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A transcription factor network model explains heterogeneity and reveals a novel hybrid phenotype in Small-Cell Lung Cancer

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Abstract

Small cell lung cancer (SCLC) is a devastating disease because of its tendency to early invasion and refractory relapse after initial treatment response. These aggressive traits have been associated with phenotypic heterogeneity, which however remains incompletely understood. To fill this knowledge gap, we inferred a set of 33 transcription factors (TFs) associated with gene signatures of the known neuroendocrine/epithelial (NE) and non-neuroendocrine/mesenchymal-like (ML) SCLC phenotypes. The topology of this SCLC TF network was derived from prior knowledge and simulated using Boolean modeling. These simulations predicted that the network settles into attractors (TF expression patterns) correlated with NE or ML phenotypes, suggesting that TF network dynamics underlie emergence of heterogeneous SCLC phenotypes in an epigenetic landscape. However, several cell lines and patient samples did not correlate with either the NE or ML attractors. Flow cytometry indicated that single cells within these cell lines simultaneously express surface markers of both NE and ML differentiation, revealing existence of a “hybrid” phenotype. Upon exposure to standard-of-care cytotoxic drugs or epigenetic modifiers, NE and ML cell populations converged toward the hybrid state, suggesting a possible escape route from treatment. Our findings indicate that SCLC phenotypic heterogeneity can be specified dynamically by attractor states of a master regulatory TF network. Thus, SCLC heterogeneity may be best understood as states within an epigenetic landscape. Understanding phenotypic transitions within this landscape could provide insights to clinical applications.

INTRODUCTION

Small cell lung cancer (SCLC), accounting for ~13% of lung cancers (1), is exceptionally aggressive. Patients with extensive disease die ~1 year from diagnosis, and patients with limited disease experience a dismal 20% cure rate (2–4). Standard of care (2), confined to chemo and radiotherapy for half a century, is largely ineffective as SCLC patients exhibit high initial response rates rapidly followed by treatment-refractory relapse.

Expression-based subtyping, impactful in other cancers (5), may be effective in SCLC because of phenotypic variability (2,6) with respect to neuroendocrine features of its cell of

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origin (7,8). A recent study (9) identified two transcriptional SCLC subtypes distinguishable by Notch pathway activity and aggressiveness, but without mutational differences. In genetic mouse models of SCLC, Calbo et al. showed that spontaneously occurring neuroendocrine and non-neuroendocrine cell phenotypes coexist and cooperate to promote metastasis (8).

These reports indicate that a deeper understanding of cellular phenotypes could produce insights into biology and evolution of SCLC. A limitation of previous studies (8,9) is that analyses were based on population averages, whereas variability in tumors should be considered at the single-cell level (5,10,11). It also remains unclear why this heterogeneity emerges.

To fill these knowledge gaps, we investigate SCLC phenotypic heterogeneity at the single-cell level using an integrative computational and experimental approach. Consistent with previous reports, we found two transcriptional subtypes at the population level in cell lines and patient specimens, characterized by gene co-expression modules enriched in neuroendocrine/epithelial (NE) and mesenchymal-like (ML) features. To understand how these phenotypes may arise in the absence of driving mutations (9), we hypothesized that they are attractors of a regulatory TF network. This approach is grounded in the mathematical interpretation of Waddington's epigenetic landscape (12,13), whereby attractors correspond to biological differentiation states or stable phenotypes. Based on this view, it has previously been proposed that malignant phenotypes in cancer correspond to attractors (14,15), and some have suggested "differentiation therapy" from malignant to benign attractors as a possible treatment strategy (14–17).

To this end we construct an SCLC master regulatory network of transcription factors (TFs) from NE and ML gene-expression signatures. We then adopt a discrete Boolean modeling approach to simulate the behavior of this TF network and evaluate its ability to dynamically control NE and ML phenotypes. Discrete models are well suited to provide insight into complex TF networks by identifying steady state TF patterns of expression, termed attractors. Mathematically, attractors represent the stable configurations of the dynamic TF network. Biologically, attractors correspond to transcriptional steady states of an epigenetic landscape formed by active (ON) and silent (OFF) TFs regulating each other. While discrete models are coarse approximations, they are nevertheless informative and circumvent the obstacle of unfeasible parameter acquisition (18,19).

