeutic approach for treating poorly immunogenic cancers characterized by low MHC expression [183–185].

As discussed in part 1, the EMT program is a key driver of drug resistance, which emerges during the clonal evolution of drug-resistant cells. A recent study showed that MES melanoma cells obtained from patients at the onset of immunotherapy are associated with resistance to both targeted therapies and ICB [79]. These cells are enriched for the transcription factor TCF4, which suppresses the expression of differentiation genes—recognized as antigens—along with MHC- I, other antigen presentation genes, and IFN signaling transcriptional programs. Collectively, these effects contribute to immune evasion and resistance to immunotherapy. Inhibition of BRD4, which is recruited to a TCF4 enhancer, was found to reduce chromatin accessibility at this enhancer and suppress TCF4 expression. This, in turn, enhances the expression of differentiation genes, the antigen presentation program, and IFN signaling response genes as well as increases sensitivity to BRAF/MEK inhibition. Thus, BET inhibition may represent a novel strategy to increase MES immunogenicity and improve sensitivity to both targeted and immune therapies.

Activation of the cGAS/STING pathway plays a pivotal role in detecting tumor antigens and triggering a T cell-mediated immune response, essential for the effectiveness of immunotherapies [186]. The process involves detection of cytosolic DNA by GMP-AMP synthase (cGAS), which catalyzes the production of cyclic GMP-AMP (cGAMP) [187]. cGAMP then binds to and activates STING, leading to activation of the transcription factors IRF3 and NF-κB, which drive the expression of type I interferons (IFN-α and IFN-β). Type I interferons establish a pro-inflammatory tumor microenvironment by promoting dendritic and natural killer cell activation, expression of chemokines, and enhancing antigen presentation, thereby strengthening the anti-tumor immune response. cGAS-STING agonists are being explored as potential enhancers of cancer immunotherapy and as a way to overcome resistance to existing immunotherapeutic approaches [188]. However, cGAS/STING signaling is disrupted in many tumors, reducing the efficacy of agonists as well as ICB. In melanomas, silencing of STING, achieved by promoter methylation [189], may explain why a substantial proportion of melanoma patients fail to respond or derive long-term benefits from immunotherapy [190]**.** Silencing of the cGAS/STING pathway, driven by DNMT3A and DNMT3B, was shown to be reversed by treatment with a DNA methyltransferase inhibitor, enhancing the response to a STING agonist [189]. In a mouse model of liver cancer, silencing of cGAS/STING signaling in tumors disrupts normal vasculature, thereby creating an immune suppressive microenvironment. This is mediated by low levels of TET2, which upon restoration upregulates cGAS expression in the tumor and activation of STING in endothelial cells [191]. Administration of vitamin C, a cofactor for TET2 activity, also restores tumor vasculature and enhances the efficacy of anti-PD-L1 therapy. Vitamin C has also been shown to enhance anti-PD1 therapy in other cancers [192, 193]. In contrast, TET2 depletion confers resistance to ICB in mouse models of melanoma and colon cancer. Furthermore, reduced TET activity— depicted by low levels of 5-hmC—is associated with diminished lymphocyte infiltration in human colon cancer and increased progression of colon adenomas [194]. In several other cancer types, elevated TET2 expression correlates with a more favorable prognosis, indicating its potential clinical significance [195, 196].

Activation of non-coding repetitive sequences, through DNA methylation, hydroxymethylation, and histone modifications, plays a crucial role in shaping the response to immunotherapy. Viral mimicry—a process involving the activation of endogenous retroviruses (ERVs), long interspersed nuclear elements (LINEs), and short interspersed nuclear elements (SINEs)—can profoundly impact the efficacy of ICB. These repetitive sequences generate double-stranded RNAs (dsRNAs), activate the cGAS/STING pathway, triggering an interferon (IFN) response that activates the immune system to target and eliminate cancer cells [197, 198]. DNMT1 maintains methylation of retrotransposons, thereby silencing them and suppressing viral mimicry. Suppression of viral mimicry is linked to immune'cold'tumors, which are unresponsive to immune checkpoint inhibition. In addition to DNA methylation and hydroxymethylation, the deposition of repressive histone marks and the removal of activating histone marks also play a key role in silencing ERVs. EZH2 inhibitors activate ERVs by removing the repressive histone H3 K27 me3, thereby promoting an immune response and sensitizing prostate tumors to ICB therapy [199, 200]. Inhibitors to the histone methyltransferase, G9a, which deposits the repressive H3 K9 me3 mark, also activate ERVs, and promote an immune response [201]. The histone demethylase, KDM5B, which removes activating H3 K4 me3 marks, and HDACs, which deacetylate histones, have been shown to synergize with DNMTs to suppress ERVs [202, 203]. Their inhibition promotes ERV activation, thereby stimulating an immune response, indicating the potential of these epigenetic inhibitors in enhancing immune therapies [86]. Vitamin C can also synergize with DNA hypomethylating agents to enhance anti-tumor effects. This occurs through the activation of TET enzymes, which promote the expression of ERVs [79]. Vitamin C deficiency, frequently observed in cancer patients, may contribute to immune suppression by reducing cGAS/STING activation and viral mimicry [80].

A significant challenge to the success of immunotherapy results from T cell exhaustion. T cells are major effectors of adaptive immunity and play a critical role in shaping the therapeutic outcome to ICB, but are prone to exhaustion under conditions of persistent antigen exposure as occurs in cancer. T cell exhaustion is characterized by reduced proliferative capacity, diminished effector function, and the expression of multiple inhibitory cell surface receptors. T cell exhaustion is driven by DNA methylation programs that limit T cell differentiation, expansion, reduce clonal diversity, and reduce tumor reactivity during ICB therapy [204–206]. One study found that promoter methylation and subsequent silencing of Runt-related transcription factor 3 (RUNX3) reduces differentiation of both effector and memory T cells, driving resistance to ICB across various tumor types [207]. Chromatin modulation also plays a significant role in promoting T cell exhaustion. The histone demethylase, LSD1, modulates the progenitor subset of exhausted CD8 + T cells capable of renewal and intra-tumoral expansion. LSD1 was found to bind to the transcription factor, TCF1, and repress the expression of genes that maintain the progenitor phenotype, by catalyzing the removal of the methyl groups from H3 K4 me1/2. LSD1 inhibition expands the pool of progenitor T cells, leading to a more sustained and durable response to anti-PD-1 therapy [208]. In addition to promoting T cell proliferation, LSD1 inhibition has been shown to activate ERVs and MHC class I expression and enhance tumor immunogenicity. Given its high expression across multiple cancer types and its association with poor prognosis, LSD1 represents a promising biomarker and therapeutic target [209–211].

