SUPPLEMENTARY TEXT FOR

An in vitro model of tumor heterogeneity resolves genetic, epigenetic, and stochastic sources of cell state variability

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Table of Contents

Genetic, epigenetic, and stochastic sources of tumor heterogeneity..............................................................1
Relationship between transcriptomics and epigenetics .................................................................................2
The genetic-to-epigenetic connection........................................................................................................3
Multiple possible routes to a new genetic state in DS8..............................................................................4
Table A. Glossary of relevant terms. .............................................................................................................6
Table B. List of genes associated with mutation heatmaps in Figs 3D and 4D. ..........................................8
Table C. Rate parameters used for monoclonal and polyclonal growth models........................................9
Supplementary References.........................................................................................................................10
Genetic, epigenetic, and stochastic sources of tumor heterogeneity

Based on prior work in this area\textsuperscript{1–7}, we view intratumoral heterogeneity as comprising genetic, epigenetic, and stochastic components that are broadly distinguished based on characteristic timescale of change: functional genetic mutations are acquired on the order of weeks to months\textsuperscript{8,9}, transitions between epigenetic states occur on the order of hours to weeks\textsuperscript{10}, and stochastic fluctuations in protein concentrations, and other sources of intracellular noise, operate on the order of seconds to minutes\textsuperscript{11}. Separating processes based on characteristic timescale is a common approach in physics\textsuperscript{12} and has a long history in biology\textsuperscript{13}. Below, we describe in detail each of these levels of heterogeneity, with precise definitions of terms that will be important for later analyses (see also Table A).

Genetic: Genomic instability is a hallmark of cancer\textsuperscript{14,15}. For cancer cells in culture, it has been estimated that approximately five million cell divisions are necessary to acquire at least one mutation per gene\textsuperscript{8}. With more than 20,000 genes in the genome, this amounts to one mutation every two weeks on average. These mutations can be single-nucleotide polymorphisms (SNPs), insertions/deletions (InDels), and larger structural variations. While mutations in the genome are relatively frequent, many do not have functional consequence, so-called “passenger” mutations\textsuperscript{6}. Therefore, we make a distinction between the genomic state of a cell and the genetic state: the genomic state is the full sequence of nucleotides that make up the DNA whereas the genetic state is the subset of this sequence that contributes to a specific cellular phenotype\textsuperscript{16,17}. In other words, two cells may differ genomically but be genetically identical if the mutations that differentiate them occur in portions of the genome that have no functional consequence on the phenotype of interest. As such, the timescale for generating genetic heterogeneity within a population of cancer cells can be quite slow (much slower than the acquisition of genomic heterogeneity), on the order of weeks to months\textsuperscript{8,9}. Note that enumerating the subset of the genome that defines the genetic state can be difficult (if not impossible) in practice and depends on the phenotypic context\textsuperscript{18}. Here, we use drug response as the phenotype of interest. Thus, the genetic state is, in principle, defined over all genes associated with drug response, which includes those involved in stress response, metabolism, cell cycle regulation, and many others.

Epigenetic: Conceptualized as a quasi-potential energy surface where local minima, or “basins of attraction,” correspond to cellular phenotypes, epigenetic landscapes were first proposed by Waddington as an abstract tool for understanding phenotypic plasticity and cellular differentiation during development\textsuperscript{19}. The genetic state of a cell sets the topography of the landscape\textsuperscript{2,4} and intrinsic (e.g., gene expression\textsuperscript{20}) or extrinsic\textsuperscript{21,22} sources of noise drive transitions between phenotypes. Phenotypic state transitions (between epigenetic basins) have been observed to occur on the order of hours to weeks\textsuperscript{23,24}, meaning epigenetic diversification occurs on a much faster timescale than genetic diversification. From a molecular perspective, the epigenetic landscape is the consequence of the complex biochemical interaction networks underlying cellular function\textsuperscript{25,26}. Complex dynamical networks can harbor multiple stable states, termed “attractors”, towards which a system will tend to return (relax back) in response to small perturbations\textsuperscript{27,28}. This property underlies epigenetic heritability: daughter cells inherit similar molecular contents to their parent; hence, they tend to remain within the region of influence of
the parental attractor. Here, we use the transcriptional state of a cell, revealed through single-cell transcriptomics, as a proxy for the epigenetic state, with the understanding that mRNA is only one lens through which to view epigenetics. Indeed, this definition of an epigenetic state, i.e., as a stable state of a complex biochemical network, differs from the traditional molecular biology definition in terms of “epigenetic marks”29 (e.g., DNA methylation, regions of open chromatin). The two are related, however, in the sense that the same biochemical network that defines the epigenetic landscape also sets the epigenetic marks on the DNA3 (see “Relationship between transcriptomics and epigenetics” below for further discussion).