Simulations of our SCLC TF network predict attractors corresponding to the NE and ML SCLC subtypes. Furthermore, by distilling the NE and ML states to their core driving TFs, the model highlighted a shortcoming of the two-subtype classification, as several cell lines and patient samples did not match any attractors. Western blots revealed that these samples expressed similar levels of both NE and ML markers. Flow cytometry revealed that this "double-positive" phenotype reflected the character of individual single-cells, confirming the existence of a previously unreported "hybrid" single-cell phenotype in SCLC. Exposure to cytotoxic and epigenetic drugs caused NE and ML cells to transition toward the hybrid state, implicating it as a refuge for survival of treated SCLC tumors.

MATERIALS AND METHODS

Data normalization

CCLE dataset (20) was downloaded from Broad Institute as CEL files. Data were normalized and median centered using quantile RMA normalization using Affy Bioconductor package (21) in R. Probe-level data for all the datasets was converted to gene-level data by probe merging using collapseRows (22). Probes with no known gene symbols were removed.

Consensus clustering

Consensus clustering was performed using ConsensusClusterPlus v1.24.0 package in R v3.2.3 (23) on both the 53 SCLC cell line dataset from CCLE (20) and 28 SCLC patients from CLCGP (24), with 80% sub-sampling of both genes and samples, 1000 repetitions, 1 - Pearson correlation, and k-means. Both the CCLE and GLCGP datasets were subsetting to only include genes measured in the GSE6044 (25) dataset, in order to maximize overlap with our previous work (26).

WGCNA and Network analysis

Co-expression network analysis was performed in R v3.2.3 using the WGCNA package v1.49 (27). As with the consensus clustering analysis, the CCLE SCLC dataset was subsetting to only include genes measured in GSE6044 (25). We used 1 - Pearson's correlation to build a co-expression based dissimilarity matrix. Modules were generated using unsupervised average-linked hierarchical clustering with a static height of 0.95. We required that each module contain at least 100 genes.

Comparative Pathway enrichment analysis of the Blue and Turquoise modules was performed using BINGO and Enrichment map (28) and visualized Cytoscape. Expression values were transformed to Z-scores, and the average expression of all genes that are simultaneously represented in a given pathway and a module (blue or turquoise) as the represented score for that module pathway combination.

Transcriptional regulatory network construction

To generate an SCLC-specific transcriptional network, we applied the bootstrap version of ARACNE (29) on gene expression profiles from the 53 CCLE SCLC cell lines using the following parameters: p-value = 10^{-7} , dpi = 0 and 100 bootstraps, resulting in 27,224 interactions among 8,706 nodes. To evaluate if genes in Blue and Turquoise module are enriched for targets of any specific TF, we used Fisher's exact test (FET) (30) to compute TF enrichment with the Blue and Turquoise module genes. We selected all TFs to be candidate master regulators if the FET p-value was ≤ 0.05 , leaving 96 and 207 TFs for Blue and Turquoise module, respectively. Of these, 23 TFs were common to both modules (Figure S7D).

Modeling gene regulatory networks which were inferred entirely from the available data can suffer from circular reasoning: a dataset generates a network, which then predicts the dataset. To avoid this fallacy, we built the network topology strictly using sources external to

the datasets of interest. Thus, we first filtered ARACNE TF predictions based on gold-standard TF-target binding site databases CHEA, ENCODE, TRANSFAC, JASPAR using EnrichR (31–35), and literature databases such as Pubmed and Glad4U (36). These filtration steps produced a list of 76 likely TF regulators of NE and/or ML differentiation. We took only heterogeneously expressed TFs (median absolute deviation above the 50th percentile) yielding a list of 38 TFs that we used to build a Boolean network for SCLC. Next we extracted directed interactions between these TFs using only information from the above gold-standard references and literature. Where possible, interactions were classified as activating or inhibiting by manually searching the literature. Interactions which we could not find in literature were classified as activating if the TFs are positively correlated across the CCLE dataset, or inhibitory given negative correlation. 5 TFs did not have outgoing edges and were eliminated, leaving 33 TFs.

