Integrative genomic and epigenomic analyses identify a distinct role of c-Myc and L-Myc for lineage determination in small cell lung cancer

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Abstract

Small cell lung cancer (SCLC) is the most aggressive subtype of lung cancer that has a dismal prognosis. Patients with SCLC are uniformly treated with chemotherapy plus radiotherapy and recently with immunotherapy; however, emerging molecular evidence suggests this disease represents heterogeneous groups. At the genomic level, in addition to the near universal loss of p53 and Rb1, the loci for all three Myc family members are frequently amplified in a mutually exclusive manner, suggesting their redundant oncogenic function. However, the expression of these factors is associated with specific neuroendocrine markers and distinct histopathology, and yet the contribution of Myc family members to SCLC molecular subtypes have not been clearly delineated. In this study, we explored a novel role of c-Myc and L-Myc as lineage specific factors contributing to SCLC molecular subtypes and histological classification. Integrated analyses of a Bayesian network generated from primary tumor mRNA expression and chromatin state profiling of SCLC cell lines showed that Myc family members impart distinct transcriptional programs associated with lineage state; wherein L-Myc was enriched for neuronal pathways and c-Myc for Notch signaling and epithelial-to-mesenchymal transition. We investigated the functional redundancy of c-Myc and L-Myc, and noted the insufficiency of L-Myc to induce lineage switch in contrast to the potential of c-Myc to induce trans-differentiation from ASCL1-SCLC to a NeuroD1-SCLC characterized by distinct histopathology. Collectively, our findings reveal a previously undescribed role for historically defined general oncogenes, c-Myc and L-Myc, in regulating lineage plasticity across molecular subtypes as well as histological classes.

Introduction

Small cell lung cancer (SCLC) represents about 15% of all lung cancers with a median survival time of approximately 10 months and 5-year overall survival at 6% (Siegel et al., 2019). SCLC tumors are characterized by neuroendocrine differentiation, rapid growth, early metastatic spread and poor prognosis (Gazdar et al., 2017). The standard of care has remained cytotoxic chemotherapy for decades, mainly etoposide combined with a platinum agent (Alvarado-Luna et al., 2016), that are only temporarily effective for the vast majority of patients (Pietanza et al., 2015). Even with recent developments in immune checkpoint inhibitors, activity against SCLCs when combined with chemotherapy has been marginal with a modest improvement in the median survival (10.3 months vs. 12.3 months) for extensive stage SCLCs treated with immunotherapy (Horn et al., 2018). These data reflect the urgent need for more effective therapeutics for patients with SCLC. The lack of effective therapeutics for SCLC stands in stark contrast to the breadth of targeted therapies for non-small cell lung cancer (NSCLC), particularly lung adenocarcinoma (Rudin et al., 2019). This progress in drug development for NSCLCs is largely attributable to a more comprehensive understanding of molecular subtypes and to identification of targetable driver oncogenes (Zappa et al., 2016). Therefore, better characterization of the molecular subtypes of SCLC should aid future drug development and permit patient stratification for targeted therapies, a strategy that has been remarkably effective for specific subsets of advanced lung adenocarcinoma patients.

Characterization of SCLC subtypes was first noted by morphological differences over three decades ago when human SCLC cell lines were implanted as xenografts and distinguished as two primary subtypes: classical SCLC and variant SCLC (Carney et al., 1985; Gazdar et al. 1985). The classical subtype featured relatively small cells with high nuclear:cytoplasm ratio

while the variant subtype exhibited relatively larger cells and moderate amounts of cytoplasm. However, the World Health Organization (WHO) classification, updated in 2015, histologically recognizes SCLC as a homogenous disease with neuroendocrine features defined by small cells, scant cytoplasm, nuclear morphology with fine granular chromatin and lacking prominent nucleoli, reminiscent of the features of the 'classical' SCLC. The originally described 'variant' subtype may represent combined SCLC with LCNEC in the current classification (Travis et al., 2015).

More recent efforts to distinguish SCLC molecular subtypes include profiling gene expression and genome-wide methylation in primary human tumors and patient derived xenografts (PDX). Profiling revealed three clusters, with a dichotomy between achaete-scute homologue 1 (ASCL1) and neurogenic differentiation factor 1 (NeuroD1) expression, in addition to a cluster with low expression of both (Poirier et al., 2015). The expression of ASCL1 and NeuroD1 has been implicated to confer SCLC heterogeneity by imparting distinct transcriptional profiles (Borromeo et al., 2016). The third neuroendocrine-low cluster led to further classification into two subtypes characterized by transcriptional driver YAP1 or POU class 2 homeobox 3 (POU2F3) (McColl et. Al., 2017, Huang et al., 2018). SCLC cell line xenograft histology has been correlated with these contrasting factors, where variant SCLC was positively correlated with a higher NeuroD1:ASCL1 ratio and classical SCLC was positively correlated with a higher ASCL1:NeuroD1 ratio in SCLC cell lines (Poirier et al., 2013).

Recapitulating Myc family members in SCLC genetically engineered mouse models (GEMMs) has provided insight in their contribution to histopathological characteristics (Brägelmann et al., 2017, Kim et al., 2016). The classical SCLC GEMM with conditional loss of

Rb1 and *Trp53* mouse, harbored stochastic *MYCL* amplifications or overexpression associated with classical SCLC histopathology (Meuwiseen et al., 2003). On the contrary, consistent with the original report on the variant subtype to harbor frequent *MYC* amplification (Gazdar et al. 1985), a more recent study showed that additional c-Myc overexpression, in a classical SCLC GEMM drives the progression of murine SCLC with variant histopathology and reduced neuroendocrine gene expression including ASCL1 but higher NeuroD1 expression (Mollaoglu et al., 2017). Yet, the molecular mechanisms underlying the distinction between L-Myc and c-Myc driven subsets of SCLC remain unexplored.

c-Myc (*MYC*) and L-Myc (*MYCL*) belong to the MYC family of basic helix-loop-helix (bHLH) transcription factors. The paralogs contain functionally relevant highly conserved amino acid sequences and are structurally homologous (DePinho et al., 1987b, Conacci-Sorell et al., 2014). c-Myc is a well-characterized oncogene; L-Myc although understudied is implicated to have a similar oncogenic role. Amplification of Myc family members is mutually exclusive and overall accounts for ~20% of SCLC and overexpression for ~50% of SCLC in primary human tumors (George et al., 2015). In contrast to the fact that *MYC* is commonly amplified across all three major lung cancer subtypes: lung adenocarcinomas, squamous cell lung carcinomas and SCLC (Weir et al., 2007, Hammerman et al., 2012, George et al., 2015), *MYCL* and *MYCN* are uniquely amplified in SCLC, in a manner suggestive of their role as lineage-amplified genes.

In this study, we explore a novel role of c-Myc and L-Myc as lineage specific factors to associate SCLC molecular subtypes with histological classes. We investigated the potential of L-Myc and c-Myc to regulate lineage state and identified transcriptional programs unique to each Myc family member, wherein L-Myc regulates neuronal developmental pathways and c-Myc

regulates epithelial-to-mesenchymal transition and Notch signaling, biological pathways that are associated with distinct molecular subsets. In addition, we showed the requirement of c-Myc to maintain lineage state marker NeuroD1, in NeuroD1-positive SCLC and the incompatibility of c-Myc with ASCL1-positive SCLC that ultimately leads to trans-differentiation to NeuroD1-driven SCLC characterized by variant histopathology mediated by a transcriptional repressor, Rest.

Results

SCLC network reveals unique and distinct sub-networks for c-Myc and L-Myc

To investigate the biological processes unique to c-Myc and L-Myc in primary SCLC, we built a molecular regulatory network for SCLC. Combining two independent primary SCLC transcriptomic datasets (George et al., 2015, Jiang et al., 2016), a Bayesian network was built using the software package RIMBANET (see Methods). The Bayesian network comprised of 8,451 unique genes (nodes) and 9,301 regulations (edges) among these genes. Network neighbors for genes of interest can provide insights into biological functions that genes of interest are associated with (Zhu et al., 2007, Zhu et al., 2008, Zhu et al., 2012). This enables us to discern transcriptional sub-networks associated with c-Myc and L-Myc that may reflect their unique biological role. To this end, we first aimed to generate a signature associated with each factor and then projected the signature to the SCLC Bayesian network to identify specific subnetworks (Figure 1A). To generate this signature, we examined *MYC* and *MYCL* mRNA expression in SCLC cell lines from Cancer Cell Line Encyclopedia (CCLE) and selected cell lines with either high expression of *MYC* or *MYCL*, then defined gene signatures for *MYC* expressing and *MYCL* expressing subsets (Figure S1A). We identified 457 differentially

expressed genes; 147 genes overexpressed in *MYC* and 310 overexpressed in *MYCL* in SCLC cell lines (t-test p-value < 0.01 and fold change > 1.5) (Figure S1B).

To explore the sub-networks associated with L-Myc, we projected the genes upregulated in *MYCL* expressing subset onto the network (Methods). We identified one large closed sub-network (L1, Figure 1B) comprising of 959 gene nodes that included 120 of 310 genes from the L-Myc signature. Gene ontology analysis of this L-Myc subnetwork revealed enrichments of two biological processes: cell cycle progression and neuronal development (Figure 1C). These two pathways have been previously implicated as core descriptors of classical SCLC (Gazdar et al., 2017). On the other hand, when we projected the c-Myc signature onto the network, the c-Myc network was organized into three unique sub-networks c1, c2 and c3, comprised of 95, 29 and 25 gene nodes, respectively (Figure 1D). Each individual c-Myc sub-networks were enriched in three distinct biological processes. Sub-network c1 was enriched for canonical c-Myc functions in transcriptional, translational and metabolic regulation (Figure 1E) (Conacci-Sorell et al., 2014). c-Myc sub-network c2 was enriched for Notch signaling pathway (Figure 1E), which has been implicated to mediate a transition from neuroendocrine to non-neuroendocrine fate of tumor cells in a murine SCLC model (Lim et al., 2017). Finally, sub-network c3 was enriched for pathways in epithelial-to-mesenchymal transition (Figure 1E); that has been shown to be relevant in the neuroendocrine-low subtype (Zhang et al., 2018). These functional pathways enriched in subnetworks c2 and c3 imply previously uncharacterized role of c-Myc in lineage state determination in SCLC.

Together, these findings suggest that c-Myc and L-Myc are associated with differential molecular mechanisms that have been implicated in SCLC biology. Specifically, it implicates c-Myc is associated with neuroendocrine-low differentiation state in addition to its canonical

oncogenic functions in SCLC, and by contrast, L-Myc is associated with classic neuroendocrine state of SCLC.

c-Myc and L-Myc driven SCLCs exhibit distinct chromatin states

Next, we sought to determine whether the distinct c-Myc and L-Myc networks are associated with their distinct cistromes. To select an appropriate model system to examine the role of each Myc family member, we examined copy number alteration and expression for each Myc family member in 49 SCLC cell lines from CCLE and classified them into four groups representing each Myc family member and low Myc (Figure S1C). Based on this classification, we selected representative cell lines for c-Myc (NCI-H82, NCI-H524, NCI-H2171 and NCI-H2081) and L-Myc (CORL-88, NCI-H1963 and NCI-H209) and confirmed protein expression for these transcription factors (Figure 2A). In order to define open regulatory elements potentially regulated by c-Myc and L-Myc, we performed the assay for transposase-accessible chromatin-sequencing (ATAC-seq) on those representative cell lines and filtered identified accessible chromatin regions with motif matrices for Myc (E-box) referring to as Myc accessible regions hereafter (Figure 2B). Of note, inferred Myc accessible regions reasonably recapitulated c-Myc binding sites previously determined by c-Myc chromatin-immunoprecipitation in NCI-H2171 cells (Lin et al., 2012) (Figures S3A and S3B).