Stochastic: Fluctuations, or noise, in intracellular species concentrations have long been recognized as a source of non-genetic heterogeneity in isogenic cell populations, first in bacteria11,30 and then in yeast31,32 and mammalian cells24,33,34. Intracellular noise can be “intrinsic”, i.e., due to the probabilistic nature of biochemical interactions35,36, or “extrinsic”37, affecting the rates of interactions, synthesis, and degradation within a biochemical network. Intrinsic sources of noise include transcriptional bursting38,39, translational bursting40, and randomness in mRNA/protein degradation41, oligomerization42, and post-translational modification43. Extrinsic noise includes randomness in the distribution of molecular contents upon cell division44,45, environmental factors such as inhomogeneities in cell culture media46, fluctuations in temperature47 and pH48, and spatial variations in the microenvironment49. Importantly, fluctuations at the molecular level can drive probabilistic cell fate decisions20,50–54, including division and death55,56, at the single-cell level and phenotypic diversification at the epigenetic (population) level34,57. Experimentally, at the intracellular level, intrinsic and extrinsic noise are difficult to distinguish, generally requiring multiple fluorescent reporters and the ability to fine-tune the external environment37. Theoretically, the chemical Master Equation58,59 (CME) is the construct upon which stochastic dynamical analyses are based. Since the CME is difficult to solve in general, a number of stochastic algorithms have been developed for simulating fluctuations at both the single cell and cell population levels36,60 (see “Simple growth model of stochastic birth and death” below).

Relationship between transcriptomics and epigenetics

As mentioned above (see “Genetic, epigenetic, and stochastic sources of tumor heterogeneity: Epigenetic”), from a molecular standpoint, the Waddington epigenetic landscape is a consequence of the complex biochemical interaction networks underlying cellular function25,26. Within this framework, cell phenotypes are stable steady states, or “attractors”27,28, of this interconnected network within cells. Each attractor is defined as a point (or cycle) within an n-dimensional “phase space,” where each axis represents the concentration of a molecular species. The defining characteristic of an attractor is that the system will tend to return (relax back) to it in response to small perturbations (e.g., gene expression noise, uneven distribution of molecular contents upon division). It is this attractiveness that underlies epigenetic heritability: daughter cells inherit similar contents to their parent; hence, they tend to remain within the region of influence of the same attractor. Larger perturbations, however, can move a system into the region of influence of a neighboring attractor, resulting in a spontaneous phenotypic state change. This explains why epigenetic inheritance is often short lived61.
An “epigenetic state” within this definition is thus simply the molecular state of the associated attractor (see Table A). This molecular state can be viewed through a variety of different lenses, including mass cytometry (protein level), ATAC-seq (open chromatin regions), and RNA-seq (transcriptomics). It is the latter that we have chosen in this work. However, as stated, it is important to recognize that transcriptomics serves only as one possible view, i.e., a proxy, for the epigenetic state. In molecular biology, for example, epigenetic states are usually discussed in terms of chromosomal changes, e.g., DNA methylation and histone modification. These are simply different views of the same epigenetic states since the biochemical network that defines the Waddington landscape also regulates the epigenetic marks on the DNA.

**The genetic-to-epigenetic connection**

In the genetic/epigenetic/stochastic (G/E/S) view of tumor heterogeneity that we utilize in this work (Figs 1 and 6 of the main text; see also “Genetic, epigenetic, and stochastic sources of tumor heterogeneity” above), genetics are fundamentally tied to epigenetics. To understand how, recall that an epigenetic landscape quantifies, in terms of a quasi-potential energy, the accessible molecular states that cells can occupy. The topography of the landscape depends on the molecular species present (the axes of the state-space), the biochemical processes that can occur, and the parameters that quantify the rates at which these processes proceed. The latter means that changes to the rate parameters can change the topography of the landscape, i.e., the depths of basins, heights of barriers, etc. As mentioned in the main text (“Introduction”), for processes such as protein-protein interactions, the rate parameters are directly dependent on protein structure, which is encoded in the DNA. Thus, mutations to the DNA that, e.g., alter the accessibility of a binding domain, change the values of rate parameters and, hence, the epigenetic landscape as a whole. In this way, each genetic state can be thought of as having an associated epigenetic landscape, which may have multiple basins, or cell phenotypes.