Boolean network simulation and analysis

The TF network was simulated as a Boolean network where each node was either ON (active) or OFF (silent). Nodes were updated using the random order asynchronous method (18). We considered two distinct approaches updating TFs: 1) threshold updating, in which the total number of ON inputs are compared such that the target node is switched ON when there are more activators, and OFF when there are more inhibitors, and 2) inhibitory dominant, in which having any inhibitor ON is sufficient to turn the target node OFF. The threshold update rule is referred to as a Hopfield neural network in some literature. Because the network's state space is so large, we only simulated a random subsample of the states. For both update rules, a state transition network was seeded with 8,000 randomly generated initial states, in which each TF had a 50% chance of being ON or OFF. When TFs are updated in asynchronous random order, it is possible that the state at time t may have several possible outgoing trajectories to time $t+1$. To account for this, each state that we observed was initialized and updated 30 times to sample distinct trajectories to the next state that may be influenced by the update order. Each newly observed state was queued to be updated in this fashion, until there were no new states identified. Attractors were identified by applying the attracting components algorithm from NetworkX (<http://networkx.github.io/documentation/latest/reference/index.html>) to the state transition graph for each update rule. Using threshold updates, we found 57 fixed point attractors, and no oscillating ones. We observed the same set of attractors using synchronous updates with 2^{17} and 2^{18} initial states. Using the inhibitory dominant update rule, we found 6 fixed point attractors, and 5 two-state oscillating attractors, finding the same set of attractors using synchronous updates from 2^{12} to 2^{18} initial states.

To score the correlation between samples and attractors, CCLE and CLCGP expression data were independently scaled between 0 and 1, and Pearson's r was calculated pairwise between attractors and cell lines/patients. Statistical significance was determined by considering the highest correlation between each attractor and cell line, and comparing against a null distribution, obtained by generating 10,000 random TF vectors. The Mann-Whitney U test was used to compare the score distributions. The model attractors had higher correlation with cell lines than the random attractors (threshold: p -value = $9.5e-34$, inhibitory-dominant: p -value = $7.6e-9$), and higher correlation with patients than the random

attractors with (threshold: p -value = $1.7\text{e-}23$, inhibitory-dominant: p -value = $1.7\text{e-}6$). Individual attractors were assigned a p -value for their highest correlation with each sample by direct comparison with the null distribution (Table S5).

Robustness of NE and ML attractor states

Cells need to be able to robustly guide their differentiation choices depending on driving signals, and therefore we would expect trajectories toward cell attractors to be robust. Structural coherence (37) is a topological metric which measures how reliably an initial condition evolves toward its appropriate attractor given a perturbation. This metric requires an estimate of the total size of the basin of attraction, so we were only able to apply it to attractors for which we could reliably estimate this size (a few basins were too small to reliably estimate their size). Values of random, maximum, observed, and structural coherence are reported in Table S4. We also calculated Derrida curves (38) (Figure S9), as the average increase growth or decay of an initial perturbation after one step, for perturbation sizes ranging from 1 to 32 TFs.

Antibodies and Reagents

Antibodies used for western blotting include GAD1/2, EphA2, PDGFR (Cellsignal®), E-cadherin (BD Biosciences), Vimentin (SantaCruz), OVOL2, CBFA2T2, SOX2, ASCL1, POU5F1, GAPDH, SOX2, FYN, beta-actin (Sigma®). Fluorophore-conjugated primary antibodies were used for flow cytometry – CD56 BV605, CD151 PE, CD24 BUV395 (BD Biosciences), CD44 Pacific blue, E-cadherin Pacific blue (Cellsignal®), CADM1 A647 (MBL), EPHA2 A488 (R&D Systems), CD133 PE-Cy7 (Biolegend), Vimentin (Santacruz);

Cell culture

Authenticated cell lines were obtained from ATCC from 2012–2015, authenticated by DNA STR profiling, morphology, and mycoplasma detection. Cell lines were grown in company recommended media. New cell lines were obtained as needed every 1–2 years. Cell lines were expanded in culture for less than 2 months, then frozen in aliquots for subsequent use. Cell lines are passaged no more than 30 times before being discarded (approximately 4 months). Any contaminated cell lines were discarded and new aliquots thawed and cultured. Mycoplasma test was performed on all cell lines in culture every 2 weeks.