Principal component analysis of peaks containing the E-box motif supported cell line classification according to Myc status (Figure 2C). Additionally, a correlation matrix of pairwise Pearson correlation coefficient of Myc accessibility signal captured distinct clusters for the c-Myc and L-Myc classified cell lines (Figure S3C). We observed a fraction of peaks (cluster 1;

Figure 2D) that overlap between c-Myc classified and L-Myc classified cells, suggestive of common functional binding that the Myc family members share. We also found distinct groups of peaks with higher signal intensity unique to L-Myc classified cells (cluster 2; Figure 2D) and in c-Myc classified cell lines (cluster 3; Figure 2D). Of note, the chromatin landscape of NCI-H82 appeared to be intermediate to the L-Myc and c-Myc profiles. Together, these findings suggest that c-Myc and L-Myc impart differential transcriptional programs.

Myc family members exert differential transcriptional programs

To gain insights into the unique biological processes c-Myc and L-Myc may impart, we analyzed differentially enriched Myc accessible sites comparing between c-Myc cell lines and L-Myc cell lines. We identified 2,808 differentially accessible regions; 1,235 peaks enriched in L-Myc classified cell lines and 1,573 peaks enriched in c-Myc classified cell lines (Figure 3A).

Next, we performed GREAT analysis (McLean et al., 2010) on the differentially accessible peaks. Unique L-Myc accessible sites are enriched for neuronal pathways such as glial cell proliferation, development of the neural plate, neural fold and future midbrain (Figure 3B). We observed augmented accessibility at neuronal development associated genes such as the *FOXA2* locus in L-Myc cell lines when compared to c-Myc cell lines (Figure 3D). By contrast, unique c-Myc accessible sites are enriched for pathways involved in Notch signaling (Figure 3C). Accordingly, we observed preferential accessibility in c-Myc classified cell lines at Notch targets such as *REST* (Figure 3E). While specific Notch signaling activity in *MYC*-amplified SCLCs has not been previously noted, there have been previous reports showing Rest as a target of the Notch signaling pathway to suppress the expression of neuronal genes (George et al., 2015, Lim et al., 2017). These findings are consistent with the observations from the Bayesian

network analysis providing additional evidence to suggest distinct transcriptional programs in c-Myc and L-Myc driven SCLCs.

c-Myc and L-Myc expression is associated with distinct lineage state markers

Previous literature showed that deletion of *Mycl* (L-Myc) in the classical murine SCLC model led to the formation of mixed and NSCLC morphology instead of classical SCLC features (Kim et al., 2016) in contrast to the c-Myc expressing SCLC with 'variant' morphology (Gazdar et al.,1985; Mollaglu et al., 2017). Therefore, given our findings from the Bayesian Network and Myc accessibility profiling, we hypothesized that the distinct programs regulated by Myc family members establish these unique lineage states in SCLC.

A recent review synthesized SCLC profiling studies to identify four molecular subtypes that are driven by four transcription factors ASCL1, NeuroD1, YAP1 and POU2F3 (Rudin et al., 2019). The accessibility profile of our c-Myc and L-Myc classified cell lines at the genetic loci for these four factors revealed augmented accessibility in the c-Myc classified cell lines at the *NeuroD1* locus in contrast to L-Myc classified cell lines that had augmented accessibility at the *ASCL1* locus (Figure 3F, 3G). We observed no differential or preferential accessibility signal in both c-Myc classified and L-Myc classified cell lines at *YAP1* and *POU2F3* loci likely reflecting that these cell lines do not represent these subtypes (Figure S3A, S3B). Protein expression of these lineage state markers showed that our L-Myc classified cell lines had exclusive expression of ASCL1 while the c-Myc classified cell lines exclusively expressed NeuroD1, consistent with the chromatin profile. (Figure S3C). To understand how c-Myc and L-Myc expression correlated with the four molecular subtypes, we examined the expression of c-Myc and L-Myc in each of the molecular subtypes in CCLE cell lines as classified in Rudin et al., 2019. We observed that

the neuroendocrine low: POU2F3 and YAP1 and NeuroD1 subtypes had higher expression of c-Myc (Figure S3D). On the other hand, L-Myc expression was enriched in the neuroendocrine high ASCL1 subtype (Figure S3E). We further sought to reproduce the classification with data from primary tumors (George et al., 2015) and CCLE cell lines with additional data from 79 primary tumors (Jiang et al., 2016) (Figure S3F). Of note, while our unsupervised clustering was in general agreement with the published classification (Rudin et al., 2019), distinction between ASCL1 and NeuroD1 subtypes was relatively ambiguous. In particular, a small distinctive cluster within the NeuroD1 cluster in our dendrogram segregates other samples previously classified as NeuroD1 subtype which clustered closer to the ASCL1 cluster. In this classification, we found that the expression of c-Myc was higher in neuroendocrine low: POU2F3 and YAP1 while L-Myc was higher in ASCL1 and NeuroD1 subtype. Within neuroendocrine subtypes, the difference of c-Myc expression between ASCL1 and NeuroD1 subtypes was not statistically significant. We noted, however, c-Myc expression was significantly higher in samples categorized in NeuroD1 with a higher NeuroD1:ASCL1 ratio (as originally described in Poirier et al., 2015) (Figure S3I). The discrepancy may imply an important distinction between the biology of cell lines and primary tumors and/or the heterogeneity of primary tumors with the dual expression of ASCL1 and NeuroD1 in a significant proportion of NeuroD1 classified tumors (Figure S3F), which may indeed reflect lineage transition. Together, these data suggest the dichotomy of c-Myc and L-Myc expression in ASCL1-SCLC.

c-Myc plays a dominant role in lineage specification

Given that L-Myc and c-Myc expression are enriched in distinct subtype(s) and they regulate programs associated with its respective subtype, we hypothesized that part of the role

Myc family members play is to serve as a lineage factor. To test the potential of L-Myc to establish the neuroendocrine lineage, we modified a c-Myc expressing NeuroD1 classified cell line NCI-H82 by exogenous overexpression of L-Myc and CRISPR-Cas9 mediated deletion of c-Myc. We found c-Myc expressing cell line (NCI-H82) was able to tolerate the over-expression of L-Myc (Figure 4A). Cells engineered to express both L-Myc and c-Myc exhibited a modest increase in proliferation rates compared to parental cells expressing only c-Myc (Figure 4B). We further demonstrated that replacement of c-Myc with the expression of L-Myc allowed the cells to retain proliferative potential in the absence of c-Myc in NCI-H82 (Figure 4B). This indicates redundant roles among the two Myc family members in regards to maintaining cell viability.

To inquire their contribution to lineage determination, we profiled the expression of known lineage state markers. Addition of L-Myc neither induced expression of neuroendocrine-marker ASCL1 nor altered the expression of variant state marker NeuroD1 (Figure 4A), suggesting that L-Myc is not sufficient to induce lineage switch to classical neuroendocrine state. This is compatible with the reported infrequency of a transition from non-neuroendocrine to neuroendocrine state (Lim et al., 2017). By contrast, the replacement of c-Myc with L-Myc led to the downregulation of NeuroD1 while ASCL1 expression was not induced even upon the loss of c-Myc. (Figure 4A). To further investigate if the downregulation of NeuroD1 expression was a combinatorial effect of L-Myc expression and c-Myc deletion, we compared with simple ablation of c-Myc in NCI-H82 (Figure 4C). Without L-Myc replacement, consistent with previous findings (Jain et al., 2002), c-Myc was essential for survival in NCI-H82 (Figure 4B), precluding us from evaluating converted lineage state. However, during the short window of survival, c-Myc ablation led to significant downregulation of lineage state marker NeuroD1 suggesting a role for c-Myc on maintaining expression of lineage state marker. The expression of

neuroendocrine-low lineage state marker, YAP1, was not induced, suggesting a lineage negative state for these cells (Figure 4A).

To investigate the state of these lineage-marker negative cells, we performed transcriptomic profiling. First, we investigated the effect of expressing of L-Myc in the presence of c-Myc in NCI-H82, which revealed 235 significantly upregulated genes and 290 downregulated genes (Figure S4A). Gene ontology analysis of upregulated genes revealed enrichment for neurogenesis and neuronal associated pathways (Figure S4B), suggesting that L-Myc imparts neuronal pathways while not fully inducing ASCL1-lineage state. Next, we investigated the profile of the lineage negative-NCI-H82 cells that with ectopic expression of L-Myc and ablation of c-Myc expression compared to NCI-H82 cells with ectopic L-Myc alone, and found genes involved in neuronal structure were enriched in the 170 upregulated genes, suggesting the cells are further committed to a neuronal state (Figure S4C and S4D). A direct comparison of the transcriptome control NCI-H82 cells with lineage negative-NCI-H82 cells, revealed significantly 458 upregulated genes enriched for neuronal associated pathways and the downregulated genes enriched for canonical Myc function (Figure 4C and 4D). Collectively, the data suggest that replacement of c-Myc with L-Myc induces a profile that represents more neural state but not fully transdifferentiates to ASCL1-positive state and remains to be negative for lineage markers used in the current consensus molecular classification (Rudin et al., 2019).

Aurora Kinase A Inhibition sensitivity is altered with change in Myc status

Previous findings suggest that *MYC* (c-Myc)-driven SCLC is more responsive to Aurora kinase A inhibition (Mollaoglu et al., 2017, Dammert et al., 2019). We confirmed previous findings that *MYC*-amplified cell lines exhibit increased sensitivity and *MYCL*-amplified cell

lines are resistant to alisertib (Figure S5A). Alisertib treatment of the NCI-H82 cells expressing both c-Myc and L-Myc revealed no changes in sensitivity to the drug nor did changes in expression of the variant state marker. However, the cells that replaced c-Myc with L-Myc became resistance to aurora kinase inhibition (Figure S5B). On the other hand, overexpression of c-Myc increased sensitivity at lower concentrations of the drug (Figure S5C). This suggests either alisertib sensitivity is attributable to c-Myc driven lineage state of the cells, or difference in molecular interaction of Aurora kinase A with each Myc protein.

c-Myc causes loss of classical neuroendocrine SCLC features

The dependency of lineage state marker NeuroD1 on c-Myc led us to hypothesize that c-Myc exerts a role in addition to its oncogenic role to regulate and establish a variant differentiation state. To this end, we genetically engineered a neuroendocrine L-Myc expressing cell lines NCI-H1963 with inducible exogenous overexpression of c-Myc (Figure 5A). Of note, exogenous expression of c-Myc led to downregulation of L-Myc in NCI-H1963.

When c-Myc expression is introduced in neuroendocrine-high *MYCL*-amplified NCI-H1963, we observed an initial phase of growth suppression (Figure 5B). This observation stands in contrast to the established role of c-Myc to promote cell cycle and cell proliferation (Dang, 2012). The growth suppression is unlikely to be due to oncogene induced senescence as these cells are deficient for Rb1 and p53 and did not stain positive for beta galactosidase (Figure S6A). Next, we investigated the cell cycle dynamics of NCI-H1963 c-Myc expressing cells in their growth suppressive phase, and found that initial c-Myc expression (day 2) modestly increased the proportion of cells in S and G2/M phase but did not significantly alter the distribution of cells or induce cell cycle arrest to explain slower cell growth (Figure S6B). Alternatively, we sought

to investigate whether NCI-H1963 cells that did not tolerate the expression of c-Myc reached cell death. We investigated the proportion of Annexin-V positive cells on day 2 and found c-Myc overexpression increased the proportion of necrotic and apoptotic cells as compared to control cells (Figure 5C).