Similar to LSD1 inhibition, inhibition of the arginine methyltransferase CARM1 enhances the response to immunotherapy by targeting both T cells and tumor cells [212]. Inhibition of CARM1 augmented the response to immunotherapy of resistant murine melanoma, prostate and colon adenocarcinoma tumors by enhancing T-cell functionality and maintaining memory-like T-cell populations within the tumors and increased numbers of not only CD8 + T cells but also dendritic cells and natural killer cells [212]. The roles of LSD1 and CARM1 in modulating both T cell function and tumor-intrinsic processes, highlight their potential as therapeutic targets to enhance the efficacy of immunotherapy. Similarly, SWI/SNF, and BRD4 also play crucial roles in regulating T cell proliferation as well as intrinsic tumor processes, making them potential therapeutic targets for enhancing the efficacy of immunotherapy [213–215].

Therapeutic opportunities

Altered metabolism in cancer produces new combinations of metabolites to generate the necessary components for cell survival and growth [216]. The intimate relationship between epigenetics and metabolism has been extensively studied and in particular the regulation of key enzymes such as TET by components of the TCA cycle or the post translational modification of histones [217]. The metabolic remodeling of epigenetics and cell cycle control in cancer therefore represent potential targets to counteract cellular adaptation and thus survival upon treatments [218].

Since many epigenetic regulators are druggable, they represent promising therapeutic targets for overcoming resistance to cancer therapies. Various epigenetic-modulating agents, including DNA hypomethylating agents (azacytidine and decitabine) and vitamin C (enhances

TET activity), have shown potential in reprogramming tumors and the tumor microenvironment to improve treatment responses. Additionally, drugs that target histone-modifying enzymes—such as HDACs [219, 220], EZH2 [221], G9a [222], LSD1 [223], and reader domains—such as BRD4 [224, 225], as well as chromatin remodeling enzymes, SWI/SNF [135] have therapeutic potential as either single agents or in combination with other drugs to enhance efficacy. As discussed previously, numerous studies have implicated these epigenetic regulators in mediating resistance to a wide range of cancer treatments, including chemotherapies, hormonal therapies, targeted therapies, and immunotherapy. By modulating these pathways, epigenetic-targeted therapies may help sensitize tumors to existing treatments, enhance immune responses, and improve long-term patient outcomes.

Epigenetic signatures show significant potential as biomarkers for predicting and monitoring treatment outcomes. As we discussed, SWI/SNF mutational status in plasma cell free DNA (cfDNA) may serve as a prognostic biomarker for VEM sensitivity in MCL patients [155]. 5 hmC in cfDNA from patients with non-small cell lung cancer (NSCLC) may be predictive for response to PD-1 therapy. In responders, 5- hmC was found to be enriched at loci linked to the IF-γ response and inflammation whereas in non-responders, it was associated with EMT. Detectable 5- hmC changes in these regions emerge even before clinical responses, suggesting their potential for early use in treatment monitoring. Furthermore, 5- hmC profiling of cfDNA has revealed novel genes and signaling pathways linked to treatment response in lung cancer, highlighting its potential as a valuable tool for guiding cancer treatment [175, 176].

Beyond pharmacological approaches, environmental factors such as diet, exercise, and aging have been demonstrated to influence the epigenome, playing crucial roles in cancer development and progression [226–229]. Understanding how these variables impact the epigenome in patients undergoing cancer treatment will enable the development of personalized, holistic lifestyle recommendations that enhance treatment effectiveness and improve patient outcomes.

Epigenetic regulation of clonal fitness

In a model of selection for function evolution, epigenetic regulation is a major determinant of clonal fitness, shaping cell fate decisions, survival, and competitive advantage within a population. Recent evidence suggest that disruption of the epigenetic regulatory network increases the tolerance of cancer cells to unfavorable environments [230]. Such adaptive reversible mechanisms that reprogram gene transcription and allow the acquisition of different transcriptional states are the landmark of persistent drug tolerant cells [83]. In this scenario, subclonal mutations in epigenetic regulators are common in cancers especially in the advanced stages and in recurrent disease and their expansion might well explain the fitness and adaption of specific subclones under the pressure of chemotherapy [230, 231]. Following the selection for function evolution model we can state that epigenetic reprogramming due to mutations in epigenetic regulators or to the altered DNA methylation/hydroxymethylation and chromatin modifications described above, is necessary to select the most fitting clones able to *death-ision* to survive in the hostile microenvironment. This is what happens in ovarian cancer cells under the pressure of cisplatinum in which, probable preexisting subclones carrying the potential to resist chemotherapy, expand and become the predominant platinum resistant population [78]. One outcome of this epigenetic reprogramming in ovarian cancer cells, is the upregulation of ITGA6 expression, which allows cancer cells to better adhere to the mesothelium improving their metastatic ability and preventing their death by anoikis [78]. Of course, the genetic background of the cell of origin is essential for the definition of the most fitted epigenetic reprogramming under stress. For instance, in TNBC, activation of the cGas-Sting pathway, which favors immune tumor infiltration, is essential for the response to PARP inhibitors especially in the presence of BRCA1 mutation [232]. These findings have clinical potential with the design of clinical trials testing the efficacy of PARP inhibitors in combination with anti-PD-L1 immunotherapy in TNBC. Initial results from this trial demonstrates that this combination therapy is tolerable and effective predominantly in patients with BRCA-mutated tumors [233]. Therefore, based on the evidence that epigenetic deregulation is essential for selection-for-function evolution, it will be expected that targeting epigenetic regulators will represent a future highway in cancer research to prevent the onset of resistant disease and/or treat drug-resistant tumors.