Flow cytometry data generation and analysis

1–2 million cells were plated in T75 or T150 flasks the previous day and collected for flow experiment the next day as described below. For drug treatment experiments, cells were plated same as above the previous day, followed by drug addition the next day. Cells were incubated with drugs at 37 degrees then collected for flow experiments.

Cells were dissociated using TrypLE (GIBCO®) for 10–15 minutes followed by staining with Alexa 700 dye (Molecular Probes®) for 5 minutes at 37 degrees. Cells were then washed and fixed with 2% paraformaldehyde (10 minutes at room temperature), followed by surface marker staining or permeabilization with ice-cold 100% methanol at -20 for 30 minutes. Cells were then stained with fluorescent conjugated antibodies for 30 minutes in dark at room temperature. Samples were washed with PBS and run on BD 5-laser instrument

at the Vanderbilt Flow cytometry core. Fluorescent channels were compensated using anti-mouse IgK beads (BD Biosciences) that were tagged with fluorescent antibodies. First intact cells were gated on the Forward Scatter Area (FSC-A) and Side-Scatter Area (SSC-A) plot, where debris has a low FSC-SSC ratio. This was followed by gating for Alexa700 negative viable cells as described previously (Box 1 in (39)), where A700 positive populations are dead/dying cells. Finally, A700 negative viable cells are further gated to include only singlet populations. At least 20000–30000 viable singlet cells were collected per sample. All subsequent gates on fluorescent markers are made on viable singlet cells depicted in the sunburst plot. The raw cytometer intensity readouts for fluorescent channels were first converted to log scale by using the $\text{asinh}()$ function with a co-factor of 150. Gating was conducted in Cytobank (40).

Western blotting

Cell lines were plated for 2 days in complete medium to equilibrate. Lysates were prepared by spinning cells at 4°C, aspirating the media, and adding M-PER lysis buffer (Pierce®) containing 1X phosphatase inhibitors 2 and 3 and protease inhibitor (Sigma-Aldrich®). Lysates were incubated for five minutes at room temperature, vortexed for 30 seconds and centrifuged at 15000 rpm for 15 minutes (at 4°C). The protein concentration was quantified using BCA assay (Pierce®). Lysates were boiled for 10 minutes at 100 degrees with 1X NuPage sample buffer (Molecular Probes®) and run on 8% or 4–12% Tris-glycine gels (Molecular Probes®). Semi-dry transfer was followed by blocking with 1X Casein-TBS. Blots were imaged by chemiluminescence.

RESULTS

Characterization of SCLC NE and ML phenotypes

We applied consensus clustering (23) to the 53 SCLC cell lines in the Cancer Cell Line Encyclopedia (CCLE) database (20), and to 28 tumor samples from the Clinical Lung Cancer Genome Project (CLCGP) (24), with the expectation to detect previously observed neuroendocrine and non-neuroendocrine phenotypes (8,9). Consensus clustering analysis, which provides a rationale for determining the number of robustly separated subtypes, indicated that the CCLE cell lines and the CLCGP patient specimens are most consistently separated into 2 distinct clusters (termed Clusters 1 and 2, Figures 1 and S1, Table S1).

To characterize these clusters, we applied weighted gene co-expression network analysis (WGCNA) (27) to the 53 CCLE cell lines. WGCNA identified 13 gene co-expression modules (Figure S2, Table S2). To summarize the overall expression of each module, eigengenes (first principal component of all genes within a module) were computed (Figure 2). The Blue module eigengene was upregulated (Bonferroni adjusted $p < 0.001$) within Cluster 2, while the Turquoise module eigengene was upregulated (Bonferroni adjusted $p < 0.05$) within Cluster 1 (Figure 2 and Figure S2B). No other module eigengenes showed statistically significant differences between the consensus clusters (Figure S2B).