We hypothesized that the cell death was a consequence of incompatibility of the neuroendocrine differentiation state with c-Myc expression and that the persisting cells eventually grown out were cells that tolerated the expression of c-Myc, potentially by switching into a differentiation state compatible with c-Myc expression (Figure 5B). Therefore, to inquire if the tolerant cells transitioned to an altered lineage state, we investigated the expression of lineage state markers and found that the overexpression of c-Myc downregulated ASCL1 and upregulated NeuroD1 in H1963 cells (Figure 5A), indicating the trans-differentiation to NeuroD1-expressing subtype of SCLC. The switch suggests that c-Myc exerts a role in dictating a distinct neuroendocrine differentiation state defined by NeuroD1 expression. To investigate if the trans-differentiation between molecular subtypes was accompanied by histological class switch *in vivo*, we injected NCI-H1963 cells genetically engineered with stable c-MYC expression. We observed that NCI-H1963 overexpressed with LacZ (control cells) had oval and elongated nuclei with a high nuclear:cytoplasm ratio, smooth granular chromatin, no nucleoli representative of classical SCLC histology and NCI-H1963 overexpressed with c-Myc showed presence of nucleoli and polygonal shaped larger cells, reminiscent of variant SCLC histology as defined by Gazdar et al., 1985 (Figure 5D, Figure S6C). The histopathology observed here, according to the current WHO classification, would likely be recognized as either “combined SCLC” that includes a component of large-cell neuroendocrine carcinomas (LCNEC) or purely LCNEC, potentially reflective of the discrepancies between clinical practice and experimental

findings (Travis et al., 2015, Rekhtman, 2010). Together, these results suggest that c-Myc expression drives the transition from ASCL1-positive state characterized by classical SCLC histology to NeuroD1-positive state SCLC characterized by SCLC/LCNEC histology.

c-Myc mediates trans-differentiation through REST

We sought to investigate the mechanism through which c-Myc mediates trans-differentiation to the ASCL1-negative/NeuroD1-positive state. To this end, we profiled the accessible chromatin profile of trans-differentiated NCI-H1963. Filtering the regions to reflect regions with Myc (E-box) motif matrices reflected a common pattern (clusters 1 to 3). c-Myc overexpression resulted in the gain of additional regulatory regions (cluster 4) and a decrease in accessibility at sites in cluster 5 (Figure 6A), revealing an alteration of the MYC (E-box) cistrome and further suggesting non-redundancy of this family of transcription factors. To identify the transcriptional programs associated with the changes in regulatory regions of the MYC cistrome, we performed RNA-seq on trans-differentiated NCI-H1963. We identified 115 differentially expressed genes (70 genes downregulated and 45 genes upregulated) (Figure S7A). Gene ontology analysis for upregulated genes revealed an enrichment for cell death and apoptosis (Figure 6B, upper panel) while gene ontology analysis for downregulated of genes revealed an enrichment for neuronal associated genes (Figure 6B, lower panel). These findings are consistent with the initial growth suppression and the loss in neuroendocrine lineage state marker (Figure 5A and Figure 5B), but did not reveal further specific mechanistic insights.

Previous works have shown the negative regulation of ASCL1 by Notch signaling during lung development (Ito et al., 2000) and activation of Notch signaling in SCLC suppresses ASCL1 expression (George et al., 2015, Augert et al., 2019, Oser et al., 2019). Additionally, our

SCLC transcriptional network analysis and Myc accessibility data revealed Notch pathway activation preferentially in c-Myc expressing tumors and cell lines (Figures 1E and 3C). Although we did not observe an enrichment for an active Notch signaling signature amongst the upregulated genes, when we investigated the expression of known Notch signaling pathway targets in trans-differentiated NCI-H1963, we identified Notch-target RE-1 Silencing transcription factor (REST) that is a transcriptional repressor of neuronal genes and ASCL1 (Lim et al, 2017) as one of the genes that were induced upon c-Myc expression (Figure S7B) and we confirmed this with protein expression (Figure S7C). Therefore, we sought to investigate the c-Myc–Notch–Rest axis in mediating trans-differentiation, we pharmacologically inhibited Notch1-4 using a γ-secretase inhibitor (GSI), DBZ (Ran et al., 2017) in combination with c-Myc expression in NCI-H1963 cells. Inhibition of Notch signaling, did not alter expression of Rest and ASCL1 (Figure 6C). Next, to investigate if Rest is required for c-Myc mediated suppression of ASCL1, we used pharmacological inhibition of Rest (Charbord et al., 2013) in c-Myc over-expressed NCI-H1963 cells. We confirmed treatment with Rest inhibitor (X5050) led to a decrease in Rest protein expression. Surprisingly, simultaneous suppression of Rest and expression of c-Myc did not result in decrease in the level of ASCL1 (Figure 6D). These data suggest a novel Notch-independent, c-Myc mediated activation of Rest and NeuroD1 and Rest mediated-suppression of ASCL1.

Discussion

In this study, we showed a novel functional distinction between c-Myc and L-Myc in SCLC, in contrast to the prevailing notion that molecular function of Myc family members are similar to each other (Kim et al., 2016). We found c-Myc is associated not only with canonical roles

including transcriptional and translational regulation, but also with lineage associated pathways such as Notch signaling and epithelial-mesenchymal transitions. Our findings are supported by a previous report that described active Notch signaling negatively regulating neuroendocrine lineage by suppressing the expression of *ASCL1* (Lim et al., 2017) as well as a study that showed mesenchymal-like features in neuroendocrine-low sub-type of SCLC (Zhang et al., 2018). By contrast, we found L-Myc is associated with neuronal pathways implicating their relevancy in determining the classical neuroendocrine state of SCLC. These findings are consistent with previous work showing a correlation of expression between L-Myc and neuronal proteins (Kim et al., 2006). Taken together, contrary to the notion that Myc family members, c-Myc in particular, exclusively act as oncogenes and general amplifiers of expression, our data suggest that they additionally exert lineage defining transcriptional programs (Lin et al., 2012). We propose that the Myc family members regulate a defined set of transcriptional programs that are essential in SCLC lineage subtype determination. Of note, although these biological pathways associated with these factors have been shown to be relevant for lineage maintenance and determination in distinct subtypes of SCLC but their association with Myc family members has not been described before.

We found L-Myc failed to induce the expression of neuroendocrine lineage state marker, ASCL1 suggesting its inability to push trans-differentiation from neuroendocrine-low/variant state to neuroendocrine high/classical state. However, based on previous work suggesting requirement of L-Myc to drive tumors of neuroendocrine origin in SCLC GEMM (Kim et al., 2016) and our findings that suggest L-Myc regulates and induces neuronal development pathways, L-Myc likely plays roles in lineage state maintenance and/or is more compatible with the neuroendocrine lineage.

We also report a previously undescribed function for c-Myc in lineage maintenance in NeuroD1-positive SCLC. c-Myc expression was incompatible with ASCL1-positive SCLC and induces trans-differentiation to NeuroD1-positive state revealing the role for c-Myc in regulating lineage plasticity. We show that this trans-differentiation between molecular subtypes of SCLC is accompanied by alterations in the transition of histopathology in SCLC from classical SCLC features to variant histopathology. These data reflect that the variant histopathology, that would typically be classified as “combined” SCLC or LCNEC, per the current guidelines of WHO, can indeed be derived from SCLC. This phenotype is reflective of biology distinct from classical SCLC and may warrant distinct therapeutic strategies.

We identified that the trans-differentiation is mediated independent of Notch signaling but through the direct activation of a Notch signaling target, Rest, a transcriptional repressor. These findings may be clinically important when evaluating the rationale for targeting SCLC by activating Notch signaling with drugs including LSD1 and KDM5A (Augert et al., 2019, Oser et al., 2019). Our data suggests that downstream actors of Notch signaling may induce the trans-differentiation to NeuroD1-positive or variant subtype. This may not be a favorable outcome since the variant subtype has been reported to be more frequent in tumors after initial response to chemotherapy (Gazdar et al., 1985, Mollaglu et al., 2017). We found that the altered differentiation state with the gain of L-Myc and loss of c-Myc cells conferred resistance to Aurora Kinase A inhibition and in contrast the trans-differentiation as a result of c-Myc gain and loss of L-Myc cells conferred sensitivity to Aurora Kinase A inhibition. The differential sensitivity associated with distinct lineage state with Myc family member status as a biomarker could translate to significant clinical implications in stratification of patients for targeted therapy.

SCLC is a recalcitrant disease typically characterized by neuroendocrine differentiation, nonetheless approximately 10-20% of SCLCs may lack expression of diagnostic neuroendocrine markers (Rekhtman, 2010). Here we report, the plasticity between these histological subtypes and molecular subtypes regulated by c-Myc and L-Myc. The role in lineage determination and maintenance for this family of transcription factors is striking since the Myc family has historically been grouped as general oncogenes. Our data suggests that the role of Myc family in SCLC tumorigenesis could be re-defined. This will enable us to categorize these subtypes to develop effective therapies to combat this highly lethal disease.

Methods

CCLE RNA-seq analysis

RNA-seq gene expression data and SNP-array copy number data for *MYC*, *MYCL* and *MYCN* were downloaded from (CCLE) web site (http://www.broadinstitute.org/ccle/home). Log_2(TPM (transcripts per million) +1) values for mRNA expression and log_2(inferred copy number/2) values for copy numbers were used to depict a heatmap for a 49 SCLC cell lines.

Bayesian Network Analysis

A comprehensive regulatory network was built using the software suite, Reconstructing Integrative Molecular Bayesian Network (RIMBANet) (Zhu et al., 2004) by combining RNAseq data from two independent SCLC primary cohorts (George et al, 2015; Jiang et al., 2016) (detailed in Yoo et al. manuscript in preparation). To identify sub-networks enriched for L-Myc and c-Myc signatures, the gene signature was overlaid on the network to include genes in the signature and its two-layered neighbors. Then, Key Driver Analysis (Liu et al., 2019) identified master regulators associated with L-Myc and c-Myc signatures in two steps. First, for each node in the network, we compared neighboring nodes within two layers for each signature to obtain a list of potential master regulators whose overlap is statistical significant (Fisher's exact test (FET) p-value<0.05/(# of nodes)). After sorting candidate genes by corresponding FET p-values, the gene with the strongest p-value was determined as a key regulator. Any candidate regulators in its two-layered neighbors were excluded from the candidate list. The process was iterated throughout the sorted candidate list. Enriched functions were identified using GO terms in Molecular Signatures Database (MSigDB). The significance of the enrichment was determined

based on FET p-value considering multiple testing (FET $p<0.05/(\#\text{ of dataset tested})$) in each category.

Cell Lines

All cells were cultured at 37°C in a humidified incubator at 5% CO_2. SCLC cell lines NCI-H2171, NCI-H82, NCI-H524, NCI-H209, NCI-H1963, CORL88, HCC33, NCI-H69, and NCI-H526 were maintained in RPMI1640 (Gibco), and supplemented with 10% Fetal Bovine Serum (FBS, Sigma) and 1mM Penicillin Streptomycin (P/S). HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), and supplemented with 10% FBS (Sigma) and 1mM P/S. Cultured cells were regularly tested for mycoplasma using the mycoAlert Detection Kit (Lonza).

ATAC-seq

ATAC-seq was performed as previously described (Buenrostro et al., 2013). Five thousand cells were harvested and pre-treated with DNase I (Thermo Fisher Scientific) at 37°C for 20 minutes to eliminate DNA contamination from dead cells. The cells were washed in cold Phosphate-buffered saline (PBS) to eliminate traces of DNase. Nuclei were isolated with nuclear lysis buffer (10 mM Tris, 10mM NaCl, 3mM $MgCl_2$ 0.1% IGEPAL-630) and centrifuged at low speeds. The nuclei were resuspended in transposase reaction mixture (10mM Tris PH 8.0, 5 mM $MgCl_2$, 10% Dimethlyformamide (DMF), 0.2 mg/ml Tn5 transposase complex). Transposition was carried out at 37°C for 30 minutes followed by DNA purification with DNA Clean and Concentrator-25 (Zymo Research) according to manufacturer's recommendation. Following purification, library fragments were PCR amplified with Nextera adapter primers. Sequencing

was performed at Tisch Cancer Institute sequencing core on the NextSeq500 (Illumina) for 38 nucleotides each from paired ends according to manufacturer's instructions.

