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Leveraging and coping with uncertainty in the response of individual cells to therapy

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Non-genetic heterogeneity fluctuates over diverse timescales, ranging from hours to months. In specific cases, such variability can profoundly impact the response of cell populations to therapy, in both antibiotic treatments in bacteria and chemotherapy in cancer. It is thus critical to understand the way phenotypes fluctuate in cell populations and the molecular sources of phenotypic diversity. Technical and analytical breakthroughs in the study of single cells have leveraged cellular heterogeneity to gain phenomenological and mechanistic insights of the phenotypic transitions that occur within isogenic cell populations over time. Such an understanding moves forward our ability to design therapeutic strategies with the explicit goal of preventing and controlling the selective expansion and stabilization of drug-tolerant phenotypic states.

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Introduction

Surveys of molecular and phenotypic states of single cells have revealed pervasive heterogeneity, both between isogenic cells in a population and within the same cell over time [1•]. One potential source of such cell-to-cell variability is at the level of the signals themselves. For instance, individual cells exposed to the same dose of ionizing radiation will effectively receive a variable number of double-stranded DNA breaks [2]. However, even when environmental inputs remain constant, variability in responses can arise as a result of differences in initial states, such as cell cycle or differentiation stage [3]. In addition, cells that populate the same initial state, and are exposed to exactly the same signal, can exhibit different responses due to the stochastic nature of the biochemical reactions that govern the production and degradation of individual molecules [4]. Variability is therefore an intrinsic property of signaling systems that cells (and more recently researchers) have evolved to harness and cope with.

Non-genetic heterogeneity has been identified as a source of molecular and phenotypic diversity that leads to variable responses to treatment within isogenic cell populations. This plasticity has emerged as a predecessor and mediator of the evolution of genetic resistance, which ultimately leads to therapeutic failure [5,6]. Here, we summarize recent evidence of the way non-genetic phenotypic variation contributes to fractional killing in specific experimental systems, from bacteria to cancer cells. Recent technical and analytical developments provide the opportunity to quantitatively understand the dynamics of interconversion between cellular states, both from phenotypic and molecular perspectives. Such an understanding will be critical for designing strategies to optimize therapeutic outcomes that account for the presence of phenotypic heterogeneity and timescales of fluctuations in cellular states.

Cell-to-cell variability limits the success of therapy

It has long been recognized that a small subpopulation of bacterial cells called 'persisters' can survive antibiotic treatment under drug concentrations that kill the vast majority of bacteria [7]. Bacterial populations that emerge from the expansion of persister cells after antibiotic removal exhibit similar drug sensitivity to that of the original cell population, arguing that persistence represents a reversible state that is maintained at low frequencies in bacterial populations [8]. Using timelapse microscopy to follow single bacteria over time, Balaban *et al.* showed that persister cells existed as a small fraction of growth arrested cells in unperturbed bacterial populations [9]. Notably, environmental and mutational perturbations can increase the fraction of persister cells in a population by engaging the stress response pathways that are stochastically activated in exponentially growing populations due to natural variability [10,11]. This suggested that the frequency of phenotypic switching and/or the lifetime of the persister state, could be subject to environmental or evolutionary modulation [12,13].

In striking resemblance to bacterial persistence, drug tolerant phenotypic states have been shown to mediate

fractional killing in cancer cell populations in response to targeted therapies. Sharma *et al.* showed that while the vast majority of lung cancer cells harboring oncogenic EGFR died within a few days upon exposure to the EGFR inhibitor gefitinib, ~0.3% of the cells survived treatment and remained in a quiescent state. Eventually, ~20% of drug tolerant cells were able to resume proliferation and could be propagated in the presence of drug concentrations that would be lethal to the drug naïve cell population. The transition from a quiescent to proliferative drug tolerant state was attributed to global changes in histone post-translational modifications and could be blocked by co-treatment with a histone demethylase inhibitor [14••]. Interestingly, such transition brought about concomitant changes in the average lifetime of the drug tolerant phenotype: while surviving quiescent cells re-gained sensitivity within ~9 doublings upon growth in the absence of drug, it took ~90 doublings for the proliferative drug tolerant cells to restore sensitivity [14••]. Thus phenotypic heterogeneity was not only present at the level of drug sensitivity versus tolerance, but also in the relative stability of the tolerant state (Figure 1a).