WGCNA modules are derived agnostically, and reveal co-expressed genes participating in similar biology. Blue and Turquoise module genes therefore provide information about the

overall character of the consensus clusters. Visual inspection reveals that hubs (highly connected genes) in the Blue and Turquoise modules are well-known neuroendocrine/epithelial or mesenchymal biomarkers, respectively (Figures S3 and S4). Statistical analyses by Gene Ontology and EnrichmentMap (28) confirm enrichment of the Blue module for neuroendocrine and epithelial differentiation processes, while the Turquoise module is enriched for pathways involved in mesenchymal phenotype and epithelial-to-mesenchymal transition (EMT) (Figure S5 and S6A). Additionally, we performed gene-set enrichment analysis (GSEA) with published signatures of pro-neural, mesenchymal and proliferative glioblastoma subtypes (30) on the 12 cell lines with highest Blue module eigengene expression, and 9 cell lines with highest Turquoise module eigengene expression. The pro-neural (Figure S6B, lower panel) and mesenchymal (Figure S6B, upper panel) signatures were enriched in the cell lines with high Blue module and Turquoise module expression, respectively. As a control, the proliferative signature was significantly enriched in neither (p-value >0.1, data not shown).

Thus the Blue module genes are a signature of the canonical neuroendocrine state of SCLC, while the Turquoise module genes are a signature of non-neuroendocrine/mesenchymal cells. We therefore refer to Cluster 2 (in which Blue module genes are high) as the SCLC neuroendocrine/epithelial (NE) subtype, and to the Cluster 1 (in which Turquoise module genes are high) as the SCLC mesenchymal-like (ML) subtype (Figure 1B, C).

Boolean simulations of TF network predict attractors corresponding to NE and ML states

Since NE and ML subtypes are not associated with driver mutations (9), we hypothesized they may be regulated epigenetically, as during normal cell differentiation. Cell identity in differentiation is largely controlled by regulatory networks of transcription factors (TFs) that coordinate expression of each other and of target genes (41,42). To understand specification of NE or ML cell identity, we derived a network of TFs which regulate expression of genes within the Blue and Turquoise modules (see Methods; Figure S7). By pruning nodes which had no outgoing edges, we reduced this network to a core set of 33 TFs and 361 interactions (Figure S8, Table S3). We used random asynchronous order Boolean simulation with both threshold and inhibitory dominant update rules to identify attractors. Using the threshold update rule, we found 57 stable fixed-point attractors (Figure 5A) and no oscillating attractors. With the inhibitory dominant rule we found only 6 fixed point attractors, but 5 two-state oscillating attractors as well. Both the threshold and inhibitory dominant attractors revealed similar features in all subsequent analyses, suggesting that these results are robust to the precise nature of the regulatory interactions. We also inferred network robustness to perturbations using structural coherence (37) (Table S4) and Derrida curves (38) (Figure S9). These results indicate that the basins of attraction are more robust than those of a random network, and that the network dynamics are ordered, rather than chaotic. Together, these observations suggest that the internal structure of interactions imposed to the network (Figure 3A) is non-random and naturally leads to well-regulated stationary states, and that our coarse modeling approach is acceptable for this system.

Each attractor is a 33-dimensional vector of TFs, differing by the overall TF ON-OFF expression pattern (columns in Figures 3B). Many of the attractors differ only by one or a

few TFs. Hierarchical clustering segregated the threshold attractors into four distinct clusters (Figure 3B). Results from the inhibitory dominant network are qualitatively similar, and are shown in Figure S8. Based on the TF ON-OFF patterning in each attractor within the clusters, attractors 49 to 25 (along the dendrogram, Figure 3B) have active TFs known to be either neuroendocrine (INSM1, POU3F2, SOX2, SOX11) or epithelial (FOXA2, OVOL2). In contrast, attractors 35 to 34 (along the dendrogram, Figure 3B) contain active TFs (MYC, NFKB1, SMAD3) involved in mesenchymal differentiation or EMT.

We computed a correlation score between attractors and both cell lines and patients by scaling the CCLE and CLCGP expression data from 0 to 1 (Figures 3C) and calculating Pearson's correlation coefficient pairwise between each attractor and sample. Several attractors exhibited high correlation with cell lines within the NE consensus cluster, while others exhibited high correlation with cell lines within the ML consensus cluster (Figure 3D). Similar results were observed with patients (Figure 3E), and in both cases the model's attractors were found to be significantly more correlated to the samples than random (Figure S10, Table S5).