Apoptosis, necroptosis, ferroptosis, pyroptosis, entosis, autophagy, cellular senescence, and mitotic catastrophe in cancer: all roads lead to roam for cancer cells

For most of the twentieth century (and most likely before), cancer has been classically viewed as a disordered proliferation of abnormal cells. However, cancer can be more accurately defined as the disruption of cellular homeostasis, i.e. the balance between cell proliferation and cell death. In the early 1990's several studies have established that cell death was a major hallmark of cancer and that the combination of proliferation and decreased cell death was associated with aggressiveness in most

cancers [234]. The introduction of the limited or no cell death occurrence in cancer was a revolutionary concept as this explains why most treatments inducing cell death would necessarily be inefficient. The following years were rich in discoveries of the importance of cell death in cancer resistance and evolution upon treatments, but practical uses of treatments specifically designed to modulate cell death in various diseases are still scarce [235]. The link between cell death and resistance to treatment in cancer is well established and has been extensively reviewed in seminal reviews [236–239].

Pathways to death are many, interconnected and mingled with other basic cellular functions

Apoptosis is a major cell death program, which prevails under normal physiological situations such as embryogenesis and normal cellular life [239]. Although at the tissues level apoptosis is a sporadic event, it remains massive at the organism level, as a huge number of cells are eliminated every day by this process [239]. Two major pathways lead to the initiation of apoptosis: an extrinsic pathway, activated at the plasma membrane by the positive or negative interactions between ligand and receptors (i.e. TNF/TNFR; FasL/Fas…) and the intrinsic pathway, which involves numerous pathways [239]. The core mechanisms of apoptosis rely on the combination of a vast array of pro and anti-apoptotic factors. The perfect example of this complex relationship is the BCL-2 family; a group of proteins, with at least one homology domain in common (e.g. BH3 domain), which interact together upon induction of cell death with the main function to promote (or inhibit) the mitochondrial release of deadly factors [240]. The nature and the duration of this interaction is instrumental for the life and death decision [241]. These proteins are pre-existing in living cells, for the most part, and are implicated in basic mechanisms such as elimination of unnecessary or excessive cells during development and/or differentiation. Apoptosis is a process that is essential not only for "normal" life but also in preventing numerous physio-pathological conditions being linked to various diseases where cell death is either excessive (neurodegenerative diseases for example) or insufficient (cancer for example). Another landmark linking cell death to cancer was the discovery that p53 (one of the most dysregulated/mutated gene in cancers) is a key player in apoptosis versus growth arrest decisions following limited external or internal injuries. P53 can induce both cell-cycle arrest through the transcription of other proteins (i.e. p21) and apoptosis through pro-apoptotic BH3-only proteins (i.e. Noxa, PUMA). The p53 function leads to either cell death or to the return to normal cell life through a complex quality control process. However, it is still unclear if this feature is indispensable for the anti-cancer role of p53 [242, 243].

Several different mechanisms of cell death, other than apoptosis, have been found and it is now certainly more accurate to discriminate "regulated" cell death programs (RCD) (apoptosis being only one of them) from "accidental" cell death mechanisms (ACD) (necrosis being the main perpetrator) [244]. Nonetheless, since many anticancer treatments trigger some kind of cell death, its inhibition or dysregulation remains a valid and major target in therapies. The mechanisms that link the different cell death programs to resistance are not, nonetheless, completely understood. In particular, more investigations are needed on the time, concentration and spatial dependency of the cell death response to any given stimulus.

Cell death implication in every step of tumorigenesis from oncogenesis, development, and resistance to treatments

Cell death programs are involved at different stages of cancer, from initiation to the development of resistance to clinical treatments [245]. Immuno-surveillance is one of the earliest strategies used by our body to eliminate malignant (or pre-malignant) cells [245]. Malignant transformation of normal cells depends on the accumulation of DNA damage and hence mutations that can generate new sets of antigens [246]. These neo antigens trigger T cell-based immune responses exploitable in cancer immunotherapies through both T cell checkpoint blockade and adoptive T cell therapy [247]. Elimination of pre-cancer cells by the immune system depend on the induction of cell death in target cells [245]. Thus, at the time of diagnosis, most cancer cells must have successfully evaded the patient immune system. Quite interestingly, a recent report has suggested that sepsis-induced immune reaction prevented, by "ricochet", de novo cancers by the induction of antitumoral tissue-resident T cells [248]. In contrast, immune-suppression after organ transplantation leads to an increased frequency of malignant disease [249]. This suggests that the immune system represents a major protective barrier against cancer and that its modulation in healthy individuals may be an important preventive strategy. However, there are numerous tactics used by pre-cancerous cells and cancer cells to either repel immune attacks and/or to prevent it, resistance to cell death is one of them [250]. The implication of apoptosis in immune-induced cell death has been among the first indications of the importance of this cell death program. In contrast, immunogenic cell death (ICD), a form of RCD which results in the release of damage-associated molecular patterns (DAMPs), promotes dendritic cell maturation and tumor antigen presentation and thus triggers a T-cell-mediated anti-tumor immune

response [251]. Resistance to cell death provides two major advantages for cancer cells: survival and prevention of further assaults by the immune system. However, some treatments are potent inducers of ICD and as such could be associated with classical anti-checkpoint inhibitors in a more efficient anti-cancer immunotherapy [252].

p53 is the prototypical tumor suppressor as it provides quality controls which are major safeguards against expansion of malignant transformation [243]. Most normal cells are eliminated through p53 dependent mechanisms but failure in this process promotes oncogenesis. Cancer cells exhibit frequent alterations of p53, through dysregulated regulations or mutations, of p53 itself or to its upstream or downstream partners [243]. In addition, it has been widely shown that dysfunctional p53 not only participates in oncogenesis but also causes resistance in cancer cells to certain chemo or radiotherapies, modifies metabolism, promotes migration as well as metastasis and drives immune resistance [253]. One important limitation of the anti-oncogenic activity of p53 is due to the inhibition of death-related pathways. Indeed, the extent of mitochondrial apoptotic priming is linked to the anti-tumor efficiency of p53 activity [254]. Like p53, many oncogenes in cancer cells, such as MYC or RAS, are associated with a global reprogramming of gene expression which in turn promotes metabolism linked pathways, cell growth, and proliferation and the inhibition of cell death [255, 256]. Numerous efforts on the "re-activation" of dysregulated p53 have been made but therapeutic success has not yet been achieved [257].

