# Integrating ENCODE data to interpret regulatory changes in cancer

## Abstract

The majority of catalogued mutations are within coding genes. However, the preponderance of mutations in tumors occur in non-coding regions throughout the genome. While coding mutations yield easily inferred mis-sense, non-sense or frameshift events that lead to altered proteins, non-coding mutations are often exceptionally difficult to characterize.

Functional mapping of the non-coding genome in efforts such as the ENCODE Project provide an opportunity to assess non-coding mutations throughout the human genome in light of diverse genomic assay profiles. For a variety of cancer-derived cell lines, as well as non-cancerous cell lines derived from the same tissues (allowing in some cases for tissue-matched non-coding background mutation rate), ENCODE provides diversity of genome-wide assays to measure genomic characteristics like chromatin state and transcription factor binding (e.g., ChIP-seq, DNase-seq, Enhancer-seq, Hi-C, and ChIA-PET). ,f, The resulting data and functional maps of the human genome provide a framework for assessing the potential for cancer mutations in the non-coding genome to dysregulate genes.

Here we integrate diverse ENCODE data to define high-confidence regulatory elements and their target genes. This allows us to define extended gene neighborhoods for mutation recurrence analysis. This approach identified novel genes, such as BCL6 in leukemia, that are recurrently mutated in cancers and associated with patient prognosis. We also integrated the ENCODE data to build hierarchical regulatory networks, including both transcription factors (TFs) and RNA-binding proteins (RBPs). Intriguingly, TFs with higher mutation burden tend to be located at the bottom of the hierarchy (e.g., EZH2 and NR2C2), whereas those with dysregulated expression tend to reside at the top. Furthermore, by comparing tumor and normal network, we identified highly “rewired” TFs with changed targets and prognostic value, such as IKZF1 and MYC. Our results indicate that such rewiring events are mainly attributable to epigenetic changes, rather than mutations that disrupt TF motifs.

Finally, we proposed a prioritization scheme for key non-coding elements, as well as the mutations they contain, according to their position in regulatory networks and potential to drive expression changes in cancer. In particular, we prioritize CTCF as a key TF for blood cancer and SUB1 as a key RBP for liver and lung cancers, and validated them through siRNA knockdown experiments. Finally, we identify active enhancers and seven high impact mutations therein in breast cancer and validated their functional effects through luciferase assays.

## Introduction

Coding mutations associated with cancer have been the focus of extensive study. However, the overwhelming bulk of mutations in cancer genomes – particularly those discovered from recent large-scale cancer genomics initiatives – lie within non-coding regions. Whether these mutations drive cancer development or progression, or simply emerge as byproducts of genomic instability,, remains an open question. Newly-released data from the ENCODE Consortium can help address this question by providing comprehensive characterization of non-coding genomic elements, as well as by linking such elements to well-known cancer associated genes.

Here we endeavor to provide a companion resource to the main ENCODE encyclopedia by building a “cancer encyclopedia”. The main encyclopedia is oriented toward breadth of annotation, describing elements over hundreds of cell lines. In contrast, we focus on cell lines with wide variety of profiles (the “tier 1 & 2” lines) . Most of these cell lines are associated with cancers of the blood, liver, lung, cervix, and breast. We show that these cell lines can be used to provide a better understanding of the portion of the non-coding genome that is affected during cancer development and progression, and we provide a resource for interpreting the wealth of mutational and transcriptional profiles produced by the cancer community.

## Data for comprehensive functional characterization in ENCODE

The most comprehensive set of assays for ENCODE are available for tier 1 cell lines. These cell lines therefore provide good models for studying gene regulation in detail. Five tier 1 cell lines are derived from cancers, including cancer of the blood (K562), breast (MCF-7), liver (HepG2), lung (A549), and cervix (HeLa-S3).

For four of these five cell lines, there is another immortalized cell line from corresponding healthy tissue. This provides an approximate ‘normal’ match to these tumor-derived cell lines. It is worth noting that both the matching of cell lines to cancers and matching of tumor-normal pairs are very approximate in nature, as these matchings are not intended to substitute data from real tumor and normal tissues. Nonetheless, they can be used to integrate a wide variety of available omics data to determine significant differences between the tumor-derived and immortalized ‘normal’ cells. Importantly, this exercise provides a resource that can then be used to better interpret more limited volume of data derived from true cancer tissue.

(Fig 1A).