These results confirm that the simulated dynamics of the 33 TF network agree well with the NE and ML nature of the cell lines, suggesting that the gene expression signatures of these phenotypes is driven by these underlying NE and ML TF attractors. Nevertheless, a few attractors showed no significant correlation with any cell line or tumor. These attractors were not further pursued, as we do not observe them biologically. More significantly, however, several cell lines and patient samples did not have significant correlation with any attractor. These samples may represent mixed populations of NE and ML cells, leading to poor correlation with either subtype at the population level, or alternatively may represent populations of "hybrid" cell types.

Experimental validation of network attractors reveals a hybrid single-cell SCLC phenotype

We probed the expression of cell lines for 10 of the 33 TFs. In general, NE TFs were expressed at a higher level in NE than ML cell lines, and vice versa (Figure 4A). Consistent with the finding that some cell lines did not correlate with either the NE or ML attractors (Figures 3D, S8B), we observed cell lines which simultaneously expressed both NE and ML TFs. Similarly, CD56 (NE marker) and CD44 (ML marker) were found co-expressed in 3 out of 10 patient samples (pt1112-1, pt216-1, and pt460-1, Figure 4B). The other tumor samples had mutually exclusive expression of either CD56 or CD44.

These double-positive cell lines may be either composed of mixed populations of NE and ML cells, or hybrid single-cells simultaneously co-expressing features of both phenotypes. We investigated this using single-cell flow cytometry with well-established neuroendocrine/epithelial and mesenchymal differentiation biomarkers (Figure 5 legend). To aid visualization, we defined overall NE and ML scores as the unweighted sum of NE and ML biomarkers, respectively.

In biaxial plots of these scores, NE cell lines consist primarily of NE⁺ML⁻ single cells, while ML cell line single cells were NE⁻ML⁺ (Figure 5A, C). However, in several cell lines, single cells simultaneously expressed similar levels of both NE and ML biomarkers (Figure

5B). These results confirm the existence of a novel SCLC NE⁺ML⁺ hybrid phenotype comprised of both neuroendocrine/epithelial and mesenchymal-like characteristics.

Other reports of hybrid phenotypes in cancer have associated the hybrid cells with a more stem-like phenotype (43,44). We measured single-cell expression of CD133, a cancer stem-cell marker, and found no significant difference between the NE, ML, and hybrid cell lines (Figure S11).

Modulation of SCLC phenotypes with chemotherapy or epigenetic drugs

Next, SCLC cell lines were treated with etoposide and cisplatin, and with epigenetic modulators valproic acid (HDAC inhibitor) and 5-azacytidine (DNA methylation inhibitor). Flow cytometry measurement of NE and ML markers (see Figure 5 legend) was used to characterize how treatment shifted the phenotypic identity of SCLC cells. Biaxial plots of NE vs ML scores showed phenotypic shifts at the single-cell level for all perturbations (Figure 6), converging toward hybrid populations. The hybrid phenotype was thus an end-state for SCLC cells subjected to stress.

DISCUSSION

In this work, we identified signatures of phenotypic heterogeneity in SCLC, and a set of TFs regulating expression of these genes. Through discrete Boolean model simulations, we showed that a master SCLC TF network naturally settles into states which were identified as NE or ML. This suggests that the NE and ML cell identities can naturally emerge from regulatory dynamics of TF networks, rather than being driven by genomic mutations. Most significantly, by distilling the NE and ML phenotypes to their essential TF drivers, the model also exposed some cell lines which had been improperly classified as NE or ML through transcriptome based clustering. *In vitro* validation confirmed existence of SCLC cell lines and patient samples that simultaneously express both NE and ML markers and TFs. We verified this hybrid phenotype at the single-cell level in cell lines, and showed that cell lines transition toward this hybrid state for survival upon drug treatment.