Targeting of the core program of apoptosis has been achieved by mimetics of BCL-2 family BH3 only domain proteins that can inhibit the activity of major anti-apoptotic proteins such as Bcl-2, Bcl-xl and Mcl-1 [258]. BH3-mimetic drugs against Bcl-2 (e.g. ABT199/Venetoclax), Bcl-2/Bcl-xL/Bcl-W (e.g. ABT263/Navitoclax) and Mcl-1 (e.g. AZD-5991) have proven to be efficient in some hematological malignancies but are associated with adverse side effects in normal tissues in treated patients and produce resistant cells [259]. The latter is often linked to a shift in anti-apoptotic thespians (i.e. from Bcl-2 to Mcl-1 dependence) [260]. A combination of drugs that induce apoptosis with BH3 mimetics has been shown to benefit patients that cannot be treated by either treatment alone [261, 262]. Thus, targeting apoptosis is still highly relevant in the treatment of cancer and especially, in resistant cells. Combined treatments that, on one hand, induce cell death and, on the other, puts the brake on anti-apoptotic mechanisms could clearly produce more efficient therapies. As a note of caution, it must be noted that resistance could be triggered much faster when one main target (apoptosis) is involved rather than two independent treatments [263]. Another caveat is that the induction of massive apoptosis in tumors leads to the production and release of factors from dead cells that enable an immunosuppressive environment and/or protection to neighboring cells [264]. These latter processes are likely to facilitate the selection/genesis on site of resistant cancer cells. Of course, this means that synergistic combinations of treatments would have to be adjusted to each type of cancer and/or to its malignant stage. This would be the purpose of studying and defining *death-ision*.

The relationships between cell death programs and the resistance to anti-cancer treatments: competition, complementation, reciprocation and substitution

Recent works have shown the importance of various forms of RCD, which co-exist with apoptosis in both normal and cancer cells (i.e. necroptosis, ferroptosis, pyroptosis, entosis, cell death associated with autophagy, cellular senescence or mitotic catastrophe…). These different forms of death implicate different inducers of cellular stress which lead to the elimination of cells under specific conditions. For example, pyroptosis is linked to inflammation, ferroptosis or cuproptosis to ion dysregulated homeostasis and anoikis or entosis to cell matrix-detachment [235, 239].

Ferroptosis is induced when cellular glutathione peroxidase 4 (GPX4) cannot prevent pathologic accumulation of toxic lipid peroxides [265]. However, the evidence for the implication of ferroptosis in diseases such as cancer come mostly from in vitro studies.

Entosis is a process during which a living cell enters another living cell [235]. Factors controlling entosis are not well characterized but recent results suggest that entosis requires TRAIL receptors DR4 and DR5, known inducers of apoptosis. However, induction of apoptosis and entosis diverges at caspase-8 which is sufficient for the induction of entosis while additional steps are required for the induction of apoptosis. In addition, apoptosis and entosis are morphologically and biochemically distinct but nonetheless the knockout of Bax and Bak, 2 two major pro-apoptotic proteins of the BCL-2 family, or inhibition of caspases, inhibit entotic cell death [266].

PANoptosis, an innate immune cell death/inflammatory process, presents key features of pyroptosis, apoptosis and/or necroptosis [267]. PANoptosis appears to use different elements of the 3 RCDs (i.e. caspase-1, −8 and RIPK-1 −3) along with more specific components [268]. Of note, caspase-8 can be both a pro-death and pro-survival protein as it mediates apoptosis induced by death receptors such as TNFR, TRAIL and Fas, and suppresses necroptosis mediated by the kinase RIPK3 and the pseudokinase MLKL2-4 [269]. Massive induction of