To build a “cancer-relevant encyclopedia of DNA elements” (C-ENCODE), we first constructed a comprehensive data matrix by normalizing raw signals of genomic features that severely confound somatic mutagenic processes (see Supp. File/Section(?) X ). In contrast to previous approaches that rely on single histone modification marks, we implemented an ensemble-based method called ESCAPE, which performs large-scale data integration to accurately identify active enhancers. This integration involves predictions using a diverse collection of histone mark ChIP-seq, DNase-seq and Enhancer-seq datasets. We further link these to genes by optimally investigating how the histone modification marks on enhancers help predict the gene expression of the potential target gene. This group of potential linkages is then filtered through the results of Hi-C experiments, which provide a low resolution three dimensional physical picture of inter-genomic chromatin interactions (see Supp. File/Section(?) X). To improve functional interpretation, we used these high-quality linkages to construct what we termed “extended gene neighborhoods” – coding regions matched with key regulatory elements, such as enhancers, promoters, and binding sites from regulators (Fig1 B). In addition, we also explored the binding profiles in ENCODE data, and constructed high-confidence gene regulatory networks for both TFs and RBPs (Fig. 1C and Fig. X in Supp. File/Section(?) X). Finally, we merged our integrated analysis with broader ENCODE data, and we provide consistent identifiers and definitions for the C-ENCODE resource.

In summary, our the C-ENCODE resource consists of a list of accurately determined enhancers, a list of regions with high mutation burden in cancer, the regulatory network of TFs (and for some lines RBPs), as well as the most rewired TFs in this regulatory network (see supplementary materials). Collectively, these resources allow us to prioritize a few key elements as being associated with oncogenesis, some of which are then validated using small-scale experiments (see table S1).

## Multi-level data integration better enables recurrent variant analysis in cancer

One of the most powerful ways of identifying key elements and functional mutations in cancer is through recurrence analysis, which identifies regions of the genome that are more frequently mutated than expected. However, a mutational process could be influenced by or associated with confounding factors (in the form of both external genomic factors and local context effects), which can result in many false positives or negatives in recurrence analysis (see Supp. File/Section(?) X). In addition, traditional burden tests often neglect the association among annotation categories and test regions separately. Consequently, these tests sometimes fail to identify distributed mutation signals from biologically relevant genomic regions, thereby limiting the interpretation power of burden tests.

To address these limitation of traditional recurrence analysis, we integrated the C-ENCODE resource at two levels. First, we predict an accurate local BMR by regressing out the confounding effects of features in a cancer-specific manner (see Supp. File/Section(?) X). Specifically, we prepare a covariate matrix by normalizing 475 features from ENCODE to remove those effects that may confound the BMR. We then separated the whole genome into 64 categories according to the local 3-mers, and run separate regression models to further remove confounders from intrinsic sequence context. In contrast to methods that use unmatched data \{cite MutsigCV}, our regression-based approach with matched data usually yields higher BMR prediction precision (Fig 2A, see also Supp. File/Section(?) X). In breast cancer, for example, the spearman’s correlation () between observed and predicted mutation counts over 1-megabase bins increases from XX to XXX when using replication-timing signals (from MCF-7 instead of HeLa-S3). This underlies the importance of integrating these chromatin features from matched tissues to infer BMR (Fig 1B). For example, only ranges from xxx-xxx using matched replication timing, but its range increases to xxx-xxx by adding 1 PC from the remaining covariates. It progressively increases to the xxx-xxx regime by adding PCs to the full model through forward selection (Fig 1B, see Supp. File/Section(?) X). Such noticeable improvements in BMR estimation significantly improve the burden analyses (below).

Rather than separately testing standalone annotation categories, we employ our extended gene (detailed above) as joint test units (see Supp. File/Section(?) X). Such a scheme allows for the accumulation of weak mutation signals distributed across multiple biologically relevant functional elements, which may otherwise be missed if evaluated under individual tests (Fig. Sx in Supp. section X). Furthermore, it enables to collectively assess the overall burdening associated with a well-known cancer associated genes. Burdening in the protein coding regions may be matched by apparently un-connected mutations in the regulatory regions. We demonstrate that our approach can effectively remove false positives and discover meaningful burdened regions (Fig 2C). For example, in the context of K562 cells derived from a chronic lymphocytic leukemia (CLL), our analysis identifies well-known highly mutated genes (such as TP53 and ATM) as well as other genes (such as BCL6) that are missed by the recurrence analysis of coding regions. BCL6 has strong prognostic value with respect to patient survival (Fig. 2D), indicating that the extended gene should be used as an annotation set for recurrence analysis. In addition, we can easily generalize this BMR calibration approach for cancer types apart from the five we focus on, as our model will work to pick an appropriately matched C-ENCODE signal type.