ATAC-seq data analysis

Illumina sequencing adapter was removed using Cutadapt from raw sequence files in fastq format. The reads were aligned to the hg19 reference genome using Bowtie2 with -k 1 parameters. The aligned reads were used to eliminate PCR duplicates using samtools and filtered off an ATAC blacklist for mitochondrial DNA homologs generated by *Buenostro et al., 2015*. Fragment ends were shifted +4 nucleotides for positive strand and -5 for negative strand to account for distance from Tn5 binding and helicase activity to identify cut sites. Extended Tn5 cut sites were used for peak calling with MACS2 with parameters --nomodel --extsize 100 --shift 50 --nolambda --keep-dup all. Fraction of reads in peaks (FRiP) score was calculated for each sample and was used as a normalization factor for pileup rescaled to a total number of uniquely alignable sequences by WigMath function of Java-Genomic Toolkit. Peaks were filtered by MYC motif using findMotifsGenome function in homer using four motif matrices *MA0058.2 (MAX), MA0059.1 (MAX::MYC), MA0104.4 (MYCN), and MA0147.3 (MYC).* Peaks were quantized using *k*-means clustering (*k*=8) and heatmaps for the peaks filtered with MYC motif were generated using cistrome (http://cistrome.org/) or using *k*-means clustering (*k*=6) and plotHeatmap function of deepTools (Liu et al., 2011) using wiggle files for genetically engineered NCI-H1963. Normalized ATAC-seq signals for each sample was visualized on integrative genome viewer (IGV) genome browser (Robinson et al., 2011).

Differentially bound sites comparing three c-Myc classified cell lines vs three L-Myc classified cell lines were identified using R package DiffBind with cutoffs set at fold change ≥ 5

and FDR ≤ 0.05. Functional analysis of the differentially bound regions was performed using Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010).

Western Blot and Antibodies

Protein lysates were prepared by washing cells with 1 ml of cold PBS and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 8.0, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, protease inhibitors) for 30 min at 4°C. Lysates were centrifuged at 5°C for 15 minutes at 13,000 rpm to remove insoluble debris. Protein concentrations were quantified using Pierce™ BCA (Thermo Fisher Scientific). Proteins were separated by electrophoresis on a SDS-PAGE gel (BioRad), transferred to a PVDF membrane (Thermo Fisher Scientific) and blocked with 5% milk in Tris-buffered saline with Tween-20 (TBS-T). The membranes were immunoblotted with anti-c-Myc (Santa Cruz Biotechnology), anti-L-Myc (Proteintech), anti-vinculin (Sigma), anti-ASCL1 (BD Biosciences) and anti-NeuroD1(Proteintech), anti-β-Actin antibody (Sigma), or anti-vinculin (Sigma).

Lentiviral introduction of genes

c-Myc, L-Myc or LacZ open reading frame (ORF) was cloned into pLEX_307 (a gift from David Root, Addgene #41392) using the Gateway® cloning methods according to manufacturer's recommendations. (Thermo Fisher Scientific). HEK293T cells were seeded in a 10 cm tissue culture dish and incubated at 37°C and 5% CO_2. At 80% confluency the cells were co-transfected with 10 µg of plasmid constructs, 7.5 µg of psPAX2 (a gift from Didier Trono, Addgene #12260) and 2.5 µg of pMD2.G (a gift from Didier Trono, Addgene #12259) vectors using TransIT-Lenti (Mirus) following manufacturer's recommendations. At 48h post

transfection, virus-containing supernatants were collected, filtered (0.45 μm) and stored at −80°C. Cells were infected with lentiviral supernatant supplemented with polybrene at a final concentration of 8 μg/mL or lentiBlast (OZ BioSciences) at a ratio of 1:1000. Cells were selected with puromycin (1-2 μg/mL for 4-6 days).

CRISPR-Cas9 genome editing

Cells with stable human codon-optimized S. pyogenes Cas9 expression were generated by infection with the lentiCas9-Blast plasmid (a gift from Feng Zhang, Addgene # 52962). sgRNAs targeting *MYC* and *MYCL* were selected from the Brunello library (Doench, Nat Biotechnol. 2016). Non-target sgRNA from the Gecko library v2 (Sanjana et al Nat Methods. 2014) were used as non-target sgRNAs. sgGRNA target sequences are listed in Supplementary Table S3. sgRNAs were cloned using BbsI site downstream of the human U6 promoter in a lentiviral vector containing EGFP downstream of the human PGK promoter (a kind gift of Brown laboratory, ISMMS). Lentivirus was produced as described above. Cas9 expressing cells were then infected with pLenti-GFP-sgRNA.

Cell proliferation assay

Cells were plated at a density of 5,000 cells/well with five replicates in a 96-well plate; four identical plates were prepared. Cell viability was assayed at 0, 2, 4 and 6 days after plating with alamarBlue Cell Viability Reagent (Thermo Fisher Scientific) and fluorescence at 585 nm was measured on a Spectra Max3 plate reader (Molecular Device) according to the manufacturer's protocol at excitation of 555 nm. Cell viability at 2, 4 and 6 days were corrected for the ratio to control cells from the day 0 reading to account for plating unevenness.

RNA-seq

Total RNAs from engineered NCI-H82 and NCI-H1963 cell lines were extracted using RNeasy kit (Qiagen). Poly-adenylated RNA was enriched from 1ug of RNA for each sample with the NEBNext® PolyA mRNA Magnetic Isolation Module (NEB), incubated at 94°C for 15 min and double-strand cDNA was synthesized using SuperScript III reverse transcriptase (Thermo Fisher Scientific) and NEBNext® Ultra™ II Directional RNA Second Strand Synthesis Module (NEB). Up to 10 ng of cDNA was used for the Illumina sequencing library construction using NEBNext® Ultra™ DNA Library Prep Kit (NEB). Paired ends sequencing was performed on NextSeq 500 (Illumina) for 38 nucleotides from each end according to the manufacturer's instructions.

RNA-seq analyses

Sequencing reads were pseudoaligned to the human reference transcriptome GRCh38.95 from Ensembl and transcript abundance was estimated using kallisto (v0.45.0) (Bray et al., 2016). Transcript abundance was aggregated to gene level abundance using biomaRt annotation. DEseq2 (Love et al., 2014) was used to identify the differentially expressed genes between control NCI-H82 cells (LacZ-overexpressed NCI-H82 cells, LacZ-overexpressed + nontarget sgRNA1 NCI-H82 and LacZ-overexpressed + nontarget sgRNA2 NCI-H82) and L-Myc-overexpressed NCI-H82 cells (L-Myc-overexpressed NCI-H82 cells, L-Myc-overexpressed + nontarget sgRNA1 NCI-H82 and L-Myc-overexpressed + nontarget sgRNA2 NCI-H82). The same strategy was used for L-Myc-overexpressed NCI-H82 cells (L-Myc-overexpressed NCI-H82 cells, L-Myc-overexpressed + nontarget sgRNA1 NCI-H82 and L-Myc-overexpressed + nontarget sgRNA2 NCI-H82) and c-Myc replaced with L-Myc NCI-H82 cells (L-Myc-overexpressed NCI-H82 + sgRNA-MYC1 cells, L-Myc-overexpressed + sgRNA-MYC2 NCI-

H82 and L-Myc-overexpressed + sgRNA-MYC3 NCI-H82), as well as for LacZ-overexpressed NCI-H1963 cells and c-Myc overexpressed NCI-H1963 cells.

Total differentially expressed genes were determined based on cutoffs of fold change> 2 and FDR<0.05. To identify potentially enriched functions of selected gene sets of interest, we compared these gene sets with the genes annotated by the same Gene Ontology (GO) terms curated in the Molecular Signature Database (MSigDB) (Subramanian et al., 2005). Each of 5917 GO terms included in "C5" collection (version 7.0) was compared with query gene sets using Fisher's exact test. The significance of the overlap was determined based on p-value adjusted for multiple comparisons (FDR<0.05). Any GO terms consisting of more than 2,000 genes were considered non-specific and removed from the analysis.

Beta-galactosidase staining

Cells were stained with Senescence beta-galactosidase Staining Kit (Cell signaling #9860) according to manufacturer's recommendations.

Cell cycle analysis

Cells were harvested at day 2 after selection and fixed with 70 % ethanol overnight at 4°C, were washed with PBS, then, incubated in PBS containing 100 μg/ml RNase and 50 μg/ml propidium iodide at room-temperature for 1 hour. DNA content was analyzed by FACS Canto II (BD Bioscience), and quantitative analyses for the proportions of cells in cell cycle were performed using FlowJo software (BD Bioscience).

Annexin-V staining

Cells were harvested at day 2 after selection and washed with PBS. Cells were stained with Annexin-V and propidium iodide using Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific) according to manufacturers' recommendations. Fluorescence was measured by FACS Canto II (BD Bioscience). Quantitative analyses for the cell viability proportions were performed using FlowJo software (BD Bioscience).

Xenograft model

All animal procedures and studies were approved by the Mount Sinai Institutional Animal Care Use Committee (IACUC) (protocol number, IACUC-2018-0021). H1963 cells which overexpressed LacZ or cMYC (5×10^6 cells) were injected with a 1:1 mixture of 50 µl cell suspension and 50 µl Matrigel (Corning) subcutaneously into both flank regions of 4- to 6-week-old male NOD-scid gamma mice (Jackson Laboratory). Tumor volume (length x $width^2$ /2) was measured twice a week. When tumor size reached at 1000 mm^3, mice were sacrificed and tumors were immersed in formalin for histological analysis.

Aurora Kinase Inhibition Assay

Cells were seeded in a 96-well plate at a density of 5,000 cells/well with five replicates. Cells were treated with Alisertib at concentrations ranging from 1 nM to 10 µM for 96h. Cell viability was measured using alamarBlue Cell Viability Reagent (Thermo Fisher Scientific) and fluorescence at 585nm was measured on a Spectra Max3 plate reader (Molecular Devices, CA) according to the manufacturer's protocol at excitation of 555 nm.

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Author Contribution

Conceptualization & Methodology, A.S.P. and H.W.; Data Collection: A.S.P., S.Y., R.K., T.S., M.F., A.S., and H.W.; Resources, M.B.B., C.A.P. and H.W.; Data Analysis, A.S.P., S.Y., G.N., M.B.B. and H.W.; Writing – Original Draft, A.S.P. and H.W.; Writing –Review & Editing, A.S.P., S.Y., G.N., C.A.P., M.B.B, J.Z. and H.W.; Supervision, C.A.P., J.Z. and H.W.; Project Administration & Funding Acquisition, H.W.

Declaration of Interests

Jun Zhu and Seungyeul Yoo are employees of Sema4, a for-profit organization that promotes genomic sequencing for patient-centered healthcare.

Figure legends

Figure 1: Bayesian Network analysis reveals unique L-Myc and c-Myc networks associated with distinct biological processes

A. Schematic of workflow to use SCLC Bayesian Network to identify c-Myc and L-Myc networks.
B. Sub-network showing directionality and association of genes when L-Myc gene signature (Figure S1B) is projected to SCLC Bayesian network. Circles colored in pink represent nodes from L-Myc gene signature. Size of pink circles is directly proportional to the number of outgoing nodes. Nodes indicated in larger text are Key drivers of the sub-network (Table S1).
C. Gene Ontology analysis for L-Myc neighbor subnetwork. Enriched functions for these genes are identified based on Fisher's exact test against GO terms.
D. Three sub-networks showing directionality and association of genes when c-Myc associated gene signature (Figure S1B) is projected to SCLC Bayesian network. Circles colored in blue represent nodes from c-Myc gene signature. Size of blue circles is directly proportional to the number of outgoing nodes. Nodes indicated in larger text are Key drivers of the sub-network (Table S2).
E. Gene Ontology analysis for corresponding c-Myc neighbor subnetwork. Enriched functions for these genes are identified based on Fisher's exact test against GO terms.