As resolving cancer heterogeneity can have profound impact on patient care and outcomes (45), a deeper understanding of SCLC heterogeneity, i.e., NE, ML, hybrid and beyond, may translate to benefits in the clinic. Historically, attempts to separate SCLC into subtypes in the clinic were abandoned due to poor reproducibility among pathologists and unclear clinical relevance (46). However, our findings indicate that SCLC heterogeneity is dynamic, since a core TF network specifies both the NE and ML phenotypes, which may confound static associations. The hybrid phenotype may be an additional confounder, exacerbated by the tendency of both NE and ML cells to move toward the hybrid phenotype under treatment. We argue that SCLC heterogeneity is best interpreted as states within a phenotypic landscape, and understanding phenotypic mobility within this landscape could provide ties to clinical relevance.

While SCLC has a high mutation rate, no correlations between mutations and distinct subtypes have been observed (9). In this work we show a non-mutational mechanism for regulation of distinct SCLC cell identities, as attractors of a TF regulatory network. This

framework of equating cell types to attractors was first advanced by Waddington in his eponymous epigenetic landscape (12), and has since been expounded upon by many mathematical biologists (13). It has further been argued that malignant cancer states and cancer heterogeneity are best described by the concept of attractors (14–16). Significantly, this framework has been shown to provide possible therapeutic strategies by identifying targets *in silico* which most significantly perturb the attractor states (17). We do not exclude the possibility that mutations can drive SCLC heterogeneous phenotypes, but our results indicate that epigenetic causes should also be considered.

The Boolean modeling approach used here is an established way of obtaining a coarse-grained picture of large network behavior, and is well suited to identifying the stable states of TF networks (18,19). Our approach was novel in constructing the TF network in blind fashion from SCLC gene expression datasets, TF databases and literature, and was validated by its ability to reproduce correlations with known phenotypes. Significantly our model revealed the existence of a previously unrecognized hybrid phenotype, by showing that some cells do not correlate to either the NE or ML attractors. However, we did not identify attractors correlated with the hybrid phenotype. Hybrid EMT phenotypes have been previously reported in NSCLC (47) and lung adenocarcinoma (48), and other groups have recently reported computational modeling of hybrid EMT phenotypes by driving EMT networks with external stimuli (43,49). Additionally, the Boolean modeling approach cannot capture intermediate levels of expression, and therefore attractors corresponding to the hybrid state may not be identifiable using this method. Continuum modeling approaches may be needed to better understand the hybrid state. Identification of gene co-expression modules enriched in the hybrid phenotype may also reveal additional relevant TFs. Considering all of these possibilities, current ongoing work in our laboratory is directed at identifying hybrid attractors.

Our study establishes the hybrid phenotype as a refuge for drug-treated SCLC cell lines. We anticipate that this phenotype may play a significant role in the evolution of SCLC tumors under treatment, and possibly in relapse. SCLC TF networks may serve as a guide for interventions aimed at preventing phenotypic transitions into resistant states. While we focused on SCLC here, such transcriptional regulation may play a similar role in maintaining non-mutational heterogeneity in other cancer types, and our approach should be generally useful in uncovering underlying mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Identification of two phenotypes in SCLC cell lines and patients

Consensus clustering was used to identify robust clusters of CCLE cell lines (A and C) and CLCGP tumor specimens (B and D). The consensus score is the frequency that a given pair of samples was placed in the same cluster over 1,000 iterations. The cumulative distribution of SCLC cell line (A) and patient (B) consensus scores is shown for values of k from 2 to 10. These results most strongly support 2 transcriptional subtypes in SCLC cell lines and patients, though in cell lines 4 subtypes may be a good fit. Corresponding consensus score heatmaps for SCLC cell lines (C) and patients (D) are shown for k=2 to k=4. Each point

represents a pair of samples, colored by consensus score from white (0, never co-cluster) to blue (1, always co-cluster). $k=5$ to $k=8$ are shown in Figure S1.

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Figure 2. Two anti-correlated gene co-expression networks distinguish phenotypic clusters
 (A and B) Upper panels: Heatmap view of the Blue (A) and Turquoise (B) module genes (rows) across 53 SCLC cell lines (columns). Cell lines are ordered and marked as in Figure 1C consensus clustering. Lower panels: The gene expression profile for each cell line is summarized by the eigengene. The Blue and Turquoise modules had significantly different eigengene expression between the two consensus clusters. (C) The Blue and Turquoise module eigengenes plotted for each cell line reveals anti-correlated expression of Blue vs. Turquoise module (Pearson's correlation: -0.86 , p-value: $1.6e-16$).