## Extensive rewiring events of several transcription factors in cancer

We next investigated transcription regulatory networks in a tissue-specific manner. In each cell type, we organized TF regulatory networks into a hierarchy by comparing the inbound and outbound edges of each factor, thereby enabling us to investigate the global topology of TF regulation (Fig. 1E, see also Supp. File/Section(?) X). TFs in different levels of the hierarchy reflect the extent to which they directly regulate the expression of other TFs \{cite 25880651}. For example, TFs in the top layer have more outbound than inbound edges in the network, and thus play larger roles in regulating other TFs (Supp. Fig. xx). In this representation, two patterns readily emerge. In the blood cell comparison (K562 vs. GM12878), top-level TFs tend to more strongly influence the differential expression between the CLL-derived K562 cells (tumor) and the immortalized GM12878 lymphoblastoid cells (normal). The average Pearson correlation between TF binding events and tumor-normal expression changes increases from 0.125 in the bottom layer to 0.270 in the top layer (Table Sx). TFs in the bottom layer are more frequently associated with burdened binding sites in general, perhaps reflecting their increased resilience to mutation (see Supp. Section X, Table Sx).

When comparing the common regulators in approximately matched tumor and normal regulatory networks, rewiring (i.e., target changing) analysis may help to identify cancer-associated deregulation. Hence, we investigated rewiring events in TF networks using multiple formulations (see Supp. File/Section(?) X). Specifically for the CLL example, we removed the general TFs and restricted our rewiring analysis to 61 common TF ChIP-seq in K562 and GM12878 from ENCODE (see Supp. File/Section(?) X). We first ranked TFs according to , which calculates their respective number of lost and gained edges, Oncogenes such as MYC and NRF1 are among the top edge gainers. In contrast, IKZF1, whose somatic mutations serve as a hallmark of high-risk acute lymphoblastic leukemia is the most significant edge loser, with up to xxx% of lost edges in K562 (Fig 3A). However, several ubiquitously distributed TFs, such as YY1, retain their regulatory linkages (Fig 3A). We observe a similar trend in TFs using a distal, proximal and combined network (see details in supplementary file). We also observe highly rewired TFs in lung and liver cancers (see fig XX) although we do not have as many common TFs between tumor and normal cell lines for these tissues.

Our rewiring index only considers direct connections associated with a given TF. One may also consider rewiring that includes not only direct connections, but also the whole neighborhood of connections a TF associates with through membership and topic models. In particular, we used a mixed-membership model to take a wide-view of local gene neighborhoods, and re-rank TFs accordingly (see Supp. File/Section(?) X). Similar patterns are observed using this model. We also observed that MYC (a well-known oncogene) becomes a top edge gainer (Fig 3A). To study the consequences of network rewiring under this model, we performed survival analysis on xxx AML patients and found IKZF1 to be significantly associated with tumor progression (see Supp. File/Section(?) X).

The combinatorial regulation of many TFs jointly determines the “ON” and “OFF” states of genes as part of maintaining homeostasis in healthy cells. The disruption of co-regulatory relationships for key elements in cancer cell lines ultimately results in erroneous gene expression patterns. We quantified the co-association of each TF, and observed major co-association changes in some of the key TFs when comparing the regulatory networks of K562 to GM12878. For example, ZNFXXX is a suppressor TF that shows only marginal co-binding events in GM12878, with binding site increases from xxx to xxx in K562. In addition, up to xxx% of its binding sites co-bind with other TFs (in which cell?).

A remaining uncertainty is the underlying cause of this rewiring. Is it a direct effect of mutations, which could knock out a binding site? Or is it due to indirect effects of chromatin changes, which could cover and uncover binding sites? We find that a majority of rewiring events result from changes in chromatin status, rather than from variant-induced loss or gain events (Fig. 3A). For example, JUND is a top gainer in K562 (with xxx gains and xx losses). We find that many of the gain/loss events are associated with substantial expression changes (of at least 2-fold) and changes to chromatin states. Only xxx percent of them could be potentially due to direct motif loss/gain effects. (Fig. 3D).

## Integrating ENCODE data with tumor expression profiles identifies key regulators in cancer

Using a regression-based learning method (see Supp. File/Section(?) X), we integrated the molecular profiles of 8,202 TCGA tumors with 921 ENCODE regulatory binding profiles to systematically search for TFs and RBPs that drive tumor-specific expression patterns (Table Sx). For each tumor sample, our integration framework selects a set of regulator binding profiles to best explain the expression difference between the tumor sample and normal controls. For each cancer type, our framework further tests whether the regulatory targets identified in a ENCODE experiment are sufficiently correlated with the regulator’s molecular status across tumors. The final output of our framework is the estimated fraction of patients with target genes differentially regulated between each pair of cancer type and regulator (see Supp. File/Section(?) X).