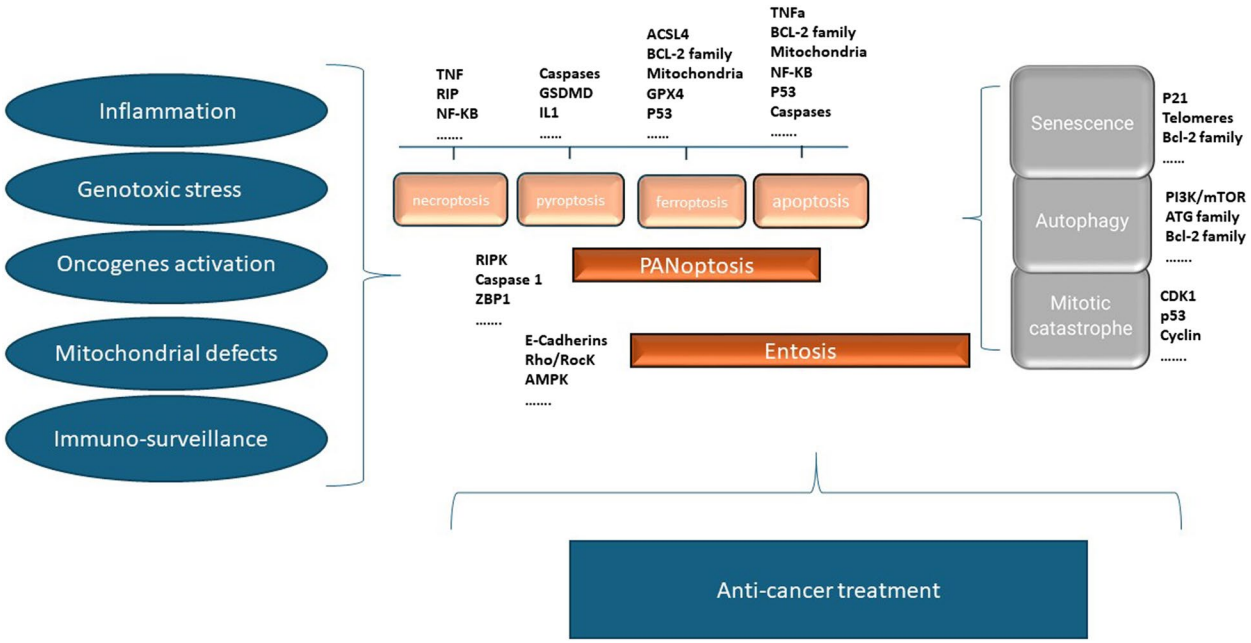

Fig. 4 Relation between RCD and senescence, autophagy and mitotic catastrophe. Cancer cells are subjected to different conditions that can trigger cell death from inflammation to environmental stress. Anticancer treatments are also potent death inducers of RCD. Within the same cell, several cell death programs co-exist and are triggered by specific signals such as DNA damage for apoptosis; inflammation for pyroptosis, dysregulated lipids oxidations for ferroptosis; TNF stimulation for necroptosis. Some programs such as entosis or PANoptosis exhibit features common with different of RCD. Alternative RCD can relay the main cell death program if it is deficient. It is not known if the activation of RCD replacement is due to specific mechanisms or co-induction at the time of the primary stimulus. More likely, since some components are common between several RCD (major genes implicated in the different pathways) and a crossover between RCD might be common in most cases

apoptosis can lead to secondary necrosis with mixed features between RCD and ACD [235].

Thus, different RCD programs can combine, substitute, or complement under specific conditions, which still have to be determined. In addition, when overwhelmed RCD can lead to ACD–like phenotypes. Interesting some actors are found at the cross roads between different RCD and more studies are needed to understand the precise regulation leading to a specific RCD (Fig. 4).

Cell death also occurs "by defect" because of flawed survival pathways. Autophagy is an evolutionarily conserved process of cell adaptation to metabolic and environmental stress and, as such, plays an essential role in normal development and physiology [235]. As such, it is a prime survival mechanism and acts through selective degradation of cellular materials and their subsequent recycling to preserve basic cellular functions. A defect in the autophagy machinery can lead to premature diseases (cancer being one of them), inflammation, fast ageing and cellular senescence [270]. However, if basic autophagy facilitates the elimination of defective or excessive materials within the cell (mitophagy being a good example), extreme autophagy leads to cell death. Moreover, autophagy modulation might represent a mechanism of survival for cancer cells to overcome the pressure of chemotherapy and its timely targeting might represent a tumor vulnerability [271]. Autophagic cell death has been associated with the activation of apoptosis and ferroptosis, through there are some components similar to those found in apoptosis (BH3 only domain proteins such as Beclin) and ferroptosis (GPX4 for example) [272]. Thus, limited autophagy is necessary to maintain baseline cellular physiology, which facilitates survival under stress conditions, while extreme autophagy leads to cell death. The latter being executed in concordance with RCD in the majority of cases (Fig. 3). The precise impact on cancer, by the acquisition of drug resistance due to defective necroptosis, pyroptosis or ferroptosis, as well as that of any other form of RCD, other than apoptosis, has not yet been completely established. However, it is tempting to assume that multiple RCD programs will interfere with cancer cell interactions with the environment. In particular, the inflammatory pathways as well as ions/metabolism regulation will be altered under these conditions leading to improved survival and time to functionally adapt to drugs through modulation of autophagy and immune response [273, 274].

Cellular senescence is a response of cells to internal or environmental stress leading to a permanent state of cell cycle arrest and pro-inflammatory secretory phenotype

(senescence-associated secretory phenotypes, SASP). Cellular senescence is a multistep process which leads to cell cycle arrest in cells at risk of or undergoing neoplastic transformation. However, many oncogenes lead to senescence (Oncogene induced senescence = OIS) and thus slow tumor growth in vitro and in vivo. Quite interestingly, p53 is associated, at least during RAS-induced senescence, with the stable proliferative arrest of cancer cells [275]. Senescence leads to the avoidance of cell death and SASP is implicated in tissue remodeling, injury, cancer, and aging. Among the therapeutic agents which target senescent cells, several of them have anti-cancer activities [276]. However, it has been shown that elimination of senescent cells such as those expressing high levels of $p16^{INK4\ A}$, could be detrimental to health [277]. Thus, like the targeting of autophagy in cancer, cautions should be considered in long-term senotherapy, especially the possible side-effects on normal cells or possible induction of secondary and/or resistant cancer that might result from inflammatory conditions induced by SASP [278]. Quite remarkably among senolytics, BCL-2 anti-apoptotic proteins has been shown to participate in the cell cycle arrest of senescent cells through limited mitochondrial-like permeabilization [279]. Thus, key players in apoptosis also act in other types of survival/death programs, although the relationships between senescent cells and RCDs have not been clearly established.

Mitotic catastrophe is a defensive barrier against aberrant mitosis, which leads to the formation of large cellular bodies that contain multiple nuclei (polyploidy) that are morphologically distinct from normal or apoptotic cells. A variety of pathways for cell elimination such as apoptosis, autophagy, senescence-associated death and necroptosis are the eventual consequence of mitotic catastrophe induced by certain classes or doses of anti-cancer drugs as well as by radiations [280]. On the other hand, it has been shown that entosis can occur in adherent cells as a result of aberrant mitotic events such as mitotic catastrophe [281].

In addition to its role in cancer resistance to treatment, RCD can be severely influenced thereby modifying the tumor microenvironment [282, 283]. Factors released from dying cells (even if death is incomplete) could trigger modifications in neighbouring cells. This includes not only proteins but also lipids, metabolites, radical oxygen species (ROS), miRNAs and ions [284]. The latter point probably constitutes a future axis of research.