Figure 2: c-Myc and L-Myc driven SCLCs exhibit distinct chromatin states

A. Protein expression of c-Myc and L-Myc as well as vinculin as a loading control in a panel of representative SCLC cell lines

B. Schematic showing the analytic approach to define Myc accessible regions.

C. Principal component analysis of open chromatin regions with E-Box motif in six SCLC cell lines. Each point represents a SCLC cell line that is colored based on Myc status (pink: L-MYC, blue: c-MYC).

D. Each heatmap depicting global Myc (E-box) accessibility in individual SCLC cell lines at 72,833 combined peaks. ATAC-seq signal intensity is shown by color shading.

Figure 3: Myc accessibility reveals distinct transcriptional programs regulated by c-Myc and L-Myc

A. Heatmap showing 2,808 differentially accessible regions (fold change ≥ 5 and FDR ≤ 0.05) between 3 L-Myc cell lines shown in pink (H209, H1963, CORL88) and 3 c-Myc cell lines shown in blue (H524, H82, H2171)

B. Enriched ontology by GREAT analyses for regions differentially accessible in L-Myc classified cells.

C. Enriched ontology by GREAT analyses for regions differentially accessible in c-Myc classified cells.

D. Genome browser view tracks of ATAC-seq signals in c-Myc cell lines shown in blue (NCI-H82, NCI-H524, NCI-H2171) and L-Myc cell lines peaks shown in pink (NCI-H209, NCI-H1963, CORL88) at the *FOXA2* locus.

E. Genome browser view tracks of ATAC-seq signals in c-Myc cell lines shown in blue (NCI-H82, NCI-H524, NCI-H2171) and L-Myc cell lines peaks shown in pink (NCI-H1963, NCI-H209, CORL88) at the *REST* locus.

Figure 4: c-Myc is required to maintain NeuroD1-positive lineage state

A. Protein expression of L-Myc, c-Myc, ASCL1, NeuroD1 and YAP1 as well as vinculin as a loading control in genetically engineered NCI-H82 cells to replace c-Myc with L-Myc.
B. Cell growth curve for NCI-H82 cells with over-expression of L-Myc and genetic deletion of c-Myc.
C. Protein expression of c-Myc and NeuroD1 with vinculin as loading control in genetically engineered NCI-H82 cells with c-Myc ablation
D. Volcano plot showing 752 differentially expressed genes (458 upregulated in pink and 294 downregulated in blue) between control NCI-H82 cells (LacZ-overexpressed cells and LacZ-overexpressed cells with nontarget sgRNA) and NCI-H82 cells (LMYC-overexpressed cells and LMYC-overexpressed cells with three sgRNA targeting MYC).
E. Gene Ontology analysis for differentially upregulated (top panel) and downregulated genes (bottom panel) for comparison in Figure S5C. Enriched functions for these genes are identified based on Fisher's exact test against GO terms.

Figure 5: c-Myc expression in ASCL1-positive SCLC cells induces trans-differentiation to histologically distinct NeuroD1-positive state

A. Protein expression of c-Myc, L-Myc, ASCL1, and NeuroD1, as well as vinculin as loading control in genetically engineered NCI-H1963 cells.
B. Cell growth curve for NCI-H1963 cells with over-expression of c-Myc and control Lac-Z cells. * $p < 0.01$.

C. Scatter plot showing Annexin-V staining for NCI-H1963 cells with control Lac-Z cells (left panel) and over-expression of c-Myc (right panel) with proportion of viable, necrotic and apoptotic cells.

D. Hematoxylin-eosin staining in LacZ-overexpressed (top panel) or c-Myc-overexpressed (bottom panel) NCI-H1963 xenograft. Original Images, x20. Inset shows individual cell morphology.

Figure 6: c-Myc mediates lineage switch through REST

A. Each heatmap depicting *k-means* clustering of global Myc (E-box) accessibility in NCI-H1963 overexpressing LacZ (left) and NCI-H1963 overexpressing c-MYC (right) at 44,722 combined peaks. ATAC-seq signal intensity is shown by color shading.

B. Gene Ontology analysis for differentially upregulated (top panel) and downregulated (bottom panel) genes. Enriched functions for these genes are identified based on Fisher's exact test against GO terms.

C. Protein expression of c-Myc, L-Myc, ASCL1, and Rest, as well as vinculin as loading control in genetically engineered NCI-H1963 cells treated with vehicle (DMSO) or 0.5 µM of DBZ for 48 hours.

D. Protein expression of c-Myc, L-Myc, ASCL1, and Rest, as well as vinculin as loading control in genetically engineered NCI-H1963 cells treated with vehicle (DMSO) or 10 µM of X5050 for 48 hours.

E. Schematic showing the role of c-Myc and L-Myc in SCLC lineage specification

Supplementary Figure Legends

Figure S1: Myc family status and signature in SCLC CCLE cell lines

A. Scatter-plot showing c-Myc expression and L-Myc expression for 49 SCLC cell lines. Cell lines selected to generate signature are highlighted L-Myc in pink and c-Myc in blue.
B. Heatmap showing 457 differentially expressed (t-test p-value < 0.01 and fold change > 1.5) between four c-Myc expressing CCLE SCLC cell lines (H1341, H2081, H211, H524, SCLC21H) and eight L-Myc expressing CCLE SCLC cell lines (HCC33, H1092, H1184, H1836, H1963, H2029, H209). 147 genes upregulated in c-Myc (condition shown in blue) and 310 genes upregulated in L-Myc (condition shown in pink).
C. Classification of 49 SCLC cell lines using copy number and expression of *MYC*, *MYCL* and *MYCN* from CCLE

Figure S2: ATAC-seq inferred c-Myc accessibility is comparable to c-Myc ChIP-seq bound regions and classifies by Myc status

A. Heatmap comparing Myc (E-box) accessibility inferred from ATAC-seq with c-Myc bound sites with E-box motif inferred from ChIP-seq (Lin et al., 2012) in NCI-H2171. ATAC-seq/ChIP-seq signal intensity is shown by color shading.
B. Genome browser view tracks of ATAC-seq signals and c-Myc ChIP-seq at *HES1* locus.
C. Heatmap showing unsupervised clustering of degree of correlation for pairwise comparisons of identified open chromatin regions with *MYC* motif in 3 L-Myc classified cell lines H209, H1963, CORL88 (condition shown in pink) and 3 c-Myc classified cell lines H524, H82, H2171 (condition shown in blue)

Figure S3: c-Myc and L-Myc expression across molecular subtypes

A - B Genome browser view tracks of ATAC-seq signals in c-Myc cell lines shown in blue (NCI-H82, NCI-H524, NCI-H2171) and L-Myc cell lines peaks shown in pink (NCI-H209, NCI-H1963, CORL88) at the (A) *NeuroD1*, (B) *ASCL1*, (A) *POU2F3, (B)* YAP1.

C. Immunoblot showing protein expression of NeuroD1 and ASCL1 in c-Myc and L-Myc classified cell lines with Vinculin as loading control.

D. Violin plot showing mRNA expression of c-Myc across four molecular subtypes in CCLE cell lines as classified in Rudin et al., 2019

E. Violin plot showing mRNA expression of L-Myc across four molecular subtypes in CCLE cell lines as classified in Rudin et al., 2019

F. Unsupervised clustering of 137 primary tumors and 51 CCLE cell lines using the expression of ASCL1, NeuroD1, POU2F3 and YAP1 distinguishing four molecular subtypes.

G. Violin plot showing mRNA expression of c-Myc across four molecular subtypes for combined primary tumor and cell line classification described in S3F.

H. Violin plot showing mRNA expression of L-Myc across four molecular subtypes for combined primary tumor and cell line classification described in S3F

I. Violin plot showing mRNA expression of c-Myc across four molecular subtypes with NeuroD1 subtype further classified into NeuroD1-N with a higher NeuroD1:ASCL1 ratio and NeuroD1-A with a lower NeuroD1:ASCL1 ratio.

Figure S4: L-Myc induces neuronal state not defined by ASCL-1

A. Volcano plot showing 525 differentially expressed genes (235 upregulated in purple and 290 downregulated in blue) between control NCI-H82 cells (LacZ-overexpressed cells and LacZ-overexpressed cells with nontarget gRNA) and NCI-H82 cells (LMYC-overexpressed cells and LMYC-overexpressed cells with nontarget gRNA).
B. Gene Ontology analysis for differentially upregulated (top panel) and downregulated (bottom panel) genes. Enriched functions for these genes are identified based on Fisher's exact test against GO terms.
C. Volcano plot showing 214 differentially expressed genes (170 upregulated in pink and 44 downregulated in purple) between NCI-H82 cells (L-Myc-overexpressed cells and L-Myc-overexpressed cells with nontarget sgRNA) and NCI-H82 cells (L-Myc-overexpressed with three sgRNA targeting MYC).
D. Gene Ontology analysis for differentially upregulated (top panel) and downregulated (bottom panel) genes. Enriched functions for these genes are identified based on Fisher's exact test against GO terms.

Figure S5: Aurora Kinase A inhibition shows differential sensitivity between c-Myc and L-Myc expressing cells

A. Heatmap showing dose-dependent anti-proliferative activity of Alisertib as screened across a panel of 3 c-Myc (shown in blue) and 3 L-Myc cell lines (shown in pink) at 96 hours. Cell viability (Alamar Blue) was calculated relative to the control. Data are means from at least $n = 3$ biological replicates.

B. Dose-response curves of H82–replacement cells treated with Alisertib for 96 hours. Viability was assessed with the Alamar Blue assay and calculated relative to the control. Data are means from at least $n = 3$ biological replicates.

C. Dose-response curves of H1963–replacement cells treated with Alisertib for 96 hours. Viability was assessed with the AlamarBlue assay and calculated relative to the control. Data are means from at least $n = 3$ biological replicates.

Figure S6: c-Myc induced growth-suppression is not a consequence of altered cell cycle dynamics

A. Beta-galactosidase staining for NCI-H1963 LacZ-overexpressed (top panel) or c-Myc-overexpressed (bottom panel) at day 2.

B. Propidium iodide staining followed by flow cytometry showing cell cycle distribution in NCI-H1963 LacZ-overexpressed control cells and c-Myc overexpressed cells at day 2.

C. Hematoxylin-eosin staining in LacZ-overexpressed (left) or c-Myc-overexpressed (right) NCI-H1963 xenograft. Original Images, ×4 (top) and x10 (bottom).

Figure S7: c-Myc induces expression of neuronal repressor REST

A. Volcano plot showing 105 differentially expressed genes (45 upregulated in purple and 70 downregulated in pink) between control NCI-H1963 cells LacZ-overexpressed cells and NCI-H1963 cells c-Myc- overexpressed.

B. Expression plot for *REST* in NCI-H1963 cells LacZ-overexpressed cells and NCI-H1963 cells c-Myc- overexpressed.

C. Protein expression of c-Myc, and Rest, as well as vinculin as loading control in genetically engineered NCI-H1963 cells.