A consequence of this final point is that phenotypic differences at the transcriptomic level do not always indicate differences at the genetic level. In other words, **cells can be genetically identical yet differ transcriptomically if they occupy different basins within a common epigenetic landscape.** In theory, we should be able to distinguish between cases in which transcriptomic differences are tied to genetic variations and those in which they are not by simply comparing, across samples, parts of the genome that are relevant to the phenotype of interest (e.g., drug response). However, in practice we do not have a full accounting of all phenotypically-relevant parts of the genome, we only have the full genomic sequences, which will invariably have differences. The question is whether those differences are functionally relevant to the phenotype of interest. If not, we can consider the samples to be genetically identical, even though they are genomics distinct (see “Genetic, epigenetic, and stochastic sources of tumor heterogeneity: Genetic” above).

This is the rationale behind the GO term analysis we perform in this work. Four of the PC9-VU sublines (DS3, DS6, DS7, DS9) display small but measurable differences in their genomic signatures (Fig 4A–E in the main text) and appear to reside in three distinct portions of the transcriptomic space (Fig 4F). Our goal is to determine whether the small genomic differences across sublines can explain the observed transcriptomic differences. A naïve approach would be to identify genes that differentiate the sublines at the transcriptomic level and look for mutations
in those genes at the genomic level. However, it is well known that a mutation in a gene does not always alter the expression level of that gene\textsuperscript{63}. Rather, if gene $X$ encodes for a protein that acts as a transcription factor for gene $Y$, then a mutation in $X$ could alter the protein structure such that it affects the expression level of $Y$. Thus, rather than making gene-by-gene comparisons of mutations and expression levels, we compare genomic variants and differentially expressed genes at the process level. Specifically, we compare Gene Ontology (GO) terms\textsuperscript{64,65} for “Biological Process” (BP; 12,272 terms), “Molecular Function” (MF; 4,165 terms), and “Cellular Component” (CC; 1,740 terms) categories at the genomic and transcriptomic levels and look for correlations between them (see “Methods” in the main text for details). High correlation means that there is evidence that variations at the genomic level are responsible for differences at the transcriptomic level, i.e., that the subline has a distinct genetic state. Conversely, lack of correlation is evidence that the cells occupy a basin within a common epigenetic landscape. As a control, we perform this analysis on the cell line versions as well (VU, MGH, BR1), which we assume are genetically distinct. This provides benchmarks against which we can compare results for the sublines.

**Multiple possible routes to a new genetic state in DS8**

As discussed in the main text, we conclude based on our genomic and transcriptomic analyses that the DS8 subline harbors a genetic resistance mutation (different from the T790M mutation present in PC9-BR1) that emerged from the PC9-VU parental population at some point in the past. It remains an open question, however, whether this mutation occurred after or before the subline was established. If it occurred after, it could be the case that DS8 actually harbors two distinct genetic states, the original PC9-VU state and the new emergent state (S11A Fig). However, if that were the case we would expect to see some DS8 cells in the same region of transcriptomic space as PC9-VU, which we do not (Fig 4F of the main text; S6C,D Fig). One explanation could be that the emergent genetic state in DS8 outgrew the PC9-VU state, leaving behind an isogenic population (i.e., a “selective sweep”). However, there does not appear to be a discernable difference between the out-of-drug proliferation rates for DS8 and the other sublines (Fig 2C of the main text), which would seem to discount this possibility. We cannot entirely preclude this prospect, however, as it is possible that the difference in proliferation rates is simply too slight to detect in our current data.

Conversely, if the mutation in DS8 arose prior to the subline being established, we would expect some mutant cells to remain within the PC9-VU parental population. We do, in fact, see a small number of PC9-VU cells in the region of transcriptomic space where DS8 cells reside (compare Fig 3F and 4F of the main text). Interestingly, we also see some DS9 cells in this region (but not DS7, despite the significant overlap between the two populations; Fig 4F of the main text and S6C,D Fig). This could indicate that DS9 actually comprises two epigenetic basins, a deep one where most of the cells reside and a shallow one in the region of transcriptomic space where DS8 cells reside. It is possible that PC9-VU cells transition frequently between these two basins and that at one point in the past one cell in the shallow basin randomly acquired a genetic mutation that caused it to get “locked into” that basin. A progeny of that cell could have been isolated to start the DS8 subline (S11B Fig). This scenario would be consistent with the findings of Shaffer et al.\textsuperscript{66}, although without the selective pressure presumed to drive the mutation in
their case. However, if this is the case, given that DS8 proliferates at essentially the same rate as the other sublines in the absence of drug, why are those cells so rare (~2%) within the PC9-VU parental population? One would expect that population to grow out in the same way that DS8 did. One explanation might be that cell-cell interactions\textsuperscript{67} destabilize the mutant cells when in culture together with PC9-VU parental cells. This is an intriguing idea that would require additional experimentation to verify, perhaps using DNA barcoding to track lineages within cocultured populations.