Figure 3. Transcription factor network predicts NE and ML attractors

(A) The 33 TF SCLC regulatory network. Green edges indicate activation, while red indicate inhibition. (B) Boolean simulations identified 57 stable attractors (columns). TF expression patterns for all attractors is displayed in rows: shaded cells represent TFs that are ON, white represents OFF. (C) TF expression in the CCLE dataset reveals similar expression patterns to *in silico* attractors. (D) Correlation score between attractors (columns) and cell lines (rows). Positive correlation is indicated in red, no correlation in yellow, and negative correlation in white. Attractors 49–25 show high correlation with the NE cell lines, while attractors 35–34 show high correlation with the ML cell lines. Nevertheless several cell lines are uncorrelated with NE or ML attractors, possibly revealing a hybrid phenotype. (E) Pearson correlation scores were computed as in (D) between the 57 attractors (columns) and 28 SCLC patient tumor specimens from the CLCGP (rows).

Figure 4. Experimental validation of TF network states in human SCLC

(A) 5 NE TFs (FOXA2, OVOL2, SOX2, ASCL1, and LEF1) and 5 ML TFs (SMAD3, MYC, NFKB1, ZEB1, MITF) validated by western blot. The NE TFs show higher expression in the NE cell lines (dark blue, as in consensus clustering) than the ML cell lines (light blue), and vice versa. Nevertheless, several cell lines showed similar levels of expression of both NE and ML TFs, including NCI-H146, NCI-H209, NCI-H1184, DMS53, NCI-H1048, NCI-H446. Many of these cell lines, such as DMS53, also showed poor correlation with any attractor in Figures 3 and S8, and were near the center in Figure 2C, suggesting a non-NE and non-ML phenotype. These cell lines are tentatively denoted as “hybrid” phenotypes. (B) CD56 (a NE marker) and CD44 (a ML marker) were probed by western blots in 10 SCLC patient samples. Most patients show expression of only one or the other, however Pt112-1, Pt216-1, and Pt460-1 show double positive expression of both markers, suggesting the hybrid phenotype may be important in patients as well.

Figure 5. Single-cell level expression of phenotypic biomarkers in SCLC cell lines reveals hybrid cells

Flow cytometry was performed using 4 NE surface markers (CD56, CD24, CADM1, and ALCAM) and 2 ML surface markers (EPHA2 and CD151). Axes represent unweighted sums of normalized NE and ML fluorescence. (A) CORL51, NCI-H146, and NCI-H2141 were classified as NE by consensus clustering, and single cells show NE⁺ML⁻ phenotype. (B) DMS53, NCI-H446, and NCI-H1048 showed comparable levels of expression of NE and ML TFs (Figure 4), Blue- and Turquoise-module eigengenes (Figure 2), and poor correlation with either NE or ML attractors (Figures 3, S8). At the single cell level, these cells also show similar levels of expression of NE and ML markers, suggesting these cells are in a hybrid NE⁺ML⁺ or NE⁻ML⁻ phenotype, not well described by a NE vs ML dichotomy. The hybrid state is characterized by cells along the diagonal of these plots. (C) SW1271, NCI-H841, and DMS114 were consistently classified as ML, and single-cells exhibit NE⁻ML⁺ phenotype.

Figure 6. Phenotypic modulation by treatment pushes cells toward hybrid state

NE, ML, and Hybrid (HY) cell lines (from Figure 5) are combined to show overall phenotypic responses. NE and ML axes are the same as defined in Figure 5. Each column represents a unique treatment condition: Untreated (column 1); 1uM Etoposide (column 2); 1uM Cisplatin (column 3); 1uM Azacitidine (column 4); 5mM Valproic Acid (column 5). Cells were treated for 2 days. (A) The NE cell lines show a shift toward the NE+ML+ or NE-ML- quadrants. (B) The HY cell lines show no significant shift. (C) The ML cell lines show a shift toward the NE+ML+ quadrant.