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Figure 1

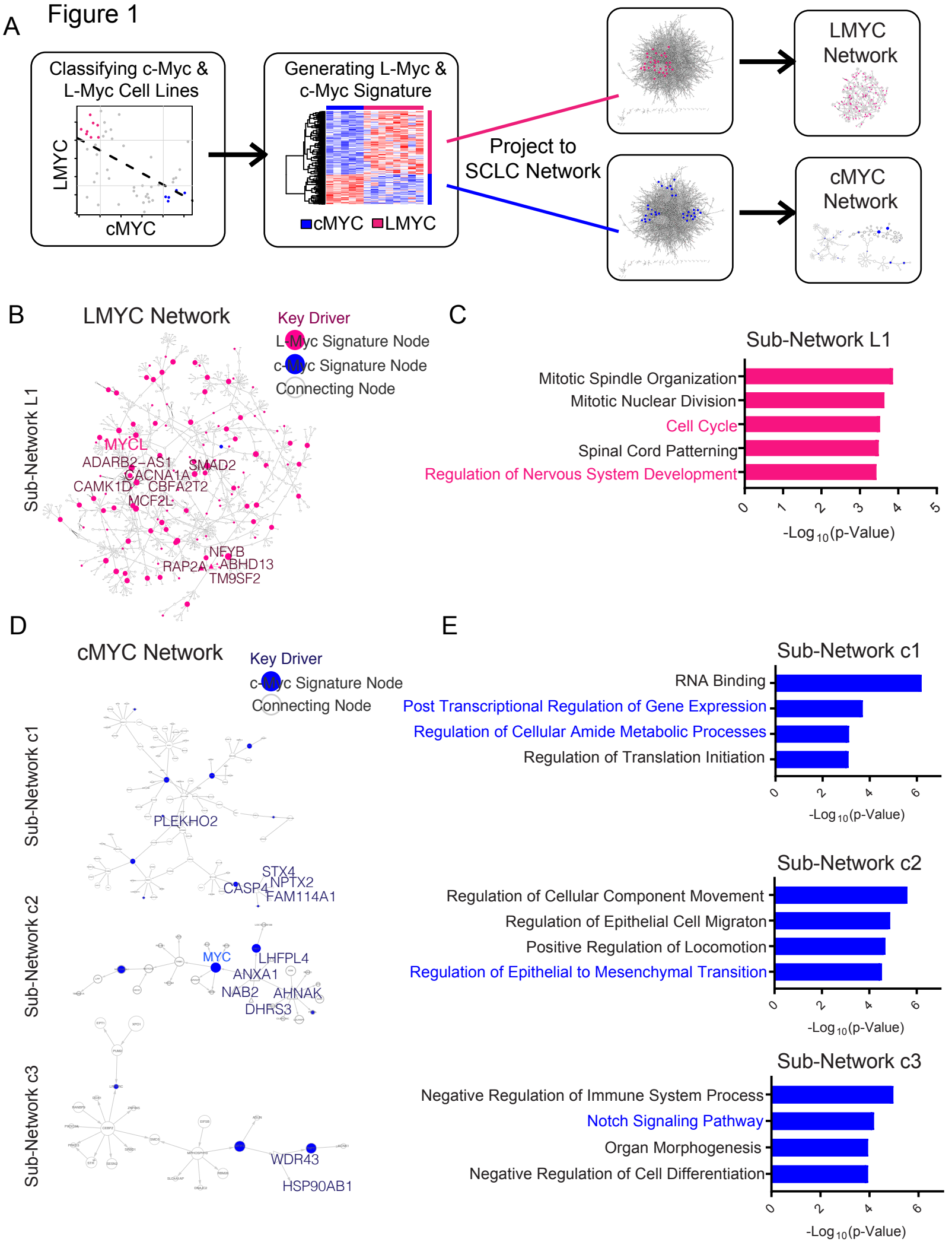
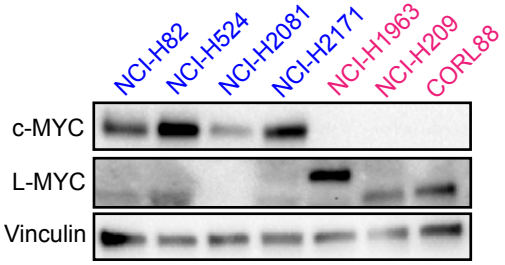
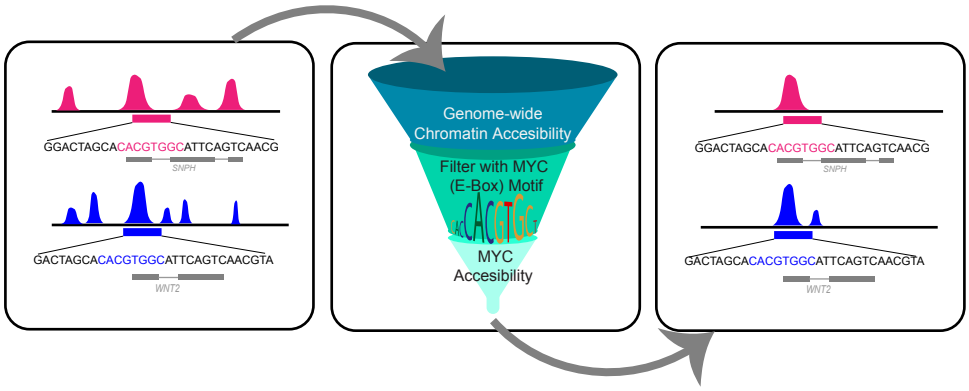

A
Classifying c-Myc & L-Myc Cell Lines
LMYC
cMYC
Generating L-Myc & c-Myc Signature
cMYC
LMYC
Project to SCLC Network
LMYC Network
cMYC Network
B
LMYC Network
Key Driver
L-Myc Signature Node
c-Myc Signature Node
Connecting Node
Sub-Network L1
MYCL
ADARB2-AS1
SMAD2
CACNA1A
CAMK1D
CBFA2T2
MCF2L
NFYB
RAP2A
ABHD13
TM9SF2
C
Sub-Network L1
Mitotic Spindle Organization
Mitotic Nuclear Division
Cell Cycle
Spinal Cord Patterning
Regulation of Nervous System Development
0
1
2
3
4
5
-Log 10(p-Value)
D
cMYC Network
Key Driver
c-Myc Signature Node
Connecting Node
Sub-Network c1
PLEKHO2
STX4
NPTX2
CASP4
FAM114A1
Sub-Network c2
MYC
LHFPL4
ANXA1
NAB2
AHNAK
DHRS3
Sub-Network c3
WDR43
HSP90AB1
E
Sub-Network c1
RNA Binding
Post Transcriptional Regulation of Gene Expression
Regulation of Cellular Amide Metabolic Processes
Regulation of Translation Initiation
0
2
4
6
-Log 10(p-Value)
Sub-Network c2
Regulation of Cellular Component Movement
Regulation of Epithelial Cell Migraton
Positive Regulation of Locomotion
Regulation of Epithelial to Mesenchymal Transition
0
2
4
6
-Log 10(p-Value)
Sub-Network c3
Negative Regulation of Immune System Process
Notch Signaling Pathway
Organ Morphogenesis
Negative Regulation of Cell Differentiation
0
2
4
6
-Log 10(p-Value)