Finally, there is also the added complication that DS8 exhibits a bimodal DIP rate distribution under EGFR\textsuperscript{i}, which we conclude from our stochastic simulation analysis (Fig 5E–H of the main text) indicates that DS8 harbors (at least) two distinct cell states. As discussed in the main text, the model is agnostic as to whether these two states are genetically identical or not, it merely requires they have distinct DIP rates. Thus, it does not preclude the possibility that DS8 is isogenic, as would be the case in two of the scenarios discussed above (i. mutation after subline establishment, followed by selective sweep; ii. mutation prior to subline establishment). However, the region of transcriptomic space in which DS8 cells reside is small compared to the region occupied by PC9-VU and there are no obvious subpopulations present (Fig 4F of the main text). It is also difficult to reconcile why the left mode of the DS8 DIP rate distribution overlaps so significantly with the PC9-VU parental and subline distributions (Fig 2D of the main text) despite the lack of overlap in transcriptomic space. These remain open questions that we hope to address in future investigations. We emphasize, however, that none of the issues raised here changes our main conclusion presented in the main text that DS8 harbors a novel genetic resistance mutation that emerged from PC9-VU at some point in the past in the absence of selective pressures (Fig 6 of the main text).
### Table A. Glossary of relevant terms.

| Term                        | Definition                                                                                                                                                                                                 |
|-----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **Phenotype**               | An observable property of a cell population, either in the absence or in response to a perturbation. In this work, our phenotype of interest is response to EGFR inhibition, which we quantify in terms of single-cell transcriptomic signatures (no drug) and drug-induced proliferation (DIP) rates. Since population-level quantities like DIP rate are driven by intracellular regulatory networks, we consider transcriptomic signatures and DIP rates to be two different presentations of the same phenotype. |
| **Genomic state**          | The full sequence of nucleotides that comprise a genome. With four types of nucleotides (A, C, T, G) and ~3.2 billion nucleotides per genome, the number of possible nucleotide configurations is massive (effectively infinite, even when excluding non-viable configurations). It is therefore exceedingly rare for two cells to have the same genomic state. In this work, we quantify genomic states using whole-exome sequencing. |
| **Functional genomic regions** | Locations in the DNA sequence that encode for genes that impact the phenotype of interest in some quantifiable way.                                                                                          |
| **Genetic state**          | The sequence of nucleotides comprising only functional genomic regions of the DNA. Because the scope is much more limited, the number of possible genetic states available to a cell is significantly smaller than the number of genomic states, i.e., many cells, up to and including an entire cell population, may share the same genetic state. |
| **Molecular state**        | The concentrations of all biochemical species (mRNAs, proteins, small molecules, etc.) associated with a phenotype of interest. Because copy numbers can be large and fluctuate due to intrinsic and extrinsic sources of noise, it is exceedingly rare for two cells to have the same molecular state. In this work, we quantify molecular states using single-cell transcriptomics. |
| **Epigenetic landscape**   | A conceptual and mathematical construct that assigns a “quasi-potential energy” to all possible molecular states of a cell. Fundamentally, the epigenetic landscape is the consequence of the complex biochemical interaction networks that underlie the phenotype of interest. Importantly, each genetic state has an associated epigenetic landscape. Mutations in the DNA can thus change the topography of the landscape. |
**Epigenetic state**

Often used interchangeably with “phenotypic state,” any heritable state of a cell that is not encoded in the DNA. Within the framework of the epigenetic landscape, an epigenetic state is the collection of states associated with each local minima, or “basin of attraction.” Cells within basis of attraction will tend to stay within the vicinity of the basin in response to small perturbations. This is the basis for epigenetic heritability since daughter cells tend to have similar molecular contents as their parent, i.e., they inherit the same basin of attraction. Conceptually, an epigenetic landscape can be seen as a collection of epigenetic states separated by barriers. Traversals of these barriers correspond to phenotypic state transitions. Importantly, cells can differ in their molecular states (e.g., due to fluctuations in gene expression) but still occupy the same epigenetic state.
Table B. List of genes associated with mutation heatmaps in Figs 3D and 4D.