Cell death and cell competition

Survival processes might produce a heterogeneous dying cell population with a large spectrum of viability aptitude (Fig. 5). Since survival of the unfit does not usually give a great selective advantage, it is possible that additional steps of selection among survivors favor cancer cells that are both capable of sustaining external and internal pressures and proliferating/invading their environment (Fig. 5). Indeed, massive cell death that can be observed during primary or secondary treatments can be linked to the incapacity of "unfit resistant cancer cells" to facing new death/life challenges.

Cell competition (another insufficiently explored phenomenon in cancer) is a major component of resistance, metastasis, and relapse in cancer cells [285]. Unfit cells usually exhibit altered levels of protein translation/turn over which in turn influence autophagy. The latter process can be used to eliminate unfit cells [217]. In addition, cell competition is not only a cell-intrinsic property but is also determined by intercellular contacts with other cancer or normal cells present in the tumor ecosystem [286]. In addition, dead or dying cells can provide signals and materials in sufficient quantities for cells to survive or thrive, although they could not do it on their own (Fig. 5). Other resistance strategies such as collectivism during which, rather than a single resistant clones, a group of cells with possible hierarchies (dominant clone vs. minor clones and/or subversion of normal cells from the environment) could survive using mutualism, amensalim (which can lead to immune suppression), commensalism, and/or parasitism processes similar to those observed in microorganism biofilms [287]. Cell competition allows cells with the best fitness phenotype to survive under specific conditions. This process appears to be implicated during all stages of cancer progression from oncogenesis to metastasis [286]. At the earlier stages, competition occurs between wild type (WT) cells and mutated (MUT) "normal" cells Depending on the mutations, WT cells or MUT cells are eliminated. This clonal competition protects against tumorigenesis in a majority of cases. However, since the "winner takes all", it does enhance the development of cancer if MUT cells prevail [286]. The mechanisms underlying cell competition are not known in precancerous states but co-existence between WT and MUT cells, might mean there are signaling imbalances between the different cell populations and thus differential growth rates. It has been shown that these mechanisms may also involve innate immune proteins, p53 and changes in protein turnover and/or protein homeostasis, all key features in cellular fitness [288]. Cell death, in combination with competition, is the key to the removal of aged or abnormal cells during physiological tissue homeostasis. Mutations that confer cell death resistance can give an obvious advantage for surviving the competition, but it is not clear how it could affect cellular fitness. As depicted in Fig. 4, resistance to cell death (and in particular apoptosis) depending on the nature of the death

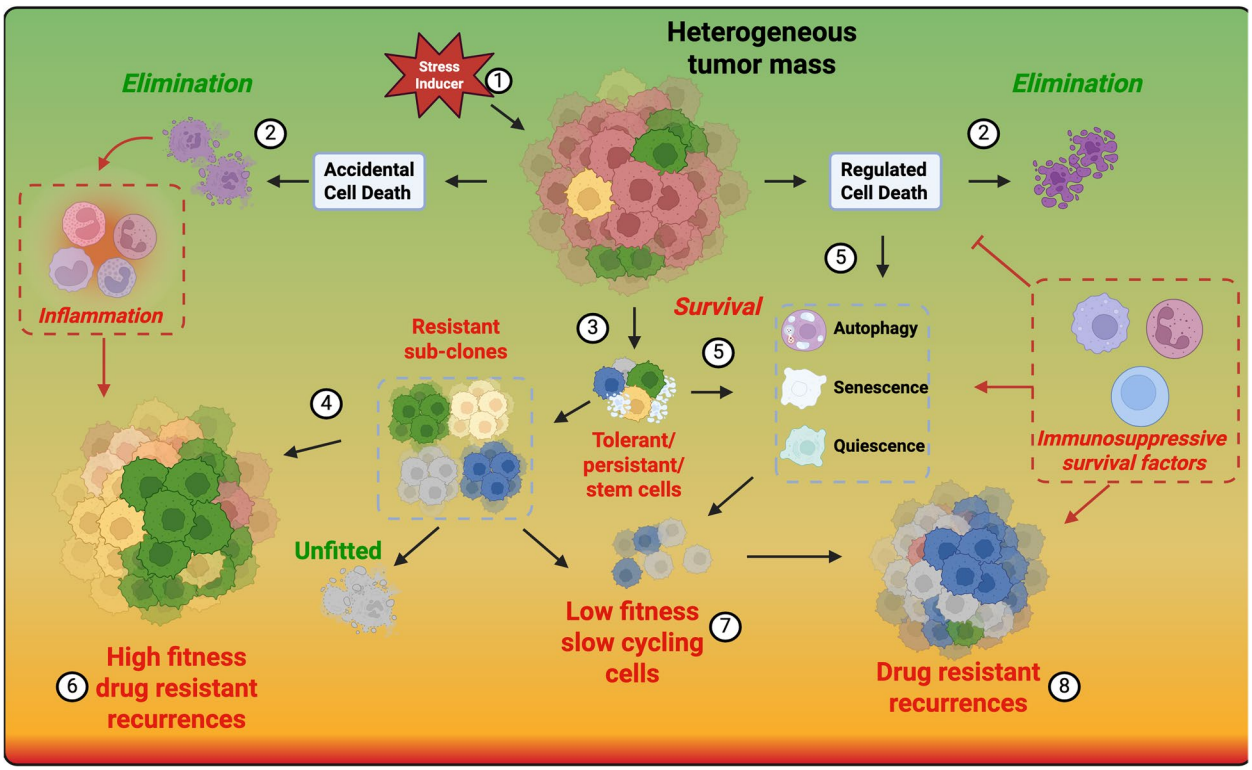

Fig. 5 Cell Death-ision. Graphical representation of the Death-ision process in which from a heterogenous drug-sensitive tumor mass (green background in the gradient color scale) drug resistant clones could emerge (red background in the gradient color scale). Cell stress is induced by external stimuli (such as treatments) in the different cancer cells populations (clones, cancer stem cells and drug tolerant/persistent) (stage 1). Depending on the nature and/or duration of the signal, stress can lead to the elimination of cancer cells through ACD, specific or combinations of RCD (stage 2). Non-responding cells can be cancer stem cells, pre-existing resistant clones or drug tolerant/persistent cells (stage 3). Cancer can survive as slow cycling cancer cells (stage 4) or acquire resistant mechanisms (stage 5). Resistant clones can either proliferate (high fitness resistant cells (stage 6) or, for some subpopulations (low fitness resistant cells) die or become slow cycling dormant cells (stage 7). Depending on external signal, dormant cells can re-enter a proliferation stage to become aggressive metastatic or recurrent cancers (stage 8). The dying or survival of cancer cells can be affected by the microenvironment which can facilitate tumor resistance and growth through various stages. The purpose of *death-ision* is to determine when and how cells survive in order to provide stage specific treatments

inducers will generate cells with distinct fitness features and growing abilities. Nonetheless, cell competition can also be countered by cooperation where unfit cells will provide components/factors that will benefit to a subset of faster growing cells. Thus, cell competition is likely to use cell death resistance in a combination of cell autonomous/non-autonomous processes.

General conclusions

Here, starting from different and complementary points of view, we have provided a provocative, although likely incomplete, summary of the emerging facts toward the understanding of how tumors evolve and resist cancer therapies. Eradication of cancer cells remains the ultimate goal for cancer biologists and clinicians. This means that we have to continue our efforts to gain understanding of the cause, the mechanisms, and the consequences of cell death under the many different conditions and states cancer cells and tumors are experiencing over time.

Most of the vulnerabilities and strengths detected during cancer progression (evolution) may become the best and the worst players during treatments. Mutations that support the initial emergence of cancers will also fuel the disorganization and intrinsic disorder characteristic of cancer cells. For instance the notion that tumor suppressor gene loss is accompanied by transcriptional reprogramming of synthetic lethal genes might have immediate clinical applications in selecting the best combination therapy for the most appropriate group of patients (e.g. PARPi for BRCA1/2 mutated patients [47]). Similarly, the clinical use of combination therapies that include immunotherapy approaches has to be tested in clinical trials that takes into account the genetic background of the cell of origin and the associated epigenetic reprogramming that may or may not favor tumor-immune infiltration and thus the efficacy of immunotherapy. In this sense, biomarker-driven studies are essential for determining whether the preclinical

Table 1 Death-ision: How Regulated Cell Death (RCD) shapes the response to therapies and drives the appearance of drug resistance: visualization and quantification

In this review we have introduced the concept of "Death-ision" as the result of multiple cell autonomous/non autonomous processes that through RCD mechanisms shape the response to drugs and eventually define if cancer cells survive or not to the pressure of treatments. We principally focused on the role of epigenetic regulation in drug response but also highlighted how genetic and epigenetic modifications contribute to the clonal evolution of targeted and conventional therapies. The major points emerging from our critical evaluation of the most recent literature suggest that under the pressure of anticancer therapies tumors use a "selection for function" model of evolution based on both genetic alterations typical of the cell of origin of each tumor type and epigenetic modifications that contribute to the phenotypic selection. The final balance of pro-survival and RCD mechanisms, defines the death-ision of cancer cells to survive and eventually overcome the pressure of therapies through the dynamic acquisition of multiple cellular states
Visualization and quantification of RCD and ACD can be achieved by using
1. annexin V label, caspase activity and cell morphology to make the distinction between some RCD and ACD
2 evaluating the level of some key RCD proteins (see Fig. 3) to assess the ratio between the different RCD and ACD. For example, cleavage of caspases and gasdermin D, using specific antibodies, to determine the apoptosis/pyroptosis ratio or antibodies raised against key components of ferroptosis to determine its ratio against apoptosis (cleaved caspase 3 or modification of the expression of members of the Bcl-2 family). Several assay kits are commercially available
3. expression of components such as EZH2 and DNMTs since apoptosis is controlled, at least partially, by epigenetics through the regulation of expression of some of its key elements [290, 291]
4. "BH3-profiling assay" to determine by either activator or sensitizer BH3 mimetics, the level of apoptotic priming (threshold to cell death) or prosurvival dependency of BCL-2 family proteins [292, 293]
5. small molecules which targets the different RCD could be exploited to improve knowledge on Death-ision and thus study its impact on cancer treatment [294] using in vitro (organoids) or in vivo (animal models) experiments
6. Single cell analyses (at transcriptomic, epigenetic and proteomic levels) before and after treatment to determine heterogeneity and plasticity of the cell death response to treatments which is the essence of "death-ision"

observations translate into clinical outcomes. Transcriptional adaptation mechanisms that drive resistance due to deleterious environmental conditions such as the pressure of chemotherapy, could be treated by drugs targeting transcriptional regulators (e.g. transcriptional Cyclin Dependent Kinases, CDK reviewed in [289]) used alone or in combination with targeted or chemo-therapies. Microenvironment modifications impairing anti-tumor immune response due to YAP1-driven resistance could be overcome by the combination of epigenetic modulators (e.g. DNMT inhibitors) combined with immune checkpoint inhibitors, as demonstrated by studies linking YAP1 expression with immune-resistance and tumor immune infiltration [168, 170]. However, cell death processes are more numerous and plastic than previously alleged and more studies are necessary to achieve practical use of "*death-ision*" therapies.

Future research aimed at understanding and overcoming resistance to therapy should consider that cancers adapt to the changing environments through functional optimization. We propose that this adaptation is based on the ability of cancer cells to make the appropriate "*death-isions*". In this context, elucidating the *death-ision* processes that allow the cell of origin, through the accumulation of genetic and epigenetic modifications, to acquire different plastic states and ultimately avoid drug-induced death will lead to the identification of specific vulnerabilities that can be exploited in the clinic. Hopefully, elucidation of *death-ision* mechanisms will allow us to move from ineffective treatments of resistant tumors to therapeutic approaches aimed at preventing the emergence of drug-resistant clones, which would be able to eradicate the diseases. Tools to visualize and quantify cell death have become widely available in recent years and it is possible to evaluate the extent of "death-ision" (see Table 1). However, several important questions remain to be answered to put this concept on a more practical basis for integration in a precision medicine perspective (Table 2).

Of course, the "*death-ision*" model has some limitations that might impact on the possibility to explore it as tumor vulnerability in clinical settings. First, unwanted toxic side effects are largely expected when targeting cell death mechanisms has to be taken into considered. The decision to live or die is shared by normal and transformed cells and thus, an indiscriminate targeting of cell death is likely not feasible. These are windows of opportunities for novel research aiming at identify specific cancer biomarkers and vulnerabilities linked to the available therapies and genetic/epigenetic context. For instance, the use of methyl-transferases inhibitors could be explored to overcome resistance to immunotherapies in EZH2 mutated follicular lymphomas while the inhibition of transcription regulators could prevent the appearance of chemo-resistant clones in selected solid tumors. In this regard, it is important to highlight that the precise and timely use of cell death inducers is of the outmost importance. From the treatment of ovarian cancer patients we have learned that exploiting the synthetic lethality between BRCA1/2 mutations and PARPi has

Table 2 Death-ision: How Regulated Cell Death (RCD) shapes the response to therapies and drives the appearance of drug resistance: pending questions

In the context of "Death-ision" we have identified some main questions raised by this concept that need to be answered in future researches:
1. What is the influence of duration and intensity of treatments on the choice of cell death(s) induced by therapies and how are pharmaco-kinetics and its metrics implicated? It would be relevant to define whether the timing and modality of drug administration (e.g. adaptive versus curative treatments) could influence the cellular death-ision and eventually contribute to the appearance of drug-resistant cell clones
2. Is the cell death response univocal and, if not, how are alternative RCDs triggered? The use of single cell and spatial analyses would likely contribute to our understanding of how heterogeneous this response may be in time and space, and if spatial distribution of cell death within the tumor mass has or not an effect on the efficacy of the treatments
3. What is the contribution of death-ision in the acquisition of clonal resistance and its implication in the recurrence of cancer shaping the extension of minimal residual disease? In this regard, it will be important to define if and how the decision to survive or die contributes to the acquisition and maintenance of different cellular states linked to clonal evolution under the pressure of anticancer therapies. These cellular states include stem-like phenotypes, drug tolerant and persistent states, dormancy or senescence characteristics
4. As a corollary of the previous point it would be relevant to establish how spatially occurring cell death (as well as its failure) influences cancer cell evolution and contributes to shaping the local tumor microenvironment, rendering it more (or less) permissive to cell survival by acting on inflammation, ECM composition, EMT and cell plasticity induction, and cellular states definition
5. Finally, could "death-ision" define common actionable mechanisms exploitable to treat cancers or are the combinations of activators of RCDs necessary to eradicate the disease? In this case, how to manage the likely side effects?

clinical benefits only when the administration of PARPi follows chemotherapy. Concomitant use of the two treatments did not improve patients' survival but significantly enhanced their toxicities. Future research should intensely investigate how *death-ision* modulators interact with other treatments not only in term of pharmacodynamic and pharmacokinetic interaction but also in time. This is particularly true in immunotherapies studies in which induction of cell death could on one side improve immunotherapy efficacy but could also hamper immune cells viability. In this regard, more researches are necessary to understand how long a patient could and should be treated with *death-ision* modulators, especially in the case of their use as maintenance therapies.

Abbreviations

5- hmC	5-Hydroxymethylcytosine
αKG	Alpha-ketoglutarate
ACD	Accidental cell death mechanisms
BH3	Bcl-2 homology domain 3
BRCA1/2	BReast CAncer gene
CAF	Cancer Associated Fibroblast
cfDNA	Plasma cell free DNA
cGAS	Cyclic GMP-AMP synthase
CNA	Copy Number Alterations
CSC	Cancer stem cell
CSG	Co-stimulatory gene
DNMT	DNA methyltransferase
dsRNA	Double-stranded RNA
ECM	ExtraCellular Matrix
EDAC	Epithelial Defense Against Cancer
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen receptor
ERV	Endogenous retroviruses
GBM	Glioblastoma multiforme
H3 K27 me3	Histone H3 K27 trimethylation
H3 K9 me3	Histone H3 K9 trimethylation
HDAC	Histone deacetylase
HDM	Histone demethylase
HER2	Human Epidermal Growth Factor Receptor 2
HMT	Histone methyl transferase
ICB	Immune checkpoint blockade
IFN	Interferon
IL-1 β	Interleukin-1β
ITGA6	Integrin α6
JNK	Jun N-terminal kinase
LINE	Long interspersed nuclear element
MAN1 A1	Mannosidase Alpha Class 1A Member 1
MAPK	Mitogen-activated protein kinase
MBP	Methylbinding Protein
MGMT	O6-methylguanine-DNA methyltransferase
NAC	Neo-Adjuvant Chemotherapy
ncRNA	Noncoding RNA
NGS	Next Generation Sequencing
PDAC	Pancreatic Ductal Adeno-Carcinoma
PgR	Progesterone Receptor
PHD	Plant homeodomain
PRC2	Polycomb repressive complex 2
RCD	Regulated cell death
RUNX3	Runt-related transcription factor 3
scRNA-seq	Single cell RNA sequencing
SINE	Short interspersed nuclear element
SMA	S-adenosylmethionine
STING	Stimulator of interferon genes
TEM	Temozolamide
TERT	Telomerase reverse transcriptase
TET	Ten-eleven translocation
TMZ	Temozolomide
TNBC	Triple Negative Breast Cancer
VEM	Vemurafenib
VEN	Venetoclax
WGS	Whole-Genome Sequencing

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Authors' contributions
GB, IDLS and FMV collected the related literature, drafted the manuscript discussed and wrote the manuscript reviewed and edited the manuscript and designed the figures. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

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