Figure 2

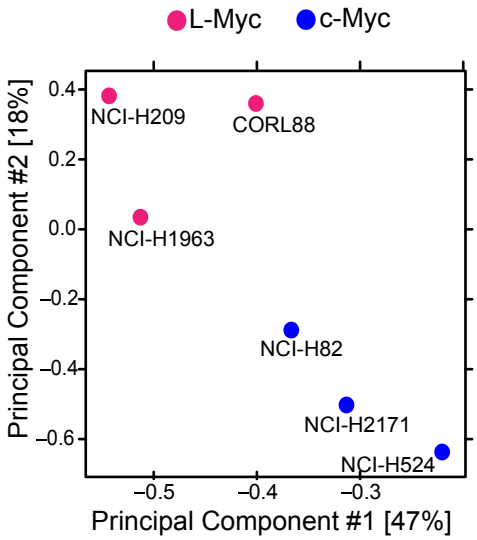
A



B



C



D

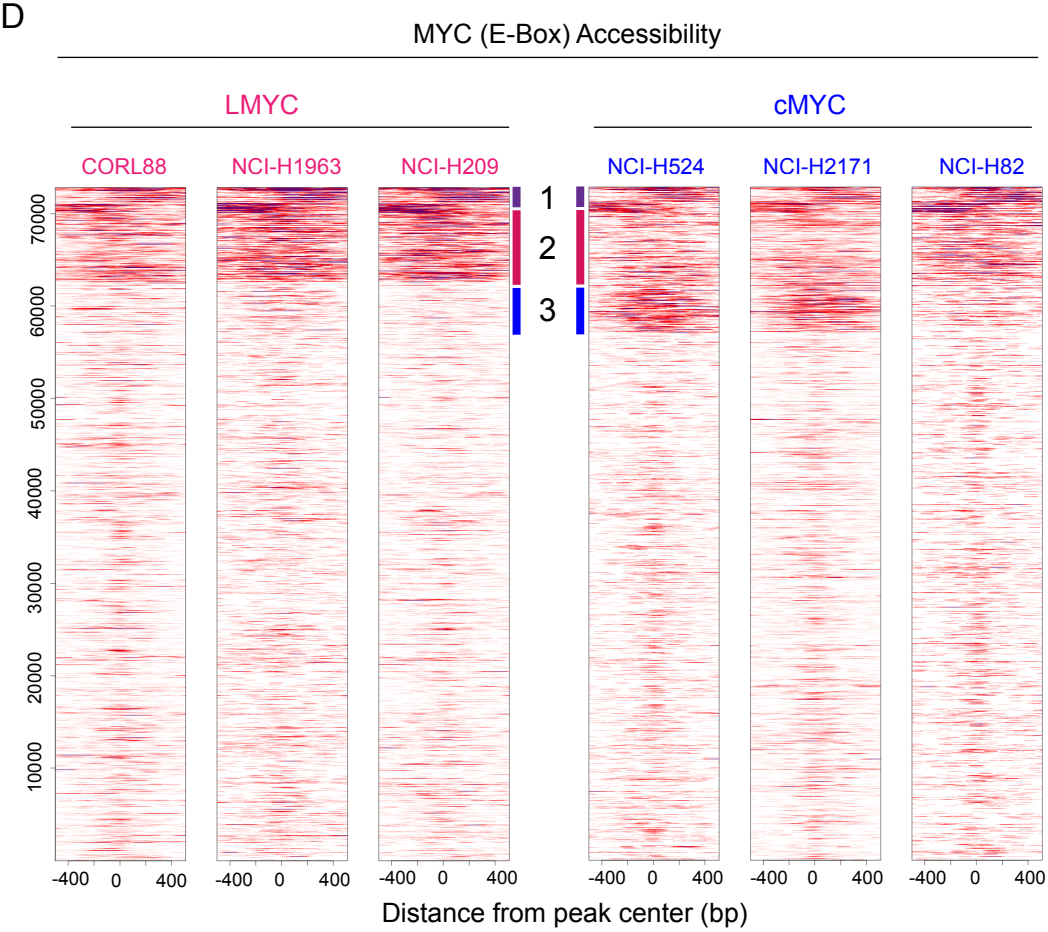


Figure 3

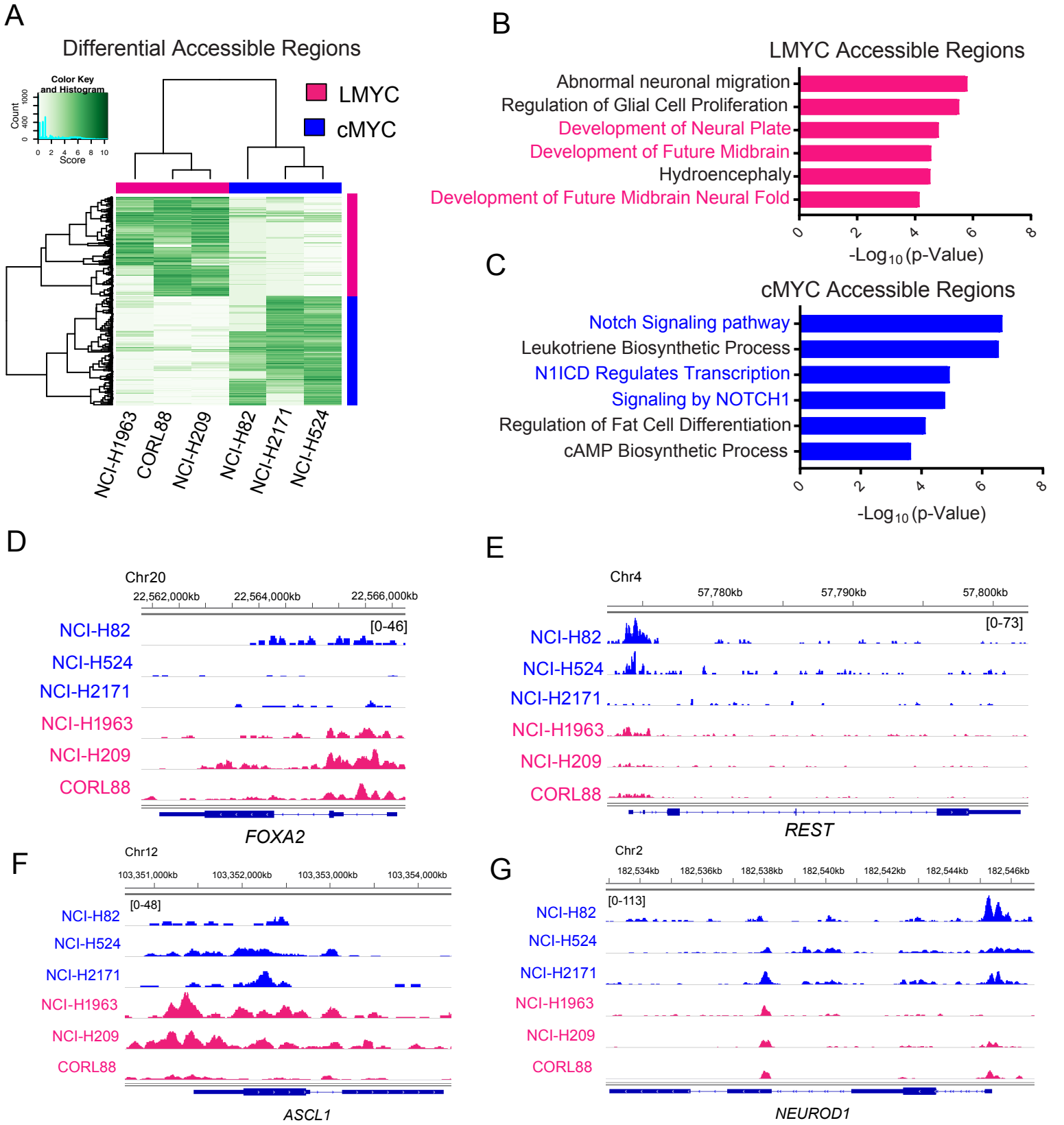

A
Differential Accessible Regions
Color Key and Histogram
Count
Score
LMYC
cMYC
NCI-H1963
CORL88
NCI-H209
NCI-H82
NCI-H2171
NCI-H524
B
LMYC Accessible Regions
Abnormal neuronal migration
Regulation of Glial Cell Proliferation
Development of Neural Plate
Development of Future Midbrain
Hydroencephaly
Development of Future Midbrain Neural Fold
-Log10 (p-Value)
C
cMYC Accessible Regions
Notch Signaling pathway
Leukotriene Biosynthetic Process
N1ICD Regulates Transcription
Signaling by NOTCH1
Regulation of Fat Cell Differentiation
cAMP Biosynthetic Process
-Log10 (p-Value)
D
Chr20
22,562,000kb
22,564,000kb
22,566,000kb
[0-46]
NCI-H82
NCI-H524
NCI-H2171
NCI-H1963
NCI-H209
CORL88
FOXA2
E
Chr4
57,780kb
57,790kb
57,800kb
[0-73]
NCI-H82
NCI-H524
NCI-H2171
NCI-H1963
NCI-H209
CORL88
REST
F
Chr12
103,351,000kb
103,352,000kb
103,353,000kb
103,354,000kb
[0-48]
NCI-H82
NCI-H524
NCI-H2171
NCI-H1963
NCI-H209
CORL88
ASCL1
G
Chr2
182,534kb
182,536kb
182,538kb
182,540kb
182,542kb
182,544kb
182,546kb
[0-113]
NCI-H82
NCI-H524
NCI-H2171
NCI-H1963
NCI-H209
CORL88
NEUROD1

Figure 4

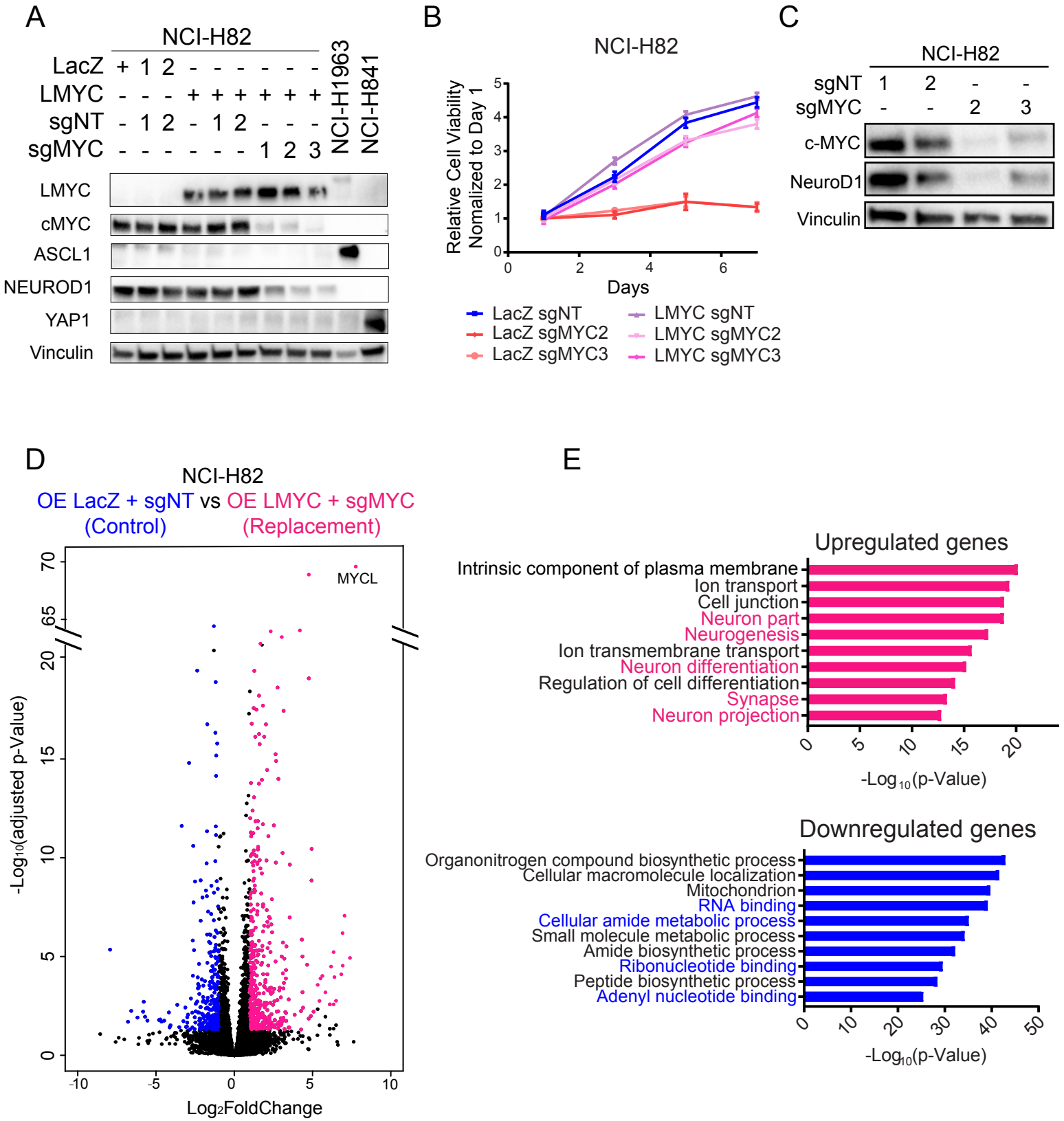
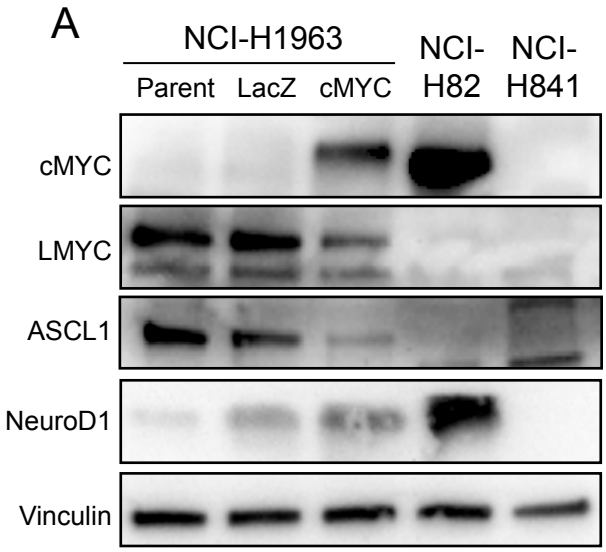
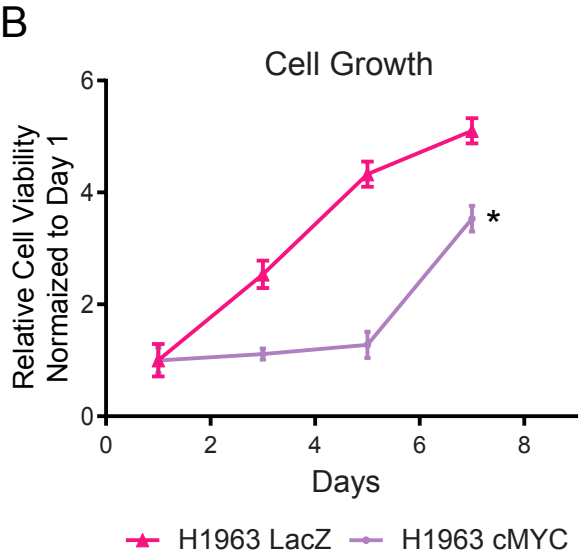
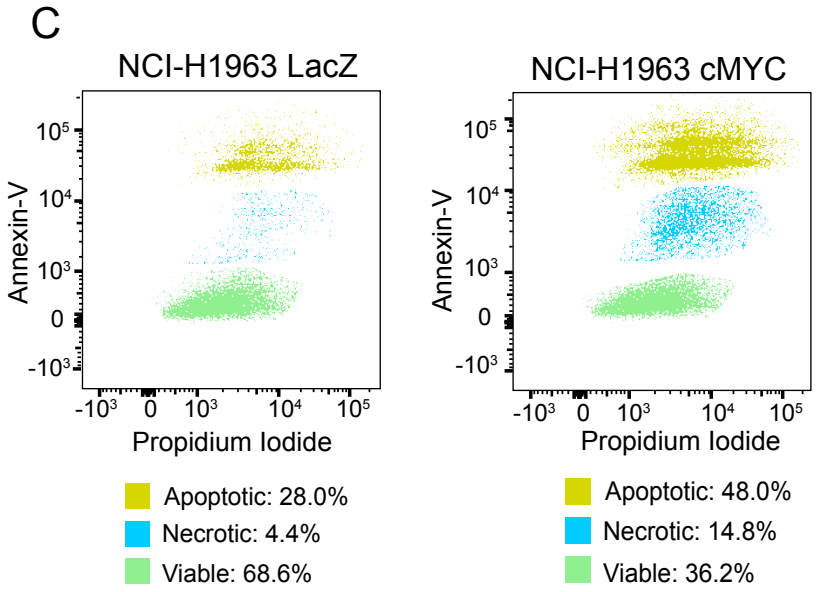