| Index | Gene   | Index | Gene   | Index | Gene     |
|-------|--------|-------|--------|-------|----------|
| 1     | BHLHA15| 35    | IL32   | 69    | AC092647.5 |
| 2     | NCR2   | 36    | SOX21  | 70    | ARID1B   |
| 3     | TMEM158| 37    | GDF11  | 71    | CNPY3    |
| 4     | JAGN1  | 38    | MECP2  | 72    | FOXD1    |
| 5     | DLX4   | 39    | VKORC1L1| 73    | TPPP     |
| 6     | RAD51B | 40    | SMAP1  | 74    | PRDM8    |
| 7     | FAU    | 41    | XBP1   | 75    | OLIG1    |
| 8     | AGAP3  | 42    | RAPH1  | 76    | DBNDD2   |
| 9     | KRTAP10-6| 43 | MMP17  | 77    | MYL1     |
| 10    | CGREF1 | 44    | TERF1  | 78    | LRP1B    |
| 11    | RPS15  | 45    | POU3F2 | 79    | CCDC74A  |
| 12    | TUBB8P12| 46 | BCHE   | 80    | ZNF257   |
| 13    | HCRT   | 47    | POU3F3 | 81    | YY1      |
| 14    | SKA3   | 48    | ZFP36L2| 82    | FOXN3    |
| 15    | OR8G1  | 49    | ZNF787 | 83    | PCDH9    |
| 16    | GPR37L1| 50    | BCL6B  | 84    | MMP1     |
| 17    | BHLHE22| 51    | TDG    | 85    | RRP8     |
| 18    | RRAGD  | 52    | IGSF21 | 86    | TCHH     |
| 19    | VEGFC  | 53    | VCX3B  | 87    | PRAMEF6  |
| 20    | NAP1L5 | 54    | OR2A1  |        |          |
| 21    | SOX10  | 55    | SLC35F1|        |          |
| 22    | IGFBP2 | 56    | CADM4  |        |          |
| 23    | ADRA2B | 57    | NPIPB3 |        |          |
| 24    | SOX11  | 58    | FAM155A|        |          |
| 25    | FAM98C | 59    | PHLDA1 |        |          |
| 26    | CEBPA  | 60    | RAB21  |        |          |
| 27    | PHOSPHO1| 61 | OR2T35 |        |          |
| 28    | PHGR1  | 62    | FOXO6  |        |          |
| 29    | BRI3BP | 63    | POU3F1 |        |          |
| 30    | CARNMT1| 64    | FMR1NB |        |          |
| 31    | TAF11L2| 65    | ASTN2  |        |          |
| 32    | EPB41L1| 66    | SNX31  |        |          |
| 33    | CAPNS1 | 67    | MNX1   |        |          |
| 34    | ACSM2A | 68    | ZNF713 |        |          |
Table C. Rate parameters used for monoclonal and polyclonal growth models. Ranges are shown in square brackets, units are in h⁻¹, $k_{DIP} = k_{div} - k_{dth}$. Note that DIP=$k_{DIP}/\ln(2)$.

| Parameter                  | Subline | DS1       | DS3       | DS4       | DS6       | DS7       | DS8       | DS9       |
|----------------------------|---------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| $k_{div,pre-drug} / \ln(2)$|         | 0.04      | 0.04      | 0.04      | 0.04      | 0.04      | 0.04      | 0.04      |
| $k_{DIP,pre-drug} / \ln(2)$|         | 0.035     | 0.035     | 0.035     | 0.035     | 0.035     | 0.035     | 0.035     |
| $k_{div1,post-drug} / \ln(2)$|         | [0.0144, 0.115] | [0.0144, 0.115] | [0.0144, 0.115] | [0.0144, 0.115] | [0.0144, 0.115] | [0.0144, 0.115] | [0.0144, 0.115] |
| $k_{DIP1,post-drug} / \ln(2)$|         | [0.000721, 0.00361] | [-0.00216, 0.000721] | [0.00361, 0.00649] | [-0.000721, 0.00216] | [0.00144, 0.00433] | [0.000721, 0.00216] | [0, 0.00289] |
| $k_{div2,post-drug} / \ln(2)$|         | N/A       | N/A       | N/A       | N/A       | N/A       | [0.0144, 0.115] | N/A       |
| $k_{DIP2,post-drug} / \ln(2)$|         | N/A       | N/A       | N/A       | N/A       | N/A       | [0.00793, 0.0108] | N/A       |
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