## The overall trends for the key TFs and RBPs detected are given in Fig. 4A. The predicted impacts of regulators on tumor gene expression are highly consistent with previous findings. For example, we find that the target genes of MYC are significantly up-regulated in numerous cancers (star in Fig Sx), which is consistent with the known role of MYC as an oncogenic TF. In addition to recapitulating existing knowledge from previous studies, our analysis also predicts previously unidentified functions for regulators in cancer. For example, the predicted targets of the RBP SUB1 were significantly up-regulated in many cancer types (Fig. 4C). Moreover, the up-regulation of SUB1 target genes is correlated with a worse patient survival in cancer types such as lung cancer (Fig. 4). Previously, SUB1 was considered as a TF. However, the ENCODE eCLIP experiment has profiled many SUB1 peaks on gene 3’UTR regions (Supp Fig. X). In HepG2 cells where SUB1 eCLIP experiment was done, the decay rate of SUB1 target genes is significantly shorter than non-targets (Fig. 4C). After knock-down of SUB1, its predicted targets are also down regulated comparing to other genes (Fig. 4D). These results indicate that SUB1 may bind gene 3’UTR regions to stabilize transcript levels. From our integrated analysis, higher SUB1 activity through regulatory binding on 3’UTR regions is likely to drive tumor specific expression patterns in many cancer types.Step-wise prioritization schemes pinpoint deleterious SNVs in cancer

The above description ofregulatory networks and mutation recurrence provide an approach to prioritize key genomic features associated with cancer. The workflow in Fig.5 A describes this prioritization scheme in a systematic fashion. First, we start by searching for key regulators that frequently rewire, locate in network hubs or at the top of a network hierarchy, or significantly drive expression changes in cancer. We then prioritize functional elements that are associated with top regulators, undergo large regulatory and chromatin changes, or (most importantly) are highly mutated in tumors. Finally, on a nucleotide level, we can pinpoint impactful SNVs for small-scale functional characterization related to their disruption or creation of specific binding sites, or induced chromatin changes, or position in areas of particularly high conservation.

Using this framework, we subject a number of key regulators (such as MYC and SUB1) to siRNA knock-down to validate their regulatory effect (Fig 4D). We then identified several active enhancers in noncoding regions, and validated their ability to influence transcription using luciferase assays (see Supp. File/Section(?) X). We further selected key SNVs within these enhancers that are important for gene expression control (table Sx). Of the eight motif-disrupting SNVs that we tested, six showed a consistent up- or down-regulation effect on expression relative to wild type (Fig. 5B and Supp. File/Section(?) X). One particularly interesting example illustrating ENCODE data integration is on chromosome 6, 13.5xxx (Fig. 5C). This enhancer is located in a noncoding region. Both histone modification and DHS signals indicate an active regulatory role active (Fig. 5C), and both our HisShape enhancer prediction method and EnhancerSeq experiment support its enhancer function (Fig. 5D). Hi-C and ChIA-PET data link this region to a downstream gene SYCP2. 21 out of the 52 ChIP-Seq experiments demonstrate that the region has high frequency chromatin interactions, and motif analysis predicts the C to G mutation significantly disrupts FOLS2 binding affinity (see Supp. File/Section(?) X). Luciferase assays demonstrate that this mutation introduces an xx-fold reduction in expression relative to wild type expression levels, indicating a strong repressive effect on this enhancer’s function.

## Conclusion

This study highlights the value of ENCODE as a resource for cancer research, and leverages ENCODE to provide a prioritization scheme to pinpoint key regulatory elements and SNVs for small-scale validations. A key aspect of our analysis, is that it scales with larger quantities of data, and more diverse data types. In particular, we anticipate that higher quality annotation of non-coding elements through progressively more accurate Enhancer-seq experiments and deeper Hi-C experiments will increase the number of known linkages between non-coding elements and target genes. Likewise, mutation recurrence analysis can be further improved by collecting better-matched data sets, and expanding the size of tumor cohorts. In that the analyses presented here improve with increasing data integration, it provides future investigations with a blueprint for similar studies going forward. By amassing ever-larger data sets, we may obtain a more accurate picture of the cancer genome through large-scale data integration.