A
NCI-H82
LacZ + 1 2 - - - - - -
LMYC - - - + + + + + +
sgNT - 1 2 - 1 2 - - -
sgMYC - - - - - - 1 2 3
NCI-H1963
NCI-H841
LMYC
cMYC
ASCL1
NEUROD1
YAP1
Vinculin
B
NCI-H82
Relative Cell Viability Nomalized to Day 1
Days
LacZ sgNT
LacZ sgMYC2
LacZ sgMYC3
LMYC sgNT
LMYC sgMYC2
LMYC sgMYC3
C
NCI-H82
sgNT 1 2 - -
sgMYC - - 2 3
c-MYC
NeuroD1
Vinculin
D
NCI-H82
OE LacZ + sgNT vs OE LMYC + sgMYC
(Control) (Replacement)
MYCL
-Log10(adjusted p-Value)
Log2FoldChange
E
Upregulated genes
Intrinsic component of plasma membrane
Ion transport
Cell junction
Neuron part
Neurogenesis
Ion transmembrane transport
Neuron differentiation
Regulation of cell differentiation
Synapse
Neuron projection
-Log10(p-Value)
Downregulated genes
Organonitrogen compound biosynthetic process
Cellular macromolecule localization
Mitochondrion
RNA binding
Cellular amide metabolic process
Small molecule metabolic process
Amide biosynthetic process
Ribonucleotide binding
Peptide biosynthetic process
Adenyl nucleotide binding
-Log10(p-Value)

Figure 5


A
NCI-H1963
Parent
LacZ
cMYC
NCI-H82
NCI-H841
cMYC
LMYC
ASCL1
NeuroD1
Vinculin


B
Cell Growth
Relative Cell Viability Normaized to Day 1
Days
0
2
4
6
8
*
H1963 LacZ
H1963 cMYC


C
NCI-H1963 LacZ
NCI-H1963 cMYC
Annexin-V
Propidium Iodide
Apoptotic: 28.0%
Necrotic: 4.4%
Viable: 68.6%
Apoptotic: 48.0%
Necrotic: 14.8%
Viable: 36.2%

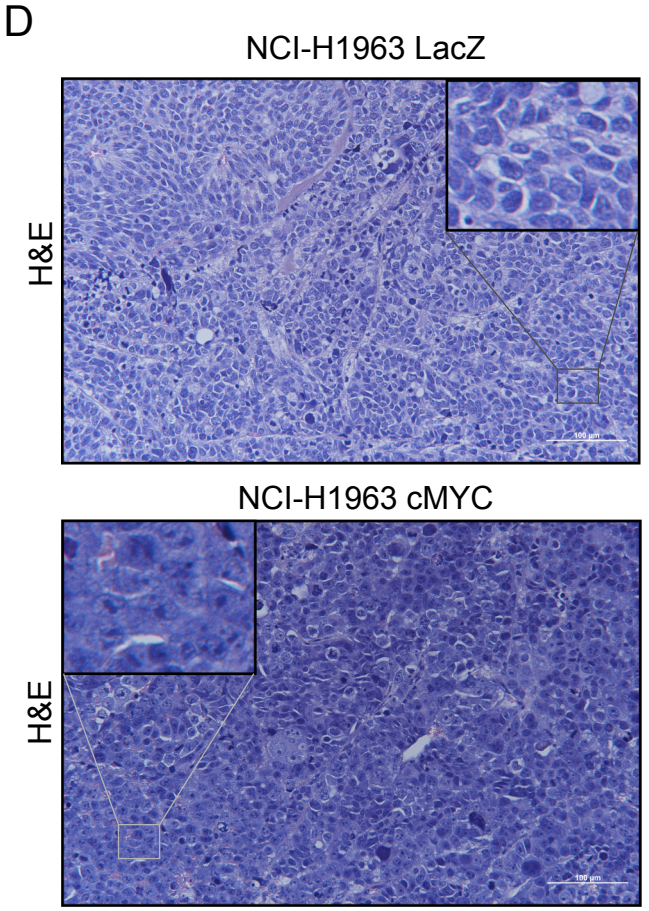
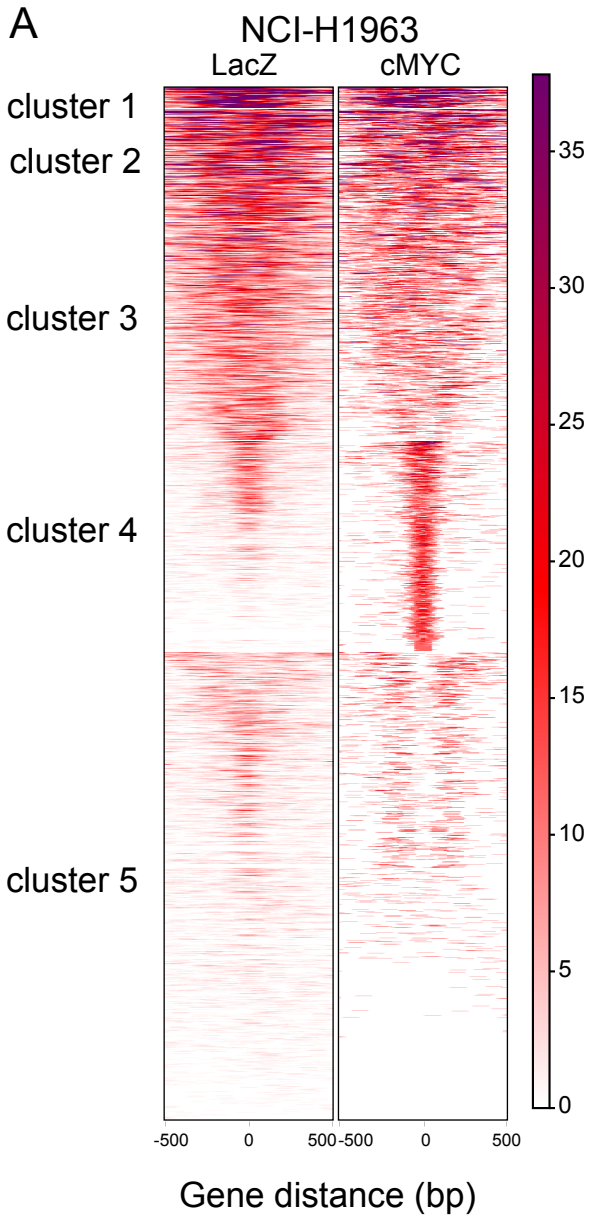
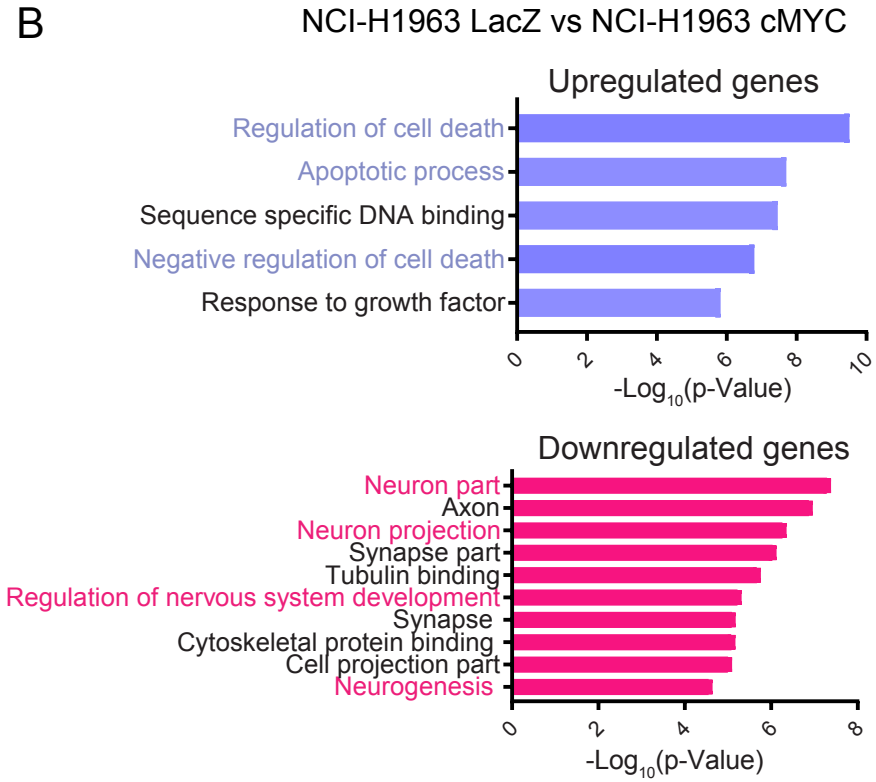
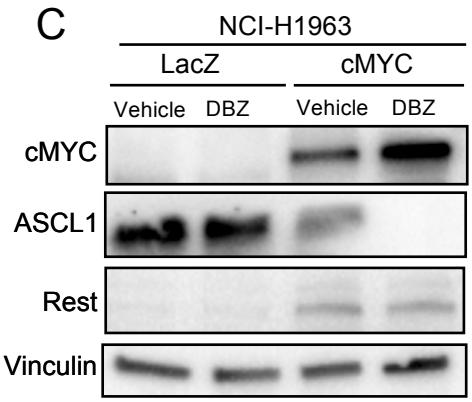
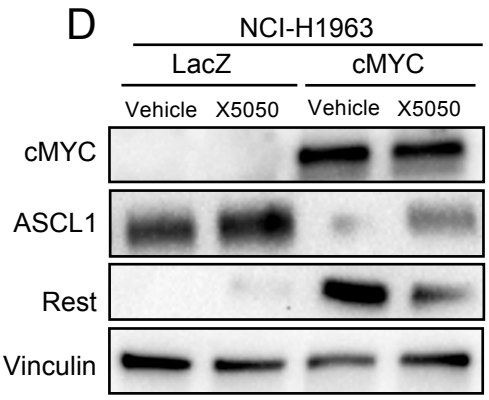

D
NCI-H1963 LacZ
H&E
NCI-H1963 cMYC
H&E

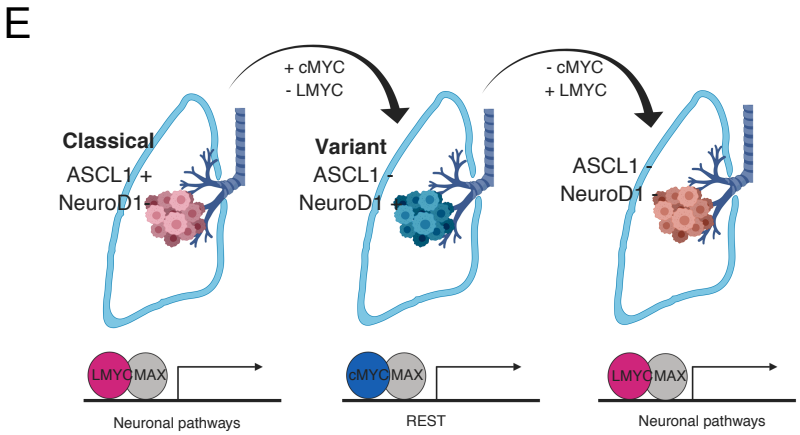
Figure 6


A
NCI-H1963
LacZ
cMYC
cluster 1
cluster 2
cluster 3
cluster 4
cluster 5
35
30
25
20
15
10
5
0
-500
0
500
-500
0
500
Gene distance (bp)


B
NCI-H1963 LacZ vs NCI-H1963 cMYC
Upregulated genes
Regulation of cell death
Apoptotic process
Sequence specific DNA binding
Negative regulation of cell death
Response to growth factor
0
2
4
6
8
10
-Log10(p-Value)
Downregulated genes
Neuron part
Axon
Neuron projection
Synapse part
Tubulin binding
Regulation of nervous system development
Synapse
Cytoskeletal protein binding
Cell projection part
Neurogenesis
0
2
4
6
8
-Log10(p-Value)


C
NCI-H1963
LacZ
cMYC
Vehicle
DBZ
Vehicle
DBZ
cMYC
ASCL1
Rest
Vinculin


D
NCI-H1963
LacZ
cMYC
Vehicle
X5050
Vehicle
X5050
cMYC
ASCL1
Rest
Vinculin


E
+ cMYC
- LMYC
- cMYC
+ LMYC
Classical
ASCL1 +
NeuroD1 +
Variant
ASCL1 -
NeuroD1 +
ASCL1 -
NeuroD1 -
LMYC
MAX
Neuronal pathways
cMYC
MAX
REST
LMYC
MAX
Neuronal pathways