Similar results have been reported in the context of $BRAF^{V600E}$ mutant melanoma cells treated with BRAF inhibitor [15••]. Shaffer *et al.* investigated the origin of rare drug resistant colonies that emerged in the presence of $BRAF^{V600E}$ inhibitor [15••]. Using long-term live cell imaging they showed that these resulted from the expansion of a small subpopulation of cells that continued cycling normally in the presence of drug. Single molecule RNA fluorescent *in situ* hybridization (FISH) revealed that heterogeneity in the expression of resistance-associated transcripts preceded drug exposure. In addition, selective expansion of these pre-resistant cells upon treatment was accompanied by a gradual epigenetic reprogramming that transformed transient transcriptional variation in drug naïve cell populations into stable resistance in the course of ~4 weeks (Figure 1a). In an independent study, Fallahi-Sichani *et al.* discovered a subpopulation of melanoma cells that cycled slowly in the presence of BRAF inhibitor. Drug tolerant cells exhibited a de-differentiated molecular profile, which could be reverted by passaging in the absence of drug and blocked by inhibition of histone modifiers [16]. The activation of proteins involved in developmental plasticity has been linked to resistance in other models [17–19], suggesting that lineage switching could be a widespread mechanism to attain resistance.

In addition to pre-existing stochastic phenotypic variation as a source of drug tolerance [14••,15••,20], cell-to-cell phenotypic variation can emerge as a direct consequence of the way individual cells respond to treatments. Paek *et al.* showed that exposure of colon cancer cells to chemotherapy led to heterogeneous activation of the pro-apoptotic tumor suppressor protein p53 and anti-apoptotic proteins. Fractional killing emerged as the result of competition between these antagonistic cellular programs: progressive accumulation of anti-apoptotic proteins gradually increased the threshold in p53 levels required to trigger cell death, limiting apoptosis to cells with early and high rate of p53 induction [21••] (Figure 1b). A similar mechanism was identified in the context of TRAIL induced apoptosis, in which the initial rate of caspase-8 activity was shown to distinguish whether a cell underwent apoptosis or survived chronic exposure to the ligand [22]. Thus, heterogeneity in signaling upon perturbations does not only contribute to variability in immediate cellular outcomes, but can also reshape the distribution of phenotypic states in the population and render cells transiently refractory to subsequent treatments.

Collectively, these and other studies have revealed a wide diversity in the timescales within which drug tolerant phenotypes fluctuate, ranging from a couple cell generations, when phenotypic variation is due to fluctuations in the levels of proteins directly involved in the response [23], to weeks or months, when phenotypic states become stabilized through engagement of self-reinforcing feedback loops or epigenomic reprogramming [14••,15••]. Short-lived states can precede the establishment of longer-lived drug tolerant states, with genetic variation ultimately conferring stable resistance [6]. It is thus critical to gain a phenomenological and mechanistic understanding of the rates at which cells enter, exit and stabilize drug tolerant states.

Leveraging uncertainty to advance mechanistic understanding of the transitions between phenotypic states

The recognition of the presence of phenotypic heterogeneity in cell populations prompted the development of experimental paradigms to unmask such variability and to understand the dynamics of diversity-generating processes. Going back to the classical fluctuation analysis developed by Luria and Delbrück's [24], clonal expansions are powerful tools to unmask heterogeneity that is otherwise missed by population averaging. Similarly to clonal expansions, population bottlenecks such as the selective growth of cells expressing specific markers [25] or fractional killing after treatments [14••,21••,23] generate homogenized cell populations. The dynamics of phenotypic diversification after population bottlenecks hold information about the stability of cellular states even when specific details of the underlying molecular circuits are unknown. While stable phenotypic states persist after prolonged culture, transient states are expected to reconstitute the phenotypic diversity of the original population with a timescale that is defined by the dynamics of phenotype interconversion (Figure 2a). However, care should be taken when using this rationale to make inferences about the genetic and non-